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Screening Targeted *Legionella pneumophila* Mutant Libraries In Vivo Using INSeq

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Abstract

Legionella pneumophila is an intracellular bacterial pathogen that can cause a severe inflammatory pneumonia in humans called Legionnaires' disease, which results from bacterial replication within alveolar macrophages. *L. pneumophila* replication within macrophages is dependent on hundreds of individual protein virulence factors. Understanding how these virulence factors contribute to disease in an animal model is important to reveal aspects of host-pathogen interactions. High-throughput sequencing (HTS)-based screens using transposon (Tn) mutagenesis are powerful approaches to identify bacterial genes important for host-pathogen interactions. Since large libraries of Tn mutants are at risk of bottleneck effects, phenotypic screening of smaller numbers of targeted mutants is an effective alternative. Insertion sequencing (INSeq) is a method that enables production of targeted Tn mutant libraries and has been used successfully to identify *L. pneumophila* virulence phenotypes. In this chapter, a protocol is described for using INSeq to generate an arrayed *L. pneumophila* Tn mutant library and for subsequent screening of targeted mutant pools in a mouse model of infection.

Keywords

Legionella pneumophila; Insertion sequencing; Transposon mutagenesis; Mouse model

1 Introduction

Transposon (Tn) mutagenesis and high-throughput sequencing (HTS) approaches are powerful methods to reveal phenotypes associated with loss-of-function mutations of bacterial genes [1]. Tn sequencing (TNSeq) and insertion sequencing (INSeq) are both massively parallel HTS techniques that enable determination of individual Tn mutant fitness in a mixed population. Both techniques have been used to successfully identify genes important for virulence of multiple clinically important bacterial pathogens [2–7]. The vast majority of these studies have been performed using genome-scale Tn mutagenesis and screening of pooled mutant libraries containing thousands of individual mutants. When performing these screens, a balance must be achieved between the quantity of individual Tn mutants and the susceptibility of the study to bottleneck effects, which decrease reliability of screen results [8, 9]. Another factor that can confound screen results is complementation of a Tn mutation by a wild-type allele provided by another strain. This trans-complementation would be especially problematic for assessing phenotypes of mutations in secreted factors

such as toxins or translocated effector proteins. For intracellular bacterial pathogens, this would result from coinfection of a single host cell with more than one independent Tn mutant. The use of animal models to screen for phenotypes associated with loss-of-function mutations presents an additional challenge since the extent of bacterial colonization is limited by factors such as the size of the organ of interest or the number of potential host cells present. However, HTS screens are a powerful and robust way to identify gene products encoded by bacterial pathogens that contribute to virulence, and it is important to conduct these experiments in vivo. To circumvent the issues associated with genome-scale screening, targeted pools of Tn mutants in genes of interest can be generated and examined. Although targeted Tn mutation can be done by various means, generation of an arrayed and mapped Tn mutant library provides a tool for generation of multiple unique pools (i.e., secreted toxins, metabolic enzymes, etc.) or a genome-wide Tn mutant pool for identifying phenotypes ex vivo or in vitro. INSeq technology enables mapping of clonal arrayed Tn mutant libraries [10], which makes this an attractive method for generation of targeted mutant libraries for phenotypic screening.

INSeq screening has provided a robust and powerful avenue to investigate the contribution of individual genes to *Legionella pneumophila* virulence in a mouse model of disease [2]. *L. pneumophila* is an accidental human pathogen that can cause disease via inhalation of the bacteria from aerosolized water droplets. Over 20 different species of *Legionella* are capable of causing human disease, but *L. pneumophila* causes over 90% of clinical cases [11]. Once inhaled, *L. pneumophila* infects and replicates within alveolar macrophages by avoidance of lysosomal degradation and establishment of a replicative niche called the *Legionella*-containing vacuole (LCV) [12]. Generation of the LCV and intracellular replication depends on translocation of bacterial effector proteins into host cells by a Dot/Icm type IVB secretion system [13, 14]. Over 300 individual effector proteins are translocated into infected host cells, and how the majority of them contribute to virulence remains unknown. Functional redundancy between effectors, subtle phenotypes associated with single loss-of-function mutation in individual effector genes, and the sheer number of effectors have made screening for effector virulence phenotypes difficult. However, the disproportionate clinical prevalence of *L. pneumophila* compared to other *Legionella* species suggests that there may be effectors unique to *L. pneumophila* that enhance replication in the mammalian lung [11, 15, 16]. Thus, INSeq was used to (1) generate an arrayed *L. pneumophila* Tn mutant library and (2) screen individual effector Tn mutants for virulence phenotypes in a mouse model of Legionnaires' disease [2].

The following protocol can be used to identify genes important for *L. pneumophila* virulence in a mouse model of infection by INSeq screening of a targeted Tn mutant pool (Fig. 1). Although originally used to screen for effector virulence phenotypes, this protocol can be used to generate other targeted pools of mutants, such as type II secretion system substrates, metabolic pathway, or bacterial surface components. The arrayed *L. pneumophila* Tn mutant library can also be used to select clonal mutants for downstream applications and validation of screening results. Thus, INSeq is a powerful and robust technique to generate clonal arrayed Tn mutant libraries and screen pools of targeted mutants in vivo.

2 Materials

Prepare all solutions using deionized distilled water ($\text{d}_2\text{H}_2\text{O}$) and analytical or molecular grade reagents. Unless otherwise specified, all buffers are stable at room temperature (20–25 °C) for up to 6 months. Use standard aseptic technique for bacterial culture and a biosafety cabinet where appropriate. Unless otherwise specified, all AYE medium for liquid culture of *L. pneumophila* should be supplemented with L-cysteine and $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (Subheading 2.1, **item 1**). All plasticware (i.e., conical tubes, culture tubes, reagent reservoirs, etc.) should be sterile unless otherwise noted. This protocol requires access to basic laboratory equipment including pipets, microcentrifuges, and electrophoresis equipment. All experiments involving vertebrate animals should be done in compliance with animal welfare and ethics guidelines at the institution where the work is being performed.

2.1 Culturing *L. pneumophila*

1. Charcoal yeast extract (CYE) plates [17]: 10 g/L N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) (Sigma; see Note 1), 10 g/L Bacto™ yeast extract (BD Difco; see Note 1), 2 g/L acid-washed charcoal powder, 15 g/L Bacto™ Agar (BD Difco), 3.3 mM L-cysteine, 0.6 mM $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$. Dissolve ACES and yeast extract in 900 mL of $\text{d}_2\text{H}_2\text{O}$. Adjust to pH 6.9 with 1 M KOH, and transfer to a 2 L flask containing 15 g agar, 2 g charcoal, and a magnetic stir bar. Autoclave media at 121 °C for 40 min, and allow to cool to ~55 °C in a water bath. Dissolve L-cysteine (0.4 g/10 mL) and $\text{Fe}_3(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (0.135 g/10 mL) separately in $\text{d}_2\text{H}_2\text{O}$ and sterilize through a 0.2 μm filter, and add 10 mL of each to 1 L autoclaved CYE (10 mL aliquots can be stored at –20 °C). Add 5 $\mu\text{g}/\text{mL}$ chloramphenicol for transposon (Tn) selection if required. Mix well using magnetic stirrer and pour media into appropriate plates. Allow the plates to dry overnight prior to use. Plates can be stored at 4 °C for up to 6 months.
2. ACES yeast extract (AYE) liquid media: 10 g/L ACES (Sigma), 10 g/L Bacto™ yeast extract, 3.3 mM L-cysteine, 0.6 mM $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$. Dissolve ACES and yeast extract in 950 mL of $\text{d}_2\text{H}_2\text{O}$. Adjust to pH 6.9 with 1 M KOH and autoclave at 121 °C for 40 min. Store AYE at room temperature for up to 2 weeks. Dissolve L-cysteine (0.4 g/10 mL) and $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (0.135 g/10 mL) separately in $\text{d}_2\text{H}_2\text{O}$, and sterilize through a 0.2 μm filter to make 100× stocks. Make 1 mL aliquots of L-cysteine and $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and store at –20 °C. Add L-cysteine and $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ separately to AYE media immediately before use.

2.2 Generation of an Arrayed Tn Mutant Library

1. Electrocompetent *L. pneumophila*.
2. 2× *Legionella*-freezing media: 4% (w/v) Bacto™ peptone (BD Difco), 10% (v/v) glycerol. Add 8 g of peptone (w/v) and 20 mL of glycerol (v/v) to 120 mL

¹The quality of media components is important for *L. pneumophila* axenic growth. Use only high-quality analytical grade chemicals.

$\text{d}_2\text{H}_2\text{O}$. Bring volume up to 200 mL with $\text{d}_2\text{H}_2\text{O}$ and autoclave at 121 °C for 30 min, and store at room temperature. Peptone will go into solution once autoclaved.

3. Chloramphenicol stock (25 mg/mL): Dissolve chloramphenicol powder in ethanol to make 25 mg/mL stock. Filter sterilize and store as 1 mL aliquots at −20 °C.
4. *Escherichia coli* S17 λ *pir* harboring pSRS_Cm1 (see Note 2).
5. Bacterial electroporator capable of 2400 V, 25 μF , 2 mm (e.g., BioRad Gene Pulser).
6. Sterile electroporation cuvettes (0.2 cm).
7. AYE medium supplemented with L-cysteine and $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$.
8. Sterile bacterial culture tubes.
9. 48-well CYE agar plates (use 500 μL CYE agar per well).
10. 15 cm CYE + Cm 5 $\mu\text{g}/\text{mL}$ plates.
11. Sterile bacterial cell spreaders.
12. Sterilized toothpicks or wooden applicator sticks.
13. Sterile 200 μL wide orifice pipette tips.
14. Sterile 96-well plates with lids containing 75 $\mu\text{L}/\text{well}$ of sterile 2 $\times$ freezing medium; make fresh when ready to use.
15. Sterile 96-well deep-well plate containing 150 μL of CYE medium; these can be made ahead of time and stored in a sealed bag at 4 °C for up to 2 months.
16. Sterilized aluminum foil to fit 96-well plate.
17. Bacteriological shaking incubator set to 37 °C.
18. Bacteriological incubator set to 37 °C.

2.3 Mapping an *L. pneumophila* Tn Mutant Library Using INSeq

1. EpMotion 5075 liquid handling robot programmed with combinatorial pooling algorithm [10].
2. *L. pneumophila* Tn mutant strains in 96-well plates.
3. Reagents for gDNA extraction from INSeq libraries (Subheading 2.8).
4. Reagents for INSeq preparation and barcoding (Subheading 2.9).
5. Reagents for purification of barcoded INSeq libraries (Subheading 2.10).
6. 10 cm CYE + 112 mM NaCl plates.

²-pSRS_Cm1 (available by request from the author) encodes an MmeI-modified mariner transposon containing the *cat* gene (chloramphenicol resistance) under the control of a *L. pneumophila* promoter. This plasmid was derived from pSAM_bt [3].

2.4 Generation of a Targeted *L. pneumophila* Tn Mutant Pool

1. Mapped and arrayed *L. pneumophila* Tn mutant library.
2. Sterile wooden applicator sticks.
3. Sterile 1 in² pieces of aluminum foil.
4. 48-well CYE agar plates (use 500 µL CYE agar per well).
5. Sterile d₂H₂O.
6. Sterile cryogenic vials.
7. 15 mL conical tubes.

2.5 Animal Infection with Targeted Mutant Pools

1. 15 cm CYE + 5 µg/mL chloramphenicol plates.
2. Bacterial spreaders.
3. Inoculum of *L. pneumophila* Tn mutants (*see* Subheading 3.4, **step 1**).
4. Spectrophotometer.
5. 6- to 8-week-old mice (*see* Note 3).
6. Ketamine (10 mg/mL)/Xylazine (1 mg/mL) mixture (*see* Note 4): Dilute stock Ketamine (100 mg/mL) and Xylazine (20 mg/mL) in PBS, and sterilize through a 0.2 µm filter. The working solution can be stored at room temperature.
7. 25 1/4" gauge needles.
8. Sterile 1 mL syringes.
9. Sterile phosphate-buffered saline (PBS).
10. Sterile d₂H₂O.

2.6 Generation of Input Libraries

1. 15 cm CYE + 5 µg/mL chloramphenicol plates.
2. Bacterial spreaders.
3. Inoculum of *L. pneumophila* Tn mutants (*see* Subheading 3.4, **step 1**).
4. 37 °C incubator.

2.7 Harvesting Output Libraries from the Lungs of Mice

1. Euthanizing chamber connected to CO₂ supply with regulator.

³The genetic background of the animals used for *L. pneumophila* infections is important. If the *L. pneumophila* parental strain used to make the Tn mutant library expresses *flaA*, animals with a functional NAIP5/NLRC4 inflammasome will be restrictive [20–22]. However, mice deficient for *Nlrc4* or *Naip5* expression, such as C57BL/6 *NLRC4*^{−/−} or A/J mice, are more permissive to *L. pneumophila* replication. Generation of a Tn mutant library in a *flaA*-deficient background will permit infection of animals with a functional NAIP5/NLRC4 inflammasome, such as C57BL/6 wild-type mice.

⁴Ketamine is a controlled substance in the USA, and a Drug Enforcement Administration license is required. 2.5% 2,2,2-tribromoethanol (TBE) solution may also be used (*see* [23]).

2. Sterile forceps and scissors.
3. Dissection tray or Styrofoam support and dissection pins.
4. 1.5 mL sterile O-ring screw cap tubes (e.g., Corning).
5. Next Advance Bullet Blender.
6. Sterilized 3.2 mm stainless steel beads.
7. Sterile d₂H₂O.
8. 15 cm CYE + 5 µg/mL chloramphenicol plates.
9. Bacterial spreaders.
10. Ice.
11. 37 °C bacteriological incubator.

2.8 Extraction of gDNA from Input and Output Libraries

1. CYE plates containing colonies from inoculum and lung homogenates.
2. Sterile d₂H₂O.
3. Large sterile cell scrapers (30 mm blade width).
4. Next Advance Bullet Blender.
5. 1.5 mL sterile O-ring screw cap tubes (e.g., Corning).
6. 20% sodium dodecyl sulfate (SDS) solution.
7. 0.15 mm zirconium oxide beads.
8. 2× buffer A: 200 mM Tris, 200 mM NaCl, 20 mM EDTA. Adjust pH to 8.0 using HCl.
9. Phenol/chloroform/isoamyl alcohol (25:24:1), pH 7.9.
10. Phase-lock gel tubes, light, 2 mL.
11. 3 M sodium acetate, pH 5.5.
12. 100% isopropanol, stored at −20 °C.
13. 100% ethanol.
14. Vacuum evaporator.
15. RNase A (100 mg/mL).
16. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.
17. Water bath set to 50 °C.
18. PCR purification kit (e.g., Qiagen QIAquick).
19. Invitrogen Qubit spectrophotometer (or other dye-based spectrophotometer) and broad-range (BR) dsDNA reagents.

2.9 INSeq Library Preparation and Barcoding

1. Magnetic particle concentrator (MPC) for 1.5 mL tubes.
2. MPC for PCR tubes.
3. Pfx DNA polymerase.
4. BioSamA (1 pmol/ μ L): 5'-Bio-TEG-CAAGCAGAA-GACGGCATAACGAAGACC-3'.
5. M12-F: 5'-CTGTCCGTTCCGACTACCCTCCCGAC-3'.
6. M12-R: 5'-GTCGGGAGGGTAGTCGGAACGGACAG-3'.
7. LIB_PCR_5: 5'-CAAGCAGAAGACGGCATAACGAAGACCGGGACTTATCATCCAACCTGT-3'.
8. LIB_PCR_3: 5'-AATGATACGGCGACCACCGAACACTCTTCCCTACACGACGCTCTTCCGATCT-3'.
9. Barcoded adaptor oligonucleotides; list and sequences associated with [2].
10. Streptavidin-coated magnetic beads (e.g., Dynabeads M280 streptavidin).
11. 2 $\times$ bind and wash (B&W) buffer: 10 mM Tris, 2 M NaCl, 1 mM EDTA, pH to 7.5.
12. 1 $\times$ B&W buffer: dilute 2 $\times$ B&W buffer 1:1 with d_2H_2O .
13. LoTE buffer: 3 mM Tris, 0.2 mM EDTA, pH to 7.5.
14. Multichannel pipette and tips.
15. Thermocycler capable of 0.1 $^{\circ}C$ s $^{-1}$ ramp rate.
16. MmeI enzyme (2000 U mL $^{-1}$).
17. T4 DNA ligase (2000,000 units mL $^{-1}$).
18. 100 bp DNA ladder.

2.10 Purification and Sequencing of Barcoded INSeq Libraries

1. Supra Sieve GPG Agarose (e.g., American Bioanalytical).
2. 100 bp DNA ladder.
3. SYBR Green I nucleic acid stain (10,000 $\times$ in DMSO).
4. 6 $\times$ Orange Loading Dye.
5. Clean razor blades.
6. Tris-acetate EDTA (TAE) buffer.
7. Blue light illuminator (e.g., Safe Imager 2.0, Invitrogen).
8. Gel extraction kit (e.g., ZymoPure gel extraction kit).

9. Invitrogen Qubit spectrophotometer (or other dye-based spectrophotometer) and high-sensitivity (HS) dsDNA reagents.
10. Genomics/sequencing facility with Illumina HiSeq instrument.
11. INSeq data analysis package [10].

3 Methods

3.1 Production of Electrocompetent *L. pneumophila*

1. Streak *L. pneumophila* strain for single colonies on CYE plates.
2. Select a single colony and generate a 2-day heavy patch. Scrape the entire patch using a loop, and resuspend in a 1.5 mL tube containing 1 mL ice-cold sterile $\text{d}_2\text{H}_2\text{O}$.
3. Centrifuge to pellet bacteria at $8000 \times g$ for 3 min at 4 °C.
4. Aspirate supernatant and resuspend bacteria in 1 mL sterile ice-cold $\text{d}_2\text{H}_2\text{O}$.
5. Repeat **steps 3 and 4** twice more for a total of three washes.
6. Aspirate supernatant, and resuspend bacterial pellet in 1 mL ice-cold sterile 10% glycerol.
7. Centrifuge for 5 min at $16,000 \times g$ at 4 °C, and resuspend pellet in 500–1000 μL of sterile ice-cold 10% glycerol (*see Note 5*).
8. Make 100 μL aliquots and either use fresh or flash freeze in liquid nitrogen, and store at -70°C .

3.2 Transposon Mutagenesis of *L. pneumophila*

1. Transform electrocompetent *L. pneumophila* with pSRS_Cm1: Thaw a 100 μL vial of electrocompetent *L. pneumophila* on ice, and add 1 μg of pSRS_Cm1 (*see Note 6*). Transfer competent cells and plasmid to a pre-chilled sterile electroporation cuvette (0.2 cm); ensure entire solution is at the bottom of the cuvette and that there are no air bubbles. Place the cuvette containing bacteria + plasmid mixture in the cuvette holder, and pulse with the parameters: 2400 V, 25 μF , 2 mm. Immediately, add 900 μL of AYE media supplemented with L-cysteine and $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, and transfer to a sterile culture tube. Incubate at 37 °C with shaking for 8 h. Several transformations can be done in parallel.
2. Plate mutagenized *L. pneumophila* for single colonies: Transfer contents of culture tube (from **step 1**) into a sterile 1.5 mL microfuge tube with volume gradations. Centrifuge bacteria at $16,000 \times g$ for 30 s to generate a pellet. Remove supernatant with a pipette but leave ~250 μL over the pellet. Mix the pellet in the remaining supernatant to get a homogeneous suspension of bacteria.

⁵The electrocompetent *L. pneumophila* need to be concentrated, so the final amount of 10% glycerol to resuspend the pellet depends on the size of the pellet. Use a lower volume for increased mutagenesis efficiency.

⁶pSRS_Cm1 should be added to electrocompetent *L. pneumophila* in a volume of 5 μL . Maxiprep of pSRS_Cm1 is efficient for generation of purified and concentrated pSRS_Cm1.

Plate the mixture on a 15 cm CYE + Cm 5 µg/mL plate. Allow plate to sit at room temperature upright for 20 min; invert and place at 37 °C for 4–6 days until macroscopic colonies are apparent.

3.3 Generation of an *L. pneumophila* Arrayed Mutant Library

1. Patch individual colonies onto 48-well agar plates using a sterile wood applicator (see Note 7), add 30 µL of sterile AYE media, swirl plate gently, and place face up in 37 °C incubator for 48–60 h.
2. Add 250 µL of sterile d₂H₂O to each well of the plate, and mix gently to resuspend bacteria.
3. Using sterile wide orifice pipette tips, transfer 75 µL of the bacterial suspension from each well into a well of a 96-well plate containing 75 µL of freezing medium.
4. Once a plate has been filled, number it and replica plate by transferring 10 µL from each well into a deep-well 96-well plate containing 150 µL of CYE (see Note 8).
5. Cover wells of 96-well plate containing bacteria in freezing media (stocked library plates) with sterile aluminum foil, and replace lid on top of foil; store at –70 °C.
6. Incubate 96-well CYE replica plates at 37 °C for 48 h; resuspend bacteria in 200 µL sterile d₂H₂O, and transfer 150 µL bacterial suspension to a fresh sterile 96-well plate (see Note 9). These plates can be stored at –20 °C until use for combinatorial pooling and INSeq mapping.

3.4 Mapping an *L. pneumophila* Arrayed Tn Mutant Library Using INSeq

The location of Tn insertions in mutagenized *L. pneumophila* clones arrayed in a 96-well plate library can be determined by combinatorial pooling combined with INSeq (Fig. 2).

1. Program the EpMotion 5075 with combinatorial pooling programs [10], and run the programs in order with five plates at a time (1–5, then 6–10, then 11–16, etc.). Label the tubes pool #1–#24, and place them in the appropriate location in the 24-well EpMotion tube holder. This will result in a set of 24 pools for each run, which should be stored at –20 °C or –80 °C.
2. Thaw all pool #1 tubes, and combine the same volume of each into a single conical tube to generate a pool #1 master pool. Repeat for pools #2–#24.
3. Harvest gDNA from each of the 24 master pools separately (see Subheading 3.7).

⁷Select well-isolated colonies to prevent inoculation of two clones into the same well.

⁸It is critical to keep the wells containing individual mutants consistent between plates. For example, bacteria from plate 1, well A1, should always be replica plated into plate 1, well A1 of subsequent plates. This ensures that mapping data can be correlated with the stocked library of mutants.

⁹Inform the sequencing facility of the concentration of your submitted DNA, and request data returned as raw sequencing reads in fastq format.

4. Prepare INSeq libraries from gDNA isolated from each master pool; include a technical replicate for each pool (48 individual unique barcoded libraries total) (Subheadings 3.8 and 3.9).
5. Submit sample for Illumina sequencing (Subheading 3.9) (*see* Note 9).
6. Download the data analysis package from [10], and run analysis using raw sequencing data in fastq format (*see* Note 10).
7. Validate mapping by identifying wells containing *L. pneumophila* mutants in the *Dot/Icm* apparatus that would not be salt sensitive and streaking on CYE + 112 mM NaCl in addition to controls that are salt sensitive [18, 19] (*see* Note 11).

3.5 Generation of a Targeted *L. pneumophila* Tn Mutant Pool

1. Identify locations of Tn mutants of interest in output files generated by INSeq data analysis (*see* Note 12).
2. Remove library plates containing your mutants of interest from the -80°C freezer (*see* Note 11).
3. Remove the plate lid and sterilize the foil cover with 70% ethanol.
4. Locate your well of interest, and poke a hole through the foil with a sterile micropipette tip (200 μL works best) to scoop up a small amount of bacterial stock from the well containing your desired mutant.
5. Make a patch of bacteria on a CYE plate and streak for single colonies.
6. Incubate plates at 37°C for 4 days to obtain single colonies.
7. Patch single colonies onto 48-well CYE agar plates, overlay with 30 μL AYE media and incubate at 37°C for 2 days.
8. Add 200 μL sterile $\text{d}_2\text{H}_2\text{O}$, and combine equal amounts of each mutant and mix well (*see* Note 13).
9. Freeze aliquots of the mutant pool in cryovials by mixing bacterial suspension 1:1 with $2\times$ freezing media.
10. Store Tn mutant aliquots at -70°C until use.

¹⁰Detailed instructions on generating mapping data for the arrayed Tn mutant library can be found in the README.txt file associated with [10]. Briefly, the data analysis package provides all scripts and instructions required to use the Illumina sequencing reads to map the location of the Tn insertions, sort the reads by associated barcode, and quantify the number of normalized reads associated with each insertion. Output files are generated which provide the number of normalized read counts associated with each Tn insertion for each barcode used. For mapping the arrayed mutant library, follow the instructions in “(B) arrayed library” under “additional features of the analysis pipeline.” The output files generated will provide information on (1) the genomic coordinate of the Tn insertions, (2) the location of the Tn within the open reading frame (ORF), (3) the location of each insertion in the library (plate and well), and (4) whether more than one independent Tn insertion was mapped to the same well of the library.

¹¹Validation of mapping can also be done by examination of Tn-mutagenized loci by PCR.

¹²Avoid freeze-thaw cycles by keeping plates on dry ice while picking mutants of interest.

¹³At this point, an arrayed sub-library can be produced by stocking mutants of interest in new 96-well plates with 1:1 mixture of $2\times$ freezing medium.

3.6 Animal Infection with Targeted Mutant Pools

1. Grow liquid culture of input pool: Thaw a vial of targeted Tn mutant library and an empirically determined amount on 15 cm CYE agar plate (*see* Note 14). Incubate plate at 37 °C for 72 h. Scrape bacteria from plate in 10 mL AYE broth, and measure the OD₆₀₀ using a spectrophotometer. Start 10 overnight cultures of the pool in AYE broth with starting OD₆₀₀ ranging from 0.1 to 1.0. Incubate at 37 °C with aeration for 16–20 h. Measure OD₆₀₀ of overnight cultures, and select culture with an OD₆₀₀ in the range of 3.2–3.8.
2. Generate a mutant pool inoculum: Quantify the amount of *L. pneumophila* in the overnight culture (OD₆₀₀ of 1 = 10⁶ bacteria/μL). Prepare a suspension of the *L. pneumophila* mutant library at a concentration of 5 × 10⁶/40 μL in PBS immediately before infection (*see* Note 15). Prepare enough for all mice being infected plus enough to plate for input library outgrowth (**step 5** below; *see* Note 16).
3. Anesthetize animals: Weigh mice to ensure proper dosage of Ketamine/Xylazine, which is commonly 0.18–0.25 mL (100 mg/kg Ketamine and 10 mg/kg Xylazine). Administer Ketamine/Xylazine mixture by the intraperitoneal (IP) route. Secure animal, and hold with head facing down such that organs move away from the site of injection. Cleanse the area of injection with 70% ethanol, and inject appropriate amount of anesthetic using a 25 ¼” syringe needle. Ensure animals are anesthetized by checking reflexes. Apply artificial tears to the eyes of anesthetized mice during the course of infection to avoid drying.
4. Intranasal mouse infection: Lay anesthetized mice face up so the nares are easily accessible. Avoid inhalation of *L. pneumophila* aerosols by wearing a mask or infecting animals in a biosafety cabinet. Mix inoculum well using a vortex. Administer 10 μL of bacterial mixture dropwise to a single nostril slowly. Wait until each drop is fully inhaled before adding the next drop. Wait at least 2 min and administer 10 μL to the opposite nostril dropwise. Repeat this twice for a total of 40 μL. Allow the mice to recover under a light bulb or on a heating pad, and transfer to an ABSL2 facility.
5. Generate input libraries: From tube of inoculum used to infect mice, plate 250 μL in duplicates on 15 cm CYE plates using sterile cell spreader or glass beads. Allow plate to dry by leaving face up for at least 30 min. Invert and incubate at 37 °C for 72 h. Add 10 mL sterile d₂H₂O and scrape bacteria using a large sterile cell scraper. Transfer bacterial suspension into a sterile conical tube and mix well. Transfer 500 μL of bacterial mixture into a sterile 1.5 mL screw cap tube and centrifuge at 16,000 × *g* for 2 min. Aspirate supernatant and bacteria until there is a pellet equivalent to ~30 μL. Pellet can be stored at –20 °C until gDNA harvest (Subheading 3.8). Remainder of bacterial suspension can be pelleted and stored at –20 °C.

¹⁴Over-plating the mutant pool will result in loss of less represented mutants. Individual colonies should be observed on plates.

¹⁵*L. pneumophila* are sensitive to salt so they should not remain in PBS for prolonged period.

¹⁶For these experiments, ten mice are sufficient to achieve enough statistical power in downstream analysis.

3.7 Harvesting Output Libraries from the Lungs of Mice

1. Add 3×3.2 mm sterile stainless steel beads and 300 μL sterile $\text{d}_2\text{H}_2\text{O}$ to a sterile 1.5 mL O-ring screw cap tube, and record the weight of each tube. Prepare one tube for each mouse.
2. Euthanize animals according to guidelines at your institution.
3. After confirmation of death, secure animals face up to dissection tray or Styrofoam support using dissection pins.
4. Spray animal with 70% ethanol, and open the chest cavity using sterile scissors and forceps.
5. Carefully remove the lungs, and transfer to a previously prepared and pre-weighed tube (**step 1**). Weigh tubes (for CFU g^{-1} calculation), and homogenize in a Bullet Blender at setting 8 for 4 min. Add 700 μL of sterile $\text{d}_2\text{H}_2\text{O}$ to each tube, and mix with a vortex on highest setting for 20 s. Transfer 120 μL of homogenate to a sterile 1.5 mL tube for serial dilutions, and plate for CFU enumeration (if desired).
6. Plate 250 μL per plate of lung homogenate on 15 cm CYE plates by spreading. Allow homogenate to dry on plates, invert plates, and incubate at 37°C for 4 days.
7. Add 8 mL of sterile $\text{d}_2\text{H}_2\text{O}$ to each plate, and scrape bacteria with a large sterile cell scraper; use a new cell scraper for each biological replicate/mouse.
8. Transfer all bacteria from the same mouse into a single 50 mL conical tube, and mix with a vortex on the highest setting for 20 s.
9. Transfer 500 μL of bacterial suspension into a sterile 1.5 mL screw cap tube, and centrifuge at $16,000 \times g$ for 2 min. Aspirate supernatant and bacteria until ~ 30 μL volume pellet remains. Pellet can be stored at -20°C until gDNA extraction (Subheading 3.8).

3.8 Isolation of gDNA from Input and Output Libraries

1. Add 2 volumes of 0.15 mm zirconium oxide beads, 500 μL of $2\times$ Buffer A, 210 μL of 20% SDS, and 500 μL of phenol/chloroform/isoamyl alcohol to each bacterial pellet.
2. Homogenize samples in Bullet Blender for 3 min at setting 8, incubate on ice for 2 min, and homogenize again in Bullet Blender for 2 min at setting 8.
3. Centrifuge the tubes at $6800 \times g$ for 3 min at 4°C .
4. Transfer the aqueous phase into a fresh sterile 1.5 mL microcentrifuge tube, and discard the organic phase.
5. Add 600 μL of ice-cold 100% isopropyl alcohol to each sample.
6. Add 60 μL of 3 M sodium acetate (pH 5.5) to each sample and vortex to mix.

7. Incubate sample at -20°C 1 h or overnight. A cloudy precipitate of DNA may form during this time.
8. Centrifuge samples at $18000 \times g$ for 20 min at 4°C , and carefully remove and discard the supernatant (the pellet may or may not be visible at this point).
9. Add 500 μL of 100% room temperature ethanol to each pellet.
10. Centrifuge sample at $18000 \times g$ for 5 min at 4°C , and carefully remove and discard the supernatant. The pellet may be loose, so exercise caution when removing supernatant.
11. Invert the tube and remove excess ethanol by blotting on laboratory tissue (e.g., Kimwipe).
12. Dry pellet in a vacuum evaporator for 5 min with no heat. If pellet is not dry after 5 min, repeat this step until dry (*see* Note 17).
13. Add 200 μL of TE buffer (pH 7.0) to each pellet.
14. Incubate samples in a 50°C water bath for 10 min, and mix using a vortex at highest setting for 10 s.
15. Repeat **step 14** three-to-four times until the pellet is fully dissolved.
16. Transfer 100 μL of sample to a clean tube, and store the remainder at -20°C or -80°C .
17. Add 0.5 μL of RNase A and incubate at room temperature for 2 min.
18. Use a PCR purification kit to clean the sample according to manufacturers' instructions; ensure all ethanol has been removed following the final wash step.
19. Elute in 52 μL of EB buffer (provided with kit).
20. Quantify the DNA using a UV or dye-based spectrophotometer. DNA can be stored at -20°C or -80°C .

3.9 INSeq Library Preparation and Barcoding (see Note 18)

1. Linear PCR to generate biotinylated Tn-adjacent DNA.
 - a. 10 μL Pfx buffer
 - b. 2 μL 10 mM dNTPs
 - c. 2 μL 50 mM MgCl_2
 - d. 5 pmol BioSam A
 - e. 0.5–2 μg clean DNA (from Subheading 3.8, **step 20**)
 - f. $\text{d}_2\text{H}_2\text{O}$ to 100 μL .

¹⁷·Avoid overdrying the pellet, which will make it difficult to dissolve.

Split into $2 \times 50 \mu\text{L}$ reactions, and run on a thermocycler: 94°C , 2 min; $[94^\circ\text{C}, 15 \text{ s}; 68^\circ\text{C}, 1 \text{ min}] \times 50$; 4°C hold.

2. Pool same reactions and purify using a PCR purification kit. Elute in $50 \mu\text{L}$ of EB.
3. Shake streptavidin-coated Dynabeads to resuspend.
4. Add $32 \mu\text{L}$ per sample of beads into a single 1.5 mL microcentrifuge tube (if volume becomes $>1 \text{ mL}$, use additional tubes).
5. Place tube on MPC for 2 min and aspirate supernatant.
6. Remove tubes from magnet and resuspend in 1 mL of $1 \times \text{B\&W}$.
7. Repeat **steps 5 and 6** for a total of three washes.
8. Add $52 \mu\text{L}$ per sample of $2 \times \text{B\&W}$ buffer, and aliquot beads into PCR strip tubes or plates (see Note 19).
9. Add entire cleaned linear PCR sample from **step 2** above to a PCR tube with beads and $2 \times \text{B\&W}$. Make note of the tube location of each sample.
10. Incubate tubes on a nutating rocker for 30 min with gentle agitation to resuspend the beads every 10 min.
11. Place tubes on a magnetic particle concentrator (MPC), and incubate for 2 min.
12. Carefully aspirate the supernatant with a pipette; be careful not to disrupt the beads on the side of the tubes.
13. Remove the tubes from the magnet, and gently resuspend beads in $100 \mu\text{L}$ of $1 \times \text{B\&W}$ buffer.
14. Repeat **steps 11–13**, but use $100 \mu\text{L}$ of LoTE buffer.
15. Using a thermocycler, denature samples by heating at 95°C for 2 min followed by quickly cooling to 4°C .
16. Prepare second strand synthesis master mix on ice by combining the indicated amounts of reagents per sample:
 - a. $2 \mu\text{L}$ $10 \times$ hexanucleotides
 - b. $1 \mu\text{L}$ 10 mM dNTPs
 - c. $1 \mu\text{L}$ Klenow (exo-)
 - d. $16 \mu\text{L}$ $\text{d}_2\text{H}_2\text{O}$.
17. Place the tubes containing beads and samples on the MPC for 2 min, and carefully aspirate the supernatant.

¹⁸. This protocol can be paused and multiple steps for sample storage at 4°C overnight. Refer to [10].

¹⁹. Use of sealing film to cover PCR tubes prevents sample loss and cross-contamination resulting from beads retained in the tube caps.

18. Add 20 μL of second strand mixture to each tube, and resuspend beads by pipetting.
19. Incubate samples for 30 min at 37 $^{\circ}\text{C}$ in a thermocycler. Vortex tubes on medium setting every 10–15 min.
20. Add 100 μL of LoTE buffer to the samples and place tubes on MPC.
21. Remove the supernatant with a pipette.
22. Repeat **steps 20** and **21**.
23. Resuspend beads in 100 μL LoTE.
24. Prepare M12 double-stranded DNA (dsDNA) (*see* Note 20): Combine 15 μL of 100 μM M12-F, 15 μL of 100 μM M12-R, and 1.5 μL of 1 M NaCl in a PCR tube. In a thermocycler, heat at 95 $^{\circ}\text{C}$ for 5 min, and cool to 4 $^{\circ}\text{C}$ at a rate of 0.1 $^{\circ}\text{C}$ per second. Aliquots of this reaction can be stored at -20°C .
25. Prepare MmeI master mix on ice by combining the indicated amounts of each of the following reagents per sample:
 - a. 2 μL 10 $\times$ NEB CutSmart Buffer
 - b. 0.08 μL 32 mM SAM
 - c. 0.2 μL M12 dsDNA
 - d. 16.8 μL $\text{d}_2\text{H}_2\text{O}$
 - e. 1 μL MmeI.
26. Place tubes on MPC for 2 min, and carefully remove and discard the supernatant.
27. Resuspend beads in 20 μL of mix prepared in **step 24**.
28. Incubate at 37 $^{\circ}\text{C}$ for 1 h in a thermocycler. Vortex tubes on medium setting every 10–15 min.
29. Add 100 μL of LoTE buffer to each tube.
30. Place tubes on MPC for 2 min, and carefully remove and discard the supernatant.
31. Repeat **steps 28** and **29**, and resuspend beads in 100 μL LoTE while preparing ligation reaction.
32. Thaw 50 μM dsDNA sequencing adapters on ice, and dilute to 5 μM with ice-cold 1 $\times$ NEB T4 ligase buffer. Prepare one adapter per sample.
33. Prepare T4 ligase master mix on ice by combining the indicated amount of each of the following reagents per sample:
 - a. 16.4 μL $\text{d}_2\text{H}_2\text{O}$
 - b. 2 μL 10 $\times$ T4 ligase buffer

²⁰M12 dsDNA is supplied in trans to ensure that MmeI cleavage of the target DNA is complete [24].

- c. 1 μ L T4 ligase.
34. Place tubes on MPC for 2 min, and carefully remove and discard the supernatant.
35. Resuspend beads in 19.4 μ L ligation master mix.
36. Add 0.6 μ L of 5 μ M sequencing adapter per sample (from **step 31**). Use a different adapter for each sample, and make note of the barcode sequence used for each sample.
37. Incubate at 16 °C for 1 h 15 min in a thermocycler. Vortex tubes on medium setting every 10–15 min.
38. Add 100 μ L of LoTE to each tube.
39. Place tubes on MPC for 2 min, and carefully remove and discard the supernatant.
40. Repeat **steps 37** and **38**, and resuspend beads while preparing PCR master mix.
41. Prepare final PCR master mix on ice by combining the indicated amounts of the following reagents per sample:
 - a. 10 μ L 10 $\times$ Pfx buffer (2 $\times$ standard concentration)
 - b. 2 μ L 10 mM dNTPs
 - c. 2 μ L 50 mM MgCl₂
 - d. 2 μ L LIB-PCR-5
 - e. 2 μ L LIB-PCR-3
 - f. 0.5 μ L Pfx polymerase
 - g. 31.5 μ L d₂H₂O.
42. Place tubes containing beads on MPC for 2 min, and carefully remove and discard the supernatant.
43. Resuspend beads in 50 μ L of final PCR master mix on ice, and run on a thermocycler with the following parameters: 94 °C, 2 min, [94 °C, 15 s; 60 °C, 1 min; 68 °C, 2 min] $\times$ 18, 4 °C hold.
44. Place the tubes on the MPC for 2 min, and transfer the supernatant to a new tube.
45. Add 100 μ L LoTE buffer to beads and store at 4 °C. A second round PCR can be performed if necessary.

3.10 Purification and Sequencing of Barcoded INSeq Libraries

1. Prepare a 4% Supra Sieve agarose gel in TAE (*see* Note 21) with one–two lanes for each sample, two controls (*see* Note 22) and molecular weight ladder. Add

²¹This takes a long time to dissolve and must be constantly monitored. Heat on 50% power for up to 2 min and swirl. Repeat until fully dissolved and cool to 55 °C in a water bath.

²²Use non-mutagenized gDNA as a negative control for non-specific bands resulting from sample prep and, if possible, an established INSeq library as a positive control.

SYBR Green I (or similar) to dissolved and cooled agarose to a 1× final concentration.

2. Add 10 µL of 6× Orange Loading Dye to each sample, and pipet up and down to mix.
3. Load 100 bp ladder into the first lane of the gel, and then load full amount of each sample into 1–2 wells of the gel.
4. Run the gel for 30–60 min at 100 V. Use the bromophenol blue in the ladder lane as an indicator of where the 125 bp sample is on the gel. The amount of time required depends on the size of the gel.
5. Use a blue light illuminator to visualize the bands at approximately 125 bp (*see* Note 23).
6. Excise the bands by cutting with clean razor blades (*see* Note 24), and purify the DNA using a gel extraction kit (e.g., ZymoPure gel extraction kit). Elute in 30 µL elution buffer.
7. Quantify the sample using a dye-based spectrophotometer. The DNA concentration is typically too low to be quantified using a UV spectrophotometer ($5 \text{ ng } \mu\text{L}^{-1}$).
8. Normalize all samples to the same concentration using elution buffer, and combine an equal volume of each sample into the same tube (*see* Note 9).
9. Submit for Illumina Sequencing and run data analysis pipelines (*see* Note 25) [10].

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References

1. van Opijnen T, Camilli A (2013) Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms. *Nat Rev Microbiol* 11:435–442 [PubMed: 23712350]
2. Shames SR, Liu L, Havey JC, Schofield WB, Goodman AL, Roy CR (2017) Multiple *Legionella pneumophila* effector virulence phenotypes revealed through high-throughput analysis of targeted mutant libraries. *Proc Natl Acad Sci U S A* 63:201708553
3. Goodman AL, McNulty NP, Zhao Y, Leip D, Mitra RD, Lozupone CA, Knight R, Gordon JI (2009) Identifying genetic determinants needed to establish a human gut symbiont in its habitat. *Cell Host and Microbe* 6:279–289 [PubMed: 19748469]

²³. Do not use UV illuminator since prolonged exposure to UV will damage DNA and decrease the quality of the extracted libraries.

²⁴. Avoid cross-contamination of the gel bands by using a fresh razor blade or clean glass coverslips to separate the bands from each other.

²⁵. The INSeq data analysis pipeline utilizes raw sequencing data and associated barcodes to quantify and assign normalized read counts to individual transposon insertions from different samples. These counts can then be used to perform statistical analysis on normalized read counts associated with individual transposon insertions from the output and input libraries [2].

4. Skurnik D, Roux D, Aschard H, Cattoir V, Yoder-Himes D, Lory S, Pier GB (2013) A comprehensive analysis of in vitro and in vivo genetic fitness of *Pseudomonas aeruginosa* using high-throughput sequencing of transposon libraries. *PLoS Pathog* 9:e1003582 [PubMed: 24039572]
5. Wang N, Ozer EA, Mandel MJ, Hauser AR (2014) Genome-wide identification of *Acinetobacter baumannii* genes necessary for persistence in the lung. *MBio* 5:e01163–e01114 [PubMed: 24895306]
6. Gao B, Lara-Tejero M, Lefebvre M, Goodman AL, Galán JE (2014) Novel components of the flagellar system in epsilonproteobacteria. *MBio* 5:e01349–e01314 [PubMed: 24961693]
7. Wong SM, Bernui M, Shen H, Akerley BJ (2013) Genome-wide fitness profiling reveals adaptations required by *Haemophilus* in coinfection with influenza A virus in the murine lung. *Proc Natl Acad Sci U S A* 110:15413–15418 [PubMed: 24003154]
8. Abel S, Abel zur Wiesch P, Davis BM, Waldor MK (2015) Analysis of bottlenecks in experimental models of infection. *PLoS Pathog* 11: e1004823 [PubMed: 26066486]
9. Hubbard TP, Chao MC, Abel S, Blondel CJ, Abel zur Wiesch P, Zhou X, Davis BM, Waldor MK (2016) Genetic analysis of *Vibrio parahaemolyticus* intestinal colonization. *Proc Natl Acad Sci U S A* 113:6283–6288 [PubMed: 27185914]
10. Goodman AL, Wu M, Gordon JI (2011) Identifying microbial fitness determinants by insertion sequencing using genome-wide transposon mutant libraries. *Nat Protoc* 6:1969–1980 [PubMed: 22094732]
11. Newton HJ, Ang DKY, van Driel IR, Hartland EL (2010) Molecular pathogenesis of infections caused by *Legionella pneumophila*. *Clin Microbiol Rev* 23:274–298 [PubMed: 20375353]
12. Horwitz MA (1983) Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. *J Exp Med* 158:1319–1331 [PubMed: 6619736]
13. Vogel JP, Andrews HL, Wong SK, Isberg RR (1998) Conjugative transfer by the virulence system of *Legionella pneumophila*. *Science* 279:873–876 [PubMed: 9452389]
14. Segal G, Purcell M, Shuman HA (1998) Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. *Proc Natl Acad Sci* 95:1669–1674 [PubMed: 9465074]
15. Doleans A, Aurell H, Reyrolle M, Lina G, Frenay J, Vandenesch F, Etienne J, Jarraud S (2004) Clinical and environmental distributions of *Legionella* strains in France are different. *J Clin Microbiol* 42:458–460 [PubMed: 14715805]
16. Burstein D, Zusman T, Degtyar E, Viner R, Segal G, Pupko T (2009) Genome-scale identification of *Legionella pneumophila* effectors using a machine learning approach. *PLoS Pathog* 5:e1000508 [PubMed: 19593377]
17. Feeley JC, Gibson RJ, Gorman GW, Langford NC, Rasheed JK, Mackel DC, Baine WB (1979) Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. *J Clin Microbiol* 10:437–441 [PubMed: 393713]
18. Catrenich CE, Johnson W (1989) Characterization of the selective inhibition of growth of virulent *Legionella pneumophila* by supplemented Mueller-Hinton medium. *Infect Immun* 57:1862–1864 [PubMed: 2722245]
19. Sadosky AB, Wiater LA, Shuman HA (1993) Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infect Immun* 61:5361–5373 [PubMed: 8225610]
20. Ren T, Zamboni DS, Roy CR, Dietrich WF, Vance RE (2006) Flagellin-deficient *Legionella* mutants evade caspase-1- and Naip5-mediated macrophage immunity. *PLoS Pathog* 2:e18 [PubMed: 16552444]
21. Molofsky AB, Byrne BG, Whitfield NN, Madigan CA, Fuse ET, Tateda K, Swanson MS (2006) Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. *J Exp Med* 203:1093–1104 [PubMed: 16606669]
22. Zamboni DS, Kobayashi KS, Kohlsdorf T, Ogura Y, Long EM, Vance RE, Kuida K, Mariathasan S, Dixit VM, Flavell RA, Dietrich WF, Roy CR (2006) The BirA cytosolic pattern-recognition receptor contributes to the detection and control of *Legionella pneumophila* infection. *Nat Immunol* 7:318–325 [PubMed: 16444259]

23. Hori JI, Zamboni DS (2013) The mouse as a model for pulmonary legionella infection. *Methods Mol Biol* 954:493–503 [PubMed: 23150416]
24. Morgan RD, Bhatia TK, Lovasco L, Davis TB (2008) MmeI: a minimal Type II restriction-modification system that only modifies one DNA strand for host protection. *Nucleic Acids Res* 36:6558–6570 [PubMed: 18931376]

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Fig. 1. Schematic overview of the protocol described in this chapter. **(a)** *L. pneumophila* is mutagenized with an INSeq-amenable *mariner* transposon, and individual colonies are archived into 96-well plates (Subheadings 3.1–3.3). **(b)** Combinatorial pooling and INSeq are used to map the location of individual Tn mutants within the archived library (Subheading 3.4). **(c)** Tn mutants of interest are selected from the arrayed library and used to generate a Tn mutant pool (Subheading 3.5). **(d)** The targeted Tn mutant pool is used to infect mice and generate input libraries for INSeq (Subheading 3.6). **(e)** Infected animals are euthanized, and lung homogenates are plated to generate output libraries for INSeq (Subheading 3.7). **(f)** Genomic DNA is extracted from input and output libraries for INSeq sample preparation, sequencing, and analysis (Subheadings 3.8–3.10)

Fig. 2. Mapping an arrayed Tn mutant library using INSeq. Each well of the library (i.e., clone) is assigned a unique binary code of presence (+) or absence (-) in each of 24 individual pools. (a) The pools are generated by programming a liquid handling robot (e.g., EpMotion 5075) to dispense bacteria from individual wells into a defined subset of the 24 pools. (b) Each pool is associated with a unique 4–6 base-pair adaptor barcode, designated here as A–X. (c) Following Illumina sequencing and INSeq analysis, the library location of the individual transposon insertions is determined, and their presence-absence (+/–) pattern in each of the 24 pools is determined. Based on this presence-absence (+/–) pattern, the individual mutants are assigned to their location in the archived strain library