Video Article

High-throughput Parallel Sequencing to Measure Fitness of *Leptospira interrogans* Transposon Insertion Mutants During Golden Syrian Hamster Infection

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Abstract

In this manuscript, we describe a transposon sequencing (Tn-Seq) technique to identify and quantify *Leptospira interrogans* mutants altered in fitness during infection of Golden Syrian hamsters. Tn-Seq combines random transposon mutagenesis with the power of high-throughput sequencing technology. Animals are challenged with a pool of transposon mutants (input pool), followed by harvesting of blood and tissues a few days later to identify and quantify the number of mutants in each organ (output pools). The output pools are compared to the input pool to evaluate the *in vivo* fitness of each mutant. This approach enables screening of a large pool of mutants in a limited number of animals. With minor modifications, this protocol can be performed with any animal model of leptospirosis, reservoir host models such as rats and acute infection models such as hamsters, as well as *in vitro* studies. Tn-Seq provides a powerful tool to screen for mutants with *in vivo* and *in vitro* fitness defects.

Video Link

The video component of this article can be found at https://www.jove.com/video/56442/

Introduction

Identification of virulence genes for some bacteria, such as *Leptospira* spp., is difficult because of the limited number of genetic tools available. One commonly used approach is the creation of a collection of mutants by random transposon mutagenesis followed by the identification of the insertion site in each mutant and virulence testing of individual transposon mutants in an animal model. This approach is time-consuming, expensive, and requires a large number of animals.

When random mutagenesis was first developed for the pathogen *Leptospira interrogans*, genes involved in virulence were identified by testing individual mutants in an animal model[1]. Mutants were selected based on criteria such as their potential roles in signaling or motility or their predicted outer membrane or surface location. As the majority of leptospiral genes encode hypothetical proteins of unknown function[2], selecting mutants based on these criteria limits the ability to discover novel leptospiral virulence genes.

More recently, pools of *L. interrogans* transposon mutants were screened for infectivity in the hamster and mouse models[3]. Each animal was challenged with a pool of up to 10 mutants. Infectivity of a mutant was scored as positive if it was detected by PCR of cultures obtained from blood and kidney. PCR testing was laborious because it required an individual PCR reaction for each mutant in the pool. Because the frequency of each mutant in the cultures was not quantified, the approach was biased towards identification of highly attenuated mutants.

We describe a transposon sequencing (Tn-Seq) technique, as a strategy to more efficiently screen for virulence genes. Tn-seq consists of the creation of a library of mutants by transposon mutagenesis followed by massive parallel sequencing[4,5,6]. Briefly, transposon mutants are pooled, inoculated into animals, and later recovered from different organs (output pools). The DNA from the output pools is extracted and digested with restriction enzymes or sheared by sonication. Two rounds of PCR targeting the junctions of the transposon insertion sites are performed. This step enables the addition of the adaptors necessary for the sequencing. The resulting PCR products are analyzed by high-throughput sequencing to identify the transposon insertion site of each mutant of the pool along with their relative abundance, which is compared to the initial composition of the pool of mutant.

The primary advantage of this approach is the ability to simultaneously screen a large number of mutants with a small number of animals. Tn-Seq does not require the prior knowledge of the transposon insertion sites which increases the chances of discovering new *Leptospira*-

specific genes involved in virulence with less time and greater efficiency. Because leptospiral burden in tissues is relatively high in rodent models susceptible to lethal infection (typically 10^4 to 10^8 bacteria/g of tissue)[7,8,9] as well as in reservoir hosts[10,11], tissues can be directly analyzed without the need to culture, reducing biases due to *in vitro* growth.

In Tn-Seq studies with most bacterial pathogens described to date, the high frequency of insertional mutagenesis allowed infection with large pools containing mutants collectively having multiple closely-spaced transposon insertions within every gene[4,12,13,14]. Tn-Seq has also been developed for a bacterium for which the mutagenesis frequency is much lower[6]. With *Leptospira*, a library of transposon mutants can be generated by introducing the transposon on a mobilizable plasmid by conjugation as described by Slamti *et al*[15]. However, the frequency of transposon mutagenesis of *L. interrogans* is low. When the *Himar1* transposon was introduced on a conjugative plasmid, the transconjugant frequency was reported to be only 8.5×10^{-8} per recipient cell with the Lai strain of *L. interrogans*[16] and is likely to be similarly poor with most other strains of *L. interrogans*. The protocol described here is in part based on that developed for *Borrelia burgdorferi*, in which the frequency of transposon insertional mutagenesis is also low[6].

For our pilot experiment with the protocol[17], we conducted transposon mutagenesis with *L. interrogans* serovar Manilae strain L495 because of the success of other groups in isolating transposon insertion mutants in the strain along with its low LD_{50} (lethal dose) for virulence[1]. We screened 42 mutants by Tn-Seq and identified several mutant candidates defective in virulence, including two with insertions in a candidate adenylate cyclase gene. Individual testing of the two mutants in hamsters confirmed that they were deficient in virulence[17].

Protocol

CAUTION: Pathogenic strains of *Leptospira* spp. must be handled under Biosafety Level 2 (BSL-2) containment procedures. Appropriate personal protective equipment (PPE) must be worn. A Class II biosafety cabinet must be used for all manipulations of pathogenic *Leptospira* spp.

1. Creation of the Transposon Mutant Library[15]

1. **Transfer of the transposon into *Leptospira* spp. by conjugation (Figure 1)**
    1. Inoculate a volume of exponential-phase culture of pathogenic *Leptospira* spp. corresponding to 10^7 cells into 10 mL of Ellinghausen-McCullough-Johnson-Harris medium (EMJH)[18,19]. Incubate at 30 °C with 150 rpm shaking until the density reaches $2 - 8 \times 10^8$ cells/mL. NOTE: The doubling time of pathogenic leptospires is 12 to 24 h, depending on the strain.
    2. Inoculate 50 µL of the donor *Escherichia coli* strain β2163[20] carrying the mobilizable transposon plasmid (pCjTKS2)[16] into 5 mL of Luria Broth (LB) supplemented with 0.3 mM of 2,6-diaminopimelicacid (DAP), 50 µg/mL of kanamycin (Km), and 50 µg/mL of spectinomycin (Spc) and place overnight in a 37 °C incubator at 255 rpm.
    3. Inoculate 60 µL of *E. coli* cells into 3 mL of EMJH supplemented with 0.3 mM of DAP (EMJH+DAP). Incubate at 37 °C at 255 rpm agitation for 3 - 4 h up to an $OD_{600nm} \approx 0.3$.
    4. To assemble the filtration unit (**Figure 1**), place the base on a 125 mL side-arm Erlenmeyer flask, position an acetate-cellulose filter (pore size 0.1 mm; diameter 25 mm) on the base, and clamp the funnel onto the base. Connect the filtration unit to a vacuum system.
    5. Add 5 mL of *Leptospira spp.* culture and 0.5 mL of *E. coli* culture into the funnel. Vacuum the liquid through the filter.
    6. Transfer the filter with the surface of bacteria facing up onto an EMJH+DAPplate. Incubate at 30 °C overnight with the filter facing up.
    7. Place the filter into a 15 mL tube containing 1 mL of EMJH and vortex for 10 s to release the bacteria into the media. Spread 200 µL of the suspension onto 5 EMJH platescontaining 50 µg/mL of Km using 10-15 1 mm sterile glass beads or a sterile disposable spreader. Wrap the plates with parafilm and incubate them upside down at 30 °C for 3 to 4 weeks until colonies are visible.
    8. Transfer colonies individually into 3 mL of EMJH containing 50 µg/mL of Km (EMJH+Km) at 30°C under 150 rpm agitation for 7 to 10 days until the culture reaches a density of $\approx 10^8$/mL. NOTE: Cultures can be stored at -80 °C or in liquid nitrogen (with 4% glycerol).
2. **Identification of the transposon insertion site by nested PCR (Figure 2)**
    1. Lyse 50 µL of each transposon mutant in PCR tubes by incubation at 95 °C for 15 min. NOTE: DNA can be purified instead, using a DNA extraction kit.
    2. Prepare the PCR mix with Deg1 and Tnk1 primers (**Table 3**) according to **Table 1**. Transfer 23.7 µL of the mix to each PCR tube and add 1.3 µL of lysed cells. Run the program: 95°C for 5 min; 40 cycles: 95 °C for 15 s, 40 °C for 1 min, 72 °C for 2 min; 72 °C for 10 min.
    3. Make the PCR mix with Tag and TnkN1 primers (**Table 3**) according to **Table 2**. Transfer 24.2 µL of the mix to each PCR tube and add 0.8 µL of PCR #1 reaction. Run the program: 95 °C for 5 min; 35 cycles: 95 °C for 15 s, 55 °C for 30 s, 72 °C for 2 min; 72 °C for 10 min.
    4. Run 3 µL of PCR products on a 1% agarose gel with 1X Tris-Acetate-EDTA (TAE) buffer at 10-15 V/cm (**Figure 2B**).
    5. Purify PCR products of positive samples using a PCR purification kit. Elute DNA with the lowest volume allowed by the kit to maximize the concentration of DNA recovered.
    6. Send purified PCR products for Sanger sequencing using the TnkN1 primer (**Table 3**).
    7. Identify the insertion sites by comparing the resulting sequence with the parental strain's genome sequence by BLASTN analysis (http://blast.ncbi.nlm.nih.gov/) or using the SpiroScope database (http://www.genoscope.cns.fr/agc/mage)[21].
    8. Confirm the insertion site of the transposon by PCR using primers annealing to the flanking host sequences. NOTE: The transposon increases the size of the wild-type sequence by ≈ 2 kb.

2. Animal Experiment (Figure 3)

1. **Culture of *Leptospira* mutants**
    1. Grow individually each selected transposon mutant in 10 mL of EMJH+Km at 30 °C at 150 rpm agitation to a density of 10^7-10^8 leptospires/mL.

2. Count the leptospires by dark-field microscopy with a Petroff-Hausser counter or as described by Miller[23].
3. Dilute each culture in EMJH to the same density, for instance 10^6 cells/mL.
4. To assemble the input pool, mix together the diluted cultures in equal volumes.
   NOTE: Include controls in the input pool by adding mutants with known fitness defects such as *loa22*[17,24] and mutants with unaltered fitness such as *ligB*[17,25], respectively. Pools of mutants can be stored with 4% glycerol at -80 °C or in liquid nitrogen.

2. **Challenge**
   NOTE: The methods described here were approved by the Veterans Affairs Greater Los Angeles Institutional Animal Care and Use Committee (protocol #09018-14).
   1. Inject intraperitoneally 1 mL of the input pool to each animal with an insulin syringe U-100 with 26G x ½" needle.
      NOTE: In the pilot experiment, 8 animals were challenged with 1 mL of the input pool, i.e., 10^6 bacteria total[17]. The infection was allowed to proceed for 4 days prior to euthanasia.
   2. Collect 10 mL of the input pool, and spin for 20 min at 3,220 x g. Carefully remove the supernatant without disturbing the pellet. Store the cell pellet at -80˚C until use (step 3.1.3).
   3. Monitor the animals daily until they are terminated at the pre-determined endpoint. Weigh the hamsters daily and look for endpoint criteria: loss of appetite, gait or breathing difficulty, prostration, ruffled fur, or loss of > 10% of the maximum weight attained.

3. *In vitro* **experiment**
   1. On the day of challenge, inoculate 3 flasks of 25 mL of EMJH+Km with 5 mL of the input pool. Grow cultures at 30 °C under 150 rpm agitation.
   2. Count the leptospires daily by using one of the methods of section 2.1.2. When the density reaches ≈ 1 x 10^8/mL, spin down each suspension for 20 min at 3,220 x g.
   3. Store the cell pellets at -80 °C until use.

4. **Harvesting and storage of the tissues**
   1. Euthanize the animals by isoflurane inhalation followed by bilateral thoracotomy[26].
   2. Immediately collect 1 to 2 mL of blood by cardiac puncture with a 3 mL syringe and a 25G x 5/8" needle. Transfer the blood into tube containing EDTA. Mix by inversion, 5 to 6 times.
   3. Collect one kidney and ⅓ to ½ of the ventral median lobe of the liver into cryotubes.
   4. Store tissues at -80 °C until use.

3. Construction of Genomic Libraries for High-throughput Sequencing (Figure 4)

1. **DNA extraction**
   1. DNA extraction from blood.
      1. Transfer 100 µL of blood from the EDTA tube to a microcentrifuge tube.
      2. Purify DNA using a DNA extraction kit. Follow the manufacturer's instructions.

   2. DNA extraction from tissues.
      1. Using scalpels and scissors, dice between 50 to 80 mg of each organ into small pieces (1 mm x 1 mm), and transfer them into a sterile screw-cap dry tube. Measure the weight of tissue with a precision balance.
      2. Add 500 µL of sterile PBS into the tube.
      3. Homogenize samples by using the disruptor for 1 min at 5 movements per second.
      4. Calculate the volume corresponding to 25 mg of tissue using the following equation:

$$Volume\ (in\ ml) = \frac{(25 \times 10^{-3})\ x\ (500 \times 10^{-3})}{Weight\ of\ tissue\ (in\ g)}$$

      5. Transfer the calculated volume into the DNA extraction kit. Proceed with DNA purification following the manufacturer's instructions.

   3. DNA extraction from the input pool and *in vitro* cultures.
      1. Thaw bacterial pellets at room temperature for 5-10 min.
      2. Proceed with DNA extraction following the instructions accompanying the kit.

   4. Store DNA at -80 °C until use.

2. **Shearing the DNA (Figure 5)**
   1. Transfer 50 µL of extracted DNA onto a 1.5 mL microcentrifuge tube.
   2. Place the tubes into the rack of the sonicator cup horn filled with cold water (4 °C).
   3. Run the sonicator for 3 min at 80% intensity with 10 s on pulse and 5 s off pulse.
      CAUTION: Wear ear muffs or ear plugs to protect hearing.
   4. Run 2.5 µL of the sheared DNA on a 2% agarose gel to confirm that most of the DNA is <600 bp in size.

3. **Addition of the C-tail**
   1. Measure DNA concentration with a small volume spectrophotometer.
   2. Calculate volume corresponding to 500 ng of DNA by using the following equation:

$$Volume\ (in\ \mu l) = \frac{500}{DNA\ concentration\ (in\ ng/\mu l)}$$

3. Prepare tailing reaction (**Table 4**). Incubate 1 h at 37 °C; inactivate at 75 °C for 20 min.
   NOTE: For samples that require adjustment to a volume greater than 14.5 µL, increase the final volume of reaction to 40 µL and scale up the remaining components accordingly.
4. Clean the samples with a PCR purification kit. Elute the DNA with 12 µL of elution buffer.

4. **Nested PCR**
   1. PCR #1
      1. Prepare PCR mixes according to **Tables 3** and **5**. Transfer 22 µL of the library mix to each PCR tube and add 3 µL of purified DNA. Proceed similarly with the control mix.
         NOTE: Primer TnkN3[17] is specific to the transposon and primer olj376[6] is specific to the C-tail. The control mix is lacking the TnkN3 primer, which specifically targets the transposon.
      2. Run the following program: 95 °C for 2 min; 24 cycles: 95 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min; 72 °C for 2 min.

   2. PCR #2.
      1. Prepare the second PCR mixes according to **Tables 3** and **6**. Transfer 49 µL of the library mix to each PCR tube and add 1 µL of PCR #1 library reaction. Transfer 24.5 µL of the control mix to each PCR tube and add 0.5 µL of PCR #1 control reaction.
         NOTE: Primer pMargent2 is specific to the transposon, and the IP primers[6] contain six-base-pair barcode and specific sequences recognized by the next-generation sequencing platform.
      2. Run the following program: 95 °C for 2 min; 18 cycles: 95 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min; 72 °C for 2 min.

   3. Run 3 µL on a 2% agarose gel. The library should show a smear with the majority of the signal between 200 to 600 bp (**Figure 6**) and no amplification for the control reaction.

5. **Purification of PCR products.**
   NOTE: Clean the genomic libraries with a PCR purification kit following the manufacturer's instructions. Elute the DNA with 30 µL of elution buffer.
6. Measure the DNA concentration using a fluorometer. Average 2 to 3 reads.
7. Calculate the volume of each library equivalent to 15 or 20 ng using the equation below:

$$Volume\ (in\ \mu l) = \frac{DNA\ concentration\ of\ the\ library\ (ng/\mu l)}{(15\ or\ 20)}$$

8. **Mix all libraries together according to the previous calculations.** Determine the molar concentration of the DNA with the following website's equation:
   http://www.molbiol.edu.ru/eng/scripts/01_07.html
   CAUTION: DNA concentration and volume requirements depend on the sequencing platform.

4. High-throughput Sequencing and Data Analysis

1. **Sequencing**
   1. Sequence libraries as 64 bp single-end reads using the custom sequencing primer pMargent3 and the standard commercial sequencing primer. The sequencing platform will provide you FastQ files with all sequencing reads.

2. **Analysis with Galaxy software**
   1. Download the genome sequence file
      1. In the SpiroScope database homepage (see step 1.2.7 for link), select the organism used for the experiment and click "LOAD INTO GENOME BROWSER".
      2. In the toolbar (near the top of the homepage), select "Search/Export > Download data", in the "Sequence (fasta)" line, click "Genome" to download the sequence.
      3. Open the file with Notepad (PC) or TextEdit (Mac) and rename the chromosome (for example "ChrI"). Maintain the fasta format.
      4. Follow the same steps to download the sequence of chromosome II. Combine both chromosome sequences into a single .txt file by copying and pasting.
         NOTE: Chromosome sequences can also be downloaded from the NCBI website (https://www.ncbi.nlm.nih.gov) and combined into a single .txt file.

   2. Upload files onto the Galaxy server
      NOTE: Galaxy is an open source, web-based platform for managing data intensive bioinformatics workflows[27,28,29,30] and can be accessed at https://usegalaxy.org/.
      1. In the Tools menu, select "Get data > Upload File from your computer". Drag and drop the .fastq file(s) generated by the sequencing platform into the window then click "Start".
      2. Follow the same steps to upload the *Leptospira* genome sequence .txt file.

   3. Groom reads
      1. Select "NGS: QC and manipulation > FASTQ Groomer" from the Tools menu.
      2. Next to File to groom, select the libraries uploaded in step 4.2.2. In Input FASTQ quality scores type, select the appropriate sequencing system. For Advanced Options, leave Hide Advanced Office selected.
      3. Click "Execute".

   4. Remove sequencing artifacts

1. Select "NGS: QC and manipulation > Remove sequencing artifacts". Next to Library to filter, select the groomed files generated in step 4.2.3. Click "Execute".

5. Remove C-tail sequences
   NOTE: Repeat these steps once or twice to ensure all C-tails are removed.
   1. Select "NGS: QC and manipulation > Clip adapter sequences".
   2. Select or enter the following:
      Library to clip: select the files generated in step 4.2.4.
      Minimum sequence length: 15
      Source: enter custom sequence
      Enter custom clipping sequence: CCCCCCC
      Enter non-zero value to keep the adapter sequences and x bases that follow it: 0
      Discard sequences with unknown (N) bases: Yes
      Output options: Output both clipped and non-clipped sequences
   3. Click "Execute".

6. Remove adaptor sequences
   1. Select "NGS: QC and manipulation > Clip adapter sequences".
   2. Select or enter the following:
   3. Library to clip: select files generated in step 4.2.5.
   4. Minimum sequence length: 15
   5. Source: enter custom sequence
   6. Enter custom clipping sequence: CGTATGCCGTCTTCTGCTTG
   7. Enter non-zero value to keep the adapter sequences and x bases that follow it: 0
   8. Discard sequences with unknown (N) bases: yes
   9. Output options: Output both clipped and non-clipped sequences
   10. Click "Execute".

7. Filter reads based on their quality
   1. Select "NGS: QC and manipulation > Filter by quality".
      NOTE: This tool selects reads based on quality scores.
   2. Select the following:
      Library to filter: select the files generated in step 4.2.6.
      Quality cut-off value: 20
      Percent of bases in sequence that must have quality equal to/higher than cut-off value: 95
   3. Click "Execute".
      NOTE: With these settings, reads shorter than 20 nucleotides or with a quality score of 20 or less for 95% of the cycles are discarded. Adapt stringency settings to your experiment.

8. Map reads[32]
   1. Select "NGS: Mapping > Bowtie2"[32].
   2. Select the following in the fields in the main window:
      Is this single or paired library: single-end
      FASTQ file: select the library filtered for quality from step 4.2.7.
      Write unaligned (in fastq format) reads to separate file(s): no
      Write aligned (in fastq format) reads to separate file(s): no
      Will you select a reference genome from your history or use a built-in index?: Use a genome from the history and build index
      Select the reference genome: select the Leptospira genome.txt file uploaded in step 4.2.2.
      Set read groups information?: do not set
      Select analysis mode: 1: default setting only
      Do you want to use presets?: No, just use defaults
      Save the bowtie2 mapping statistics to the history: No
      Job resource parameters: Use default job resource parameters
   3. Click "Execute" to align reads to the genome.

9. Convert files
   1. Select "NGS: SAMtools > BAM-to-SAM convert BAM to SAM".
   2. Select the following:
      BAM file to convert: Select mapped library from step 4.2.8.
      Header options: include header in SAM output (-h)
   3. Click "Execute".

10. Convert files
    1. Select "NGS: SAMtools > Convert SAM to interval".
    2. Select the following:
       Select dataset to convert: Select the SAM mapped library file generated in step 4.2.9.
       Print all?: Yes
    3. Click "Execute".

11. Sort reads
    1. Select "Filter and Sort > Sort data in ascending or descending order".

    2. Select the following:
       Sort dataset: Select the interval file generated in step 4.2.10.
       on column: 2
       with flavor: Numerical order
       everything in: Ascending order
    3. Click "Execute".

12. Select reads matching chromosome I
    1. Select "Filter and Sort > Select lines that match an expression".
    2. Select the following:
       Select lines from: select file with sorted reads generated in step 4.2.11.
       that: Matching
       the pattern: Enter the name of chromosome I as determined in step 4.2.1.3 (e.g., "ChrI").
    3. Click "Execute".

13. Select reads matching chromosome II
    Proceed following step 4.2.12.
14. Group reads according to the insertions sites in chromosome I
    1. Select "Join, Subtract and Group > Group data by a column and perform aggregate operation on other columns".
    2. Select the following:
       Select data: select resulting file from step 4.2.12.
       Group by column: 2
       Ignore case while grouping?: No
       Ignore lines beginning with these characters: Ø
       Operation > + insert operation
       Type: Count
       On column: 2
       Round result to nearest integer: NO
    3. Click "Execute".

15. Group reads according to the insertion sites in chromosome II
    Proceed following step 4.2.14.
16. Sort insertion sites on chromosome I
    1. Select "Filter and Sort > Sort data in ascending or descending order".
    2. Select the following:
       Sort Dataset: Select file from step 4.2.14.
       on column: 1
       with flavor: Numerical order
       everything in: Ascending order
    3. Click "Execute".

17. Sort insertion sites on chromosome II
    Proceed following step 4.2.16.

3. **Statistical analysis**
   1. Transfer the Galaxy data into spreadsheet files by copying and pasting the two columns from steps 4.2.16. and 4.2.17. into an Excel file.
    NOTE: The first column is the nucleotide coordinate of the transposon insertion site, and the second column is the number of reads at each insertion site.
   2. Identify the gene harboring the transposon using the nucleotide coordinate provided in the table.
    NOTE: For example, a transposon inserted at nucleotide #30718 of chromosome I is located in the *LIC10024* gene, which spans nucleotides 29263-31539 in chromosome I.
   3. Calculate relative frequencies (*F*) for each mutant in each tissue and in the input pool following the equation below:

$$F = \frac{number\ of\ reads\ at\ an\ insertion\ site\ (= mutant)}{total\ number\ of\ reads\ mapped\ in\ this\ genomic\ library} * 100$$

   4. Calculate output/input ratios (R) for each mutant and tissue using the equation below:

$$R = \frac{relative\ frequency\ in\ a\ tissue}{relative\ frequency\ in\ the\ input\ pool}$$

   5. Wilcoxon signed-rank test
    1. Normalize all output/input ratios by setting the median ratio for each tissue in each animal to 1.0. A ratio of 1.0 is neutral, >1.0 is disadvantageous, and <1.0 is advantageous[33].
    2. Compare output/input ratios to 1.0 (neutral fitness) by using the Wilcoxon rank test with P values <0.05 considered statistically significant.

Representative Results

Creation of a library of transposon mutants in *L. interrogans* by conjugation requires a filtration unit, as shown in **Figure 1**. We recovered 100-200 transconjugants from each mating.

The transposon insertion site is identified in each mutant by sequencing the PCR product generated by semi-random PCR that targets the end of the transposon and adjacent host sequences[15] (**Figure 2A**). An example of results of the semi-random PCR is shown in **Figure 2B**. In most cases a dominant amplicon will be observed when the Deg1 and Tnk1 primers are used for the first round of PCR and the Tag and TnkN1 for the second round. However, due to the low specificity of the pentameric sequence (…N_{10}GATAT-3') at the 3' end of the Deg1 primer, this primer will anneal at multiple locations throughout the leptospiral genome. If these sites are present near the transposon end, multiple PCR products may be obtained. When multiple amplicons are generated, they can be purified together from the PCR mix and sequenced directly; gel purification of each amplicon is not necessary because they will all contain the same sequence downstream of where TnkN1 primer anneals. On the other hand, PCR may fail if the nearest copy of the sequence targeted by the Deg1 primer is located at a great distance from the transposon end. If no PCR products are detected, the first round of semi-random PCR can be repeated with the Deg1 primer and Tnk2 primer, which targets the opposite end of the transposon. In this case TnkN2 and Tag would be used for the second round; the amplicon would be sequenced with the TnkN2 primer (**Figure 2B**). Alternatively, the first round of PCR can be done with the Deg2 primer, whose 3' end (…N_{10}TCTT-3') targets a four- rather than a five-nucleotide sequence. Four different sets of primers can be used for the first round of PCR (PCR #1): Tnk1+Deg1, Tnk1+Deg2, Tnk2+Deg1, Tnk2+Deg2. When one set of primers does not work, use another set. If this second set also fails, use another one and so on. Spontaneous kanamycin resistant mutants are very rare, and arise at a frequency of <10^{-10}[34].

During the preparation of genomic libraries (**Figure 4**), a couple of steps can be verified. Shearing the DNA by sonication should be confirmed by electrophoresis of an aliquot in an agarose gel (**Figure 5**). When DNA is sheared correctly, DNA fragments ranging from 200 to 600 bp will form a smear in a 2% agarose gel. Before sending libraries for high-throughput sequencing, the generation of PCR products by nested PCR reactions must to be confirmed by agarose gel electrophoresis (**Figure 6**).

After processing the sequencing reads following the protocol described here (section 4.2.), the frequency of each mutant in the input pool and in all tissues is determined using the equation in section 4.3.3. The output/input ratio for each mutant in each tissue is calculated with the equation in section 4.3.4. **Figure 7** shows an example of results obtained with the Tn-Seq approach. Alternatively, the MAGenTA tool can be used to process and analyze the sequencing data [31]. Mutants with statistically significantly reduced and increased fitness were identified. Mutations in known leptospiral virulence genes caused reduced fitness, validating the Tn-Seq approach.
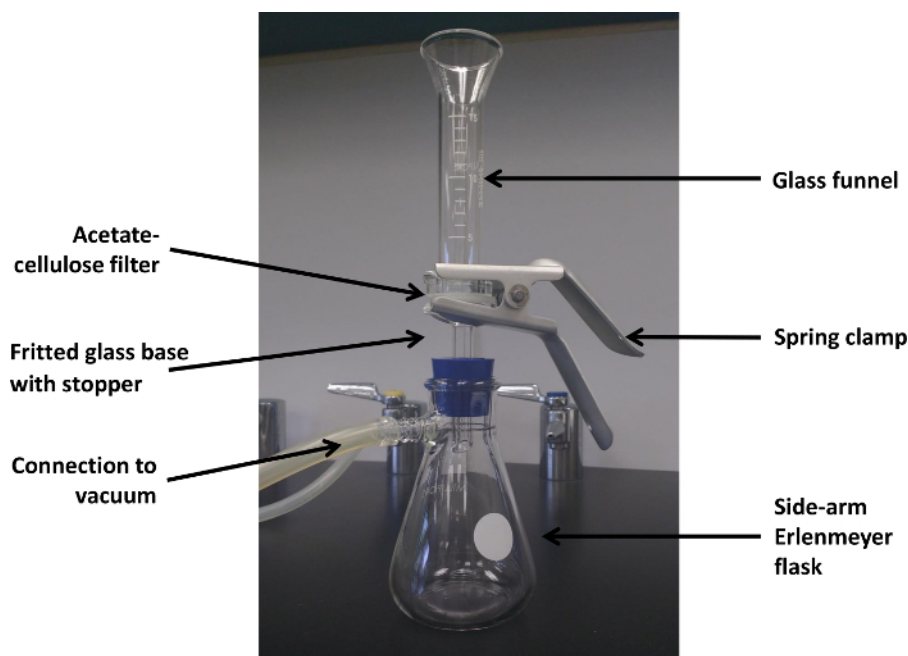

Figure 1: Filtration unit used for conjugation. The filtration unit is assembled by inserting the stopper of the filter support into a side-arm Erlenmeyer flask, positioning the acetate-cellulose filter onto the base with sterile forceps, then placing the funnel onto the base, and securing the system with a clamp. The filtration unit is then connected to the vacuum system. Please click here to view a larger version of this figure.
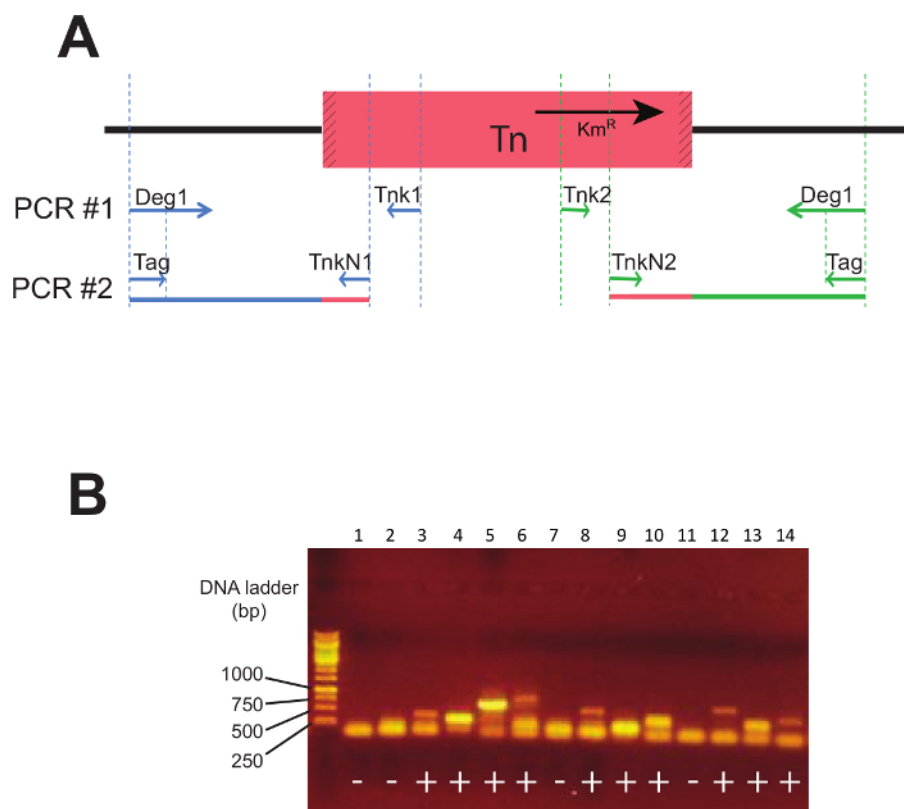
Figure 2: Identification of the transposon insertion sites. (**A**) Scheme showing annealing sites of semi-random PCR primers. Tnk1 and Tnk2 primers anneal close to the opposite ends of the transposon (Tn). Deg1 is a 35-nucleotide primer that contains a stretch of 10 degenerate nucleotides followed by the sequence GATAT at the 3' end. The first round of PCR (PCR #1) is conducted with Deg1 and Tnk1 or with Deg1 and Tnk2. TnkN1 and TnkN2 primers, used for PCR #2, anneal between the transposon ends and Tnk1 and Tnk2, respectively. The sequence of the Tag primer is identical to that of the 5' end of the Deg1 primer. (**B**) Example of agarose gel obtained after the second round of PCR with 14 transposon mutants (lanes 1 to 14). The first round of PCR was performed with Deg1 and Tnk1; the second round was done with Tag and TnkN1. Positive PCR is represented by "+" and a negative PCR by "-". "Km^R" represents the kanamycin resistance cassette. Please click here to view a larger version of this figure.
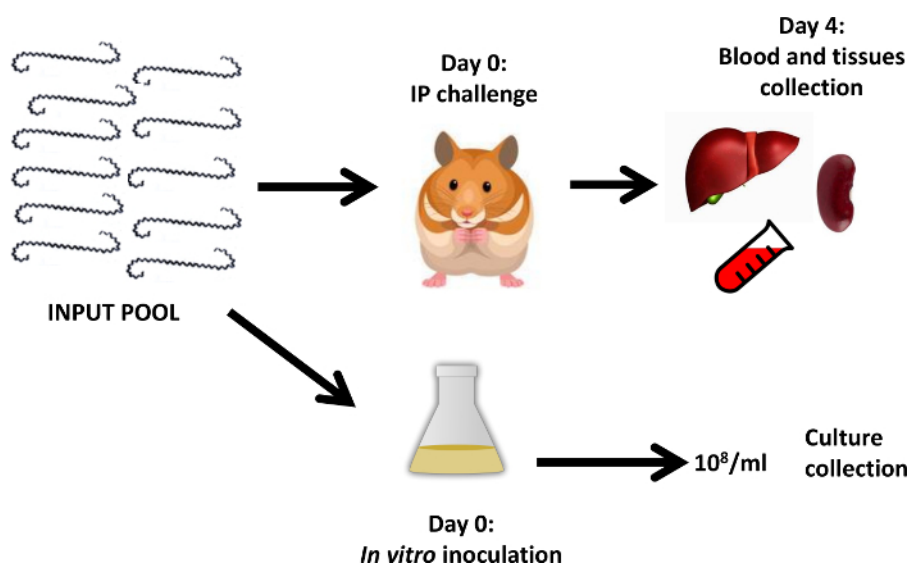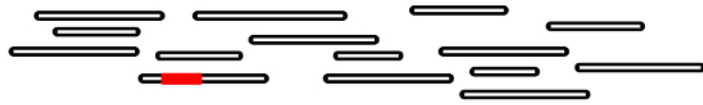

Figure 3: Flow chart for obtaining the output pool. On the day of challenge (day 0), each leptospiral mutant is counted by darkfield microscopy, diluted to the same density, and combined to form the input pool (details provided in section 2.1). Hamsters are challenged intraperitoneally with the input pool. In addition, three cultures were started with the input pool. On the day of inoculation (day 0) and when the cultures reach a density of $\approx 10^8$/mL, the input pool and cultures are collected by centrifugation and stored as pellets at -80 °C until use (see section 2.3). On the endpoint day (day preselected to terminate the animals, *e.g.*, day 4 after challenge), animals are euthanized; blood, kidney, and liver are collected and stored at -80 °C until use (see section 2.4). Please click here to view a larger version of this figure.

Figure 4: Flow chart of the genomic library preparation. (**A**) DNA is extracted from blood, tissues or cultures following the protocol described in section 3.1. The red box represents the transposon (Tn). (**B**) The DNA is sheared by sonication into fragments between 200 and 600 bp. Further details are provided in section 3.2. (**C**) C-tails (yellow boxes) are added to all DNA fragments with terminal deoxynucleotidyl transferase (TdT) as described in section 3.3 and Table 4. The genomic library is prepared for sequencing by nested PCR (**D-E**). The first round of PCR is performed with TnkN3 and olj376 primers specific to the transposon and the C-tail, respectively. See Table 3 and 5. Only fragments containing the transposon ends (red box) are amplified. Note: Use 3 times more olj376 primer than TnKN3 because all DNA fragments have C-tails. The second round of PCR is conducted with pMargent2, specific for the transposon end, and one indexing primer, containing a six-base-pair barcode sequence (in pink) allowing all samples to be multiplexed in a single sequencing lane. See Table 3 and 6. Both primers contain sequences necessary for binding the flow-cell during Illumina sequencing (green and purple box). (**F**) The resulting PCR products are cleaned, and then their concentration is measured as described in section 3.6. (**G**) All genomic libraries are pooled together; each library has a different barcode and (**H**) the pool is sent to the sequencing platform. Please click here to view a larger version of this figure.
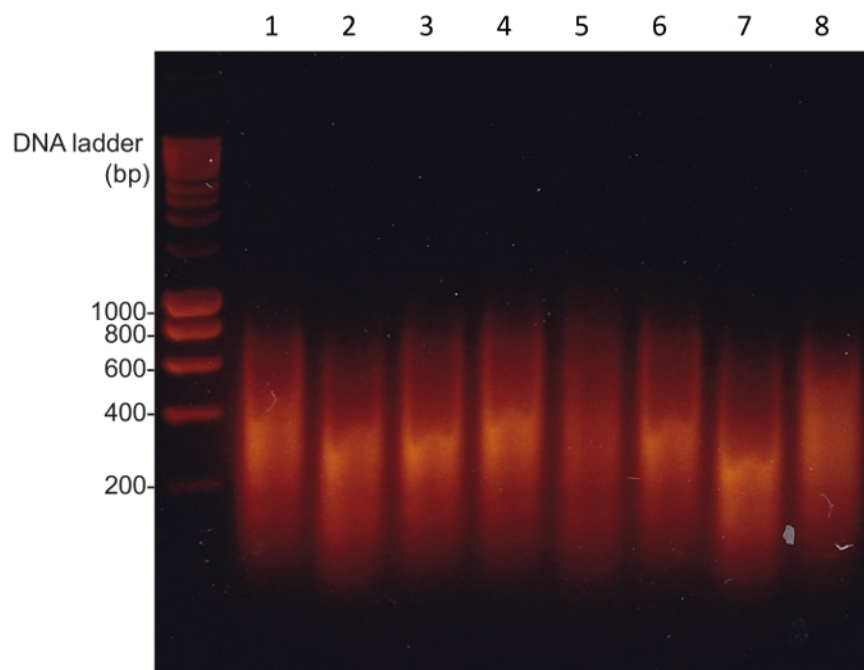
Figure 5: Sonication of DNA. DNA was purified from the livers of eight infected hamster (lanes 1 to 8), sonicated, and examined by electrophoresis in a 2% agarose gel. Sheared DNA is characterized by a smear in which the size of most of the fragments is between 200 and 600 bp. Please click here to view a larger version of this figure.
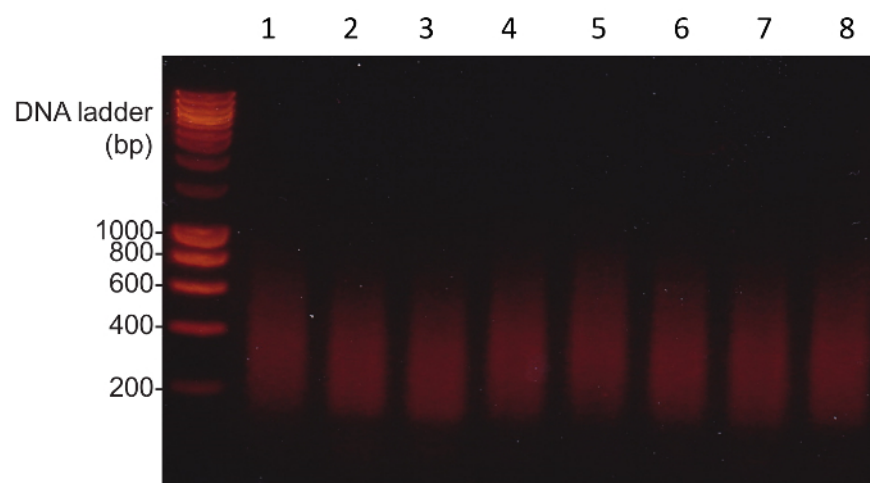
Figure 6: Genomic libraries for high-throughput sequencing. Genomic libraries were prepared by ested PCR with DNA from the blood of eight infected hamsters (lanes 1 to 8) and examined by electrophoresis in a 2% agarose gel. The size of most of the PCR products is between 200 and 600bp. Negative control (no TnkN3 primer in PCR #1 mix, see Table 5) shows no amplification (not shown). Please click here to view a larger version of this figure.
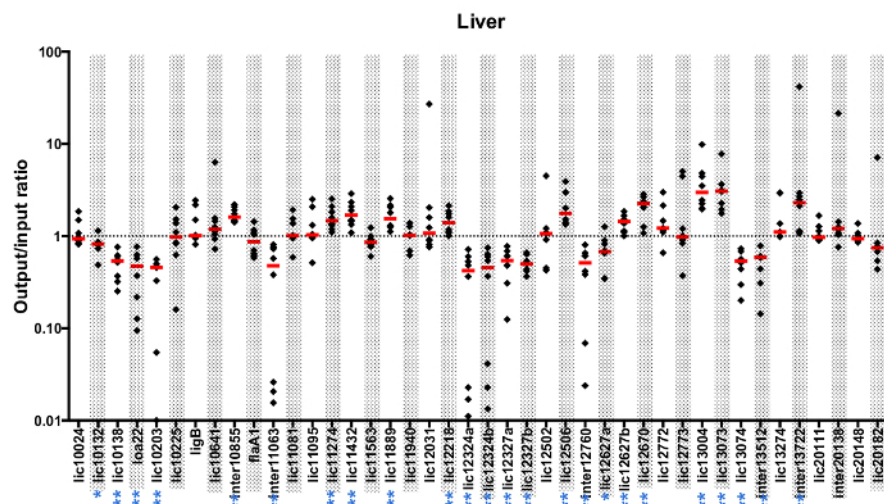
Adapted from Lourdault et al. (17)

Figure 7: Fitness of mutants from Tn-Seq experiment. Fitness of a pool of mutants in hamster's kidney 4 days after challenge (adapted from Lourdault's study[17]). The output/input ratio of all 42 mutants was determined for each animal. Each mutant is named by the gene (lic) or the intergenic region (inter) the transposon is inserted into or the name commonly used in the literature. Each ratio is represented by a black diamond. The median of ratios (red line) was determined for each mutant and compared to 1.0 using the Wilcoxon rank test. The dotted line represents fitness of 1.0, which corresponds to neutral fitness. Mutants whose fitness is significantly affected are marked by asterisks: * P <0.05; ** P <0.01. Please click here to view a larger version of this figure.

Reagents	Volume for one reaction
Master mix 2X	12.5 µL
Primer 1, 10 mM (TnK1 or TnK2)*	1.2 µL
Primer 2, 10 mM (Deg1 or Deg2)*	1.2 µL
Water	8.8 µL
Template (cells lysate or DNA)	1.3 µL
Final volume	*25 µL*
* Primer sequences in Table 3.	

Table 1: Identification of the transposon insertion site, semi-random PCR #1 mix.

Reagents	Volume for one reaction
Master mix 2X	12.5 µL
Primer 1, 100 mM (TnKN1 or TnKN2)*	0.2 µL
Primer 2, 100 mM (Tag)*	0.2 µL
Water	10.1 µL
PCR products from PCR#1	0.8 µL
Final volume	*25 µL*
* Primer sequences in Table 3.	

Table 2: Identification of the transposon insertion site, semi-random PCR #2 mix.

Name	Sequence (5'-3')	Reference
Primers used for semi-random PCR		
Tnk1	CTTGTCATCGTCATCCTTG	15
Tnk2	GTGGCTTTATTGATCTTGGG	
Deg1	GGCCACGCGTCGACTAGTAC**NNNNNNNNNNN**GATAT	
Deg2	GGCCACGCGTCGACTAGTAC**NNNNNNNNNNN**TCTT	
TnkN1	CGTCATGGTCTTTGTAGTCTATGG	
TnKN2	TGGGGATCAAGCCTGATTGGG	
Tag	GGCCACGCGTCGACTAGTAC	
Primers used for Illumina sequencing		
TnkN3	CGGGGAAGAACAGTATGTCGAGCTATTTTTTGACTTACTGGGGATCAAGCCTGATTGGG	17
olj376	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGGGGGGGGGGGGGGGG	6
pMargent2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCGGGGACTTATCAGCCAACCTGTTA	
IP 1	CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	Illumina sequencing (barcodes in bold)
IP 2	CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 3	CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 4	CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 5	CAAGCAGAAGACGGCATACGAGAT**CACTGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 6	CAAGCAGAAGACGGCATACGAGAT**ATTGGC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 7	CAAGCAGAAGACGGCATACGAGAT**GATCTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 8	CAAGCAGAAGACGGCATACGAGAT**TCAAGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 9	CAAGCAGAAGACGGCATACGAGAT**CTGATC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 10	CAAGCAGAAGACGGCATACGAGAT**AAGCTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 11	CAAGCAGAAGACGGCATACGAGAT**GTAGCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 12	CAAGCAGAAGACGGCATACGAGAT**TACAAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 13	CAAGCAGAAGACGGCATACGAGAT**TTGACT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 14	CAAGCAGAAGACGGCATACGAGAT**GGAACT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 15	CAAGCAGAAGACGGCATACGAGAT**TGACAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 16	CAAGCAGAAGACGGCATACGAGAT**GGACGG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 17	CAAGCAGAAGACGGCATACGAGAT**CTCTAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 18	CAAGCAGAAGACGGCATACGAGAT**GCGGAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 19	CAAGCAGAAGACGGCATACGAGAT**TTTCAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 20	CAAGCAGAAGACGGCATACGAGAT**GGCCAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 21	CAAGCAGAAGACGGCATACGAGAT**CGAAAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 22	CAAGCAGAAGACGGCATACGAGAT**CGTACG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 23	CAAGCAGAAGACGGCATACGAGAT**CCACTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 24	CAAGCAGAAGACGGCATACGAGAT**GCTACC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 25	CAAGCAGAAGACGGCATACGAGAT**ATCAGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 26	CAAGCAGAAGACGGCATACGAGAT**GCTCAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 27	CAAGCAGAAGACGGCATACGAGAT**AGGAAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 28	CAAGCAGAAGACGGCATACGAGAT**CTTTTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP 29	CAAGCAGAAGACGGCATACGAGAT**TAGTTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	
IP seq	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC	
pMargent3	ACACTCTTTCCGGGGACTTATCAGCCAACCTGTTA	6

Table 3: Primer sequences.

Reagents	Volume for one reaction
Sheared DNA	500 ng (see equation in 3.3.2.)
Water	Up to 14.5 µL
5x TdT buffer	4 µL
(9.5 mM dCTP + 0.5 mM ddCTP) mix*	1 µL
TdT (30 U/µL)	0.5 µL
Final volume	*20 µL*
* Mix 3 µLof 10 mM ddCTP, 5.7 µL of 100 mM dCTP and 51.3 µL of water	

Table 4: C-tailing reaction with terminal deoxynucleotidyl transferase (TdT).

Reagents	Volume for one reaction	
	Library reaction	Control reaction
Master mix 2x	12.5 µL	12.5 µL
Primer 1, 30 µM (TnkN3)*	0.5 µL	-
Primer 2, 30 µM (olj376)*	1.5 µL	1.5
Water	7.5 µL	8 µL
C-tailed DNA	3 µL	3 µL
Final volume	*25 µL*	*25 µL*
* Primer sequences in Table 3.		

Table 5: Construction of genomic library, PCR #1 mix.

Reagents	Volume for one reaction	
	Library reaction	Control reaction
Master mix 2x	25 µL	12.5 µL
Primer 1, 30 µM (pMargent2)*	0.5 µL	0.25 µL
Primer 2, 30 µM (indexing primer)*	0.5 µL	0.25 µL
Water	23 µL	11.5 µL
PCR products from PCR#1	1 µL	0.5 µL
Final volume	*50 µL*	*25 µL*
* Primer sequences in Table 3.		

Table 6: Construction of genomic library, PCR #2 mix.

Discussion

Although results from our pilot experiment for hamster challenged intraperitoneally with 42 *L. interrogans* mutants are presented[17], we expect that larger pools of mutants can be screened by Tn-Seq. Because the frequency of transconjugants is low (100-200 transconjugants/mating), several matings are necessary to generate a sufficient number of mutants for large Tn-Seq experiments. Maintaining a large number of mutants in liquid cultures presents logistical challenges that must be addressed. Cultures can be incubated in deep 96-well plates. Culture densities can be monitored by optical density readings in a spectrophotometer set to 420 nm. The determination of the number of animals to use depends in part on the size of the input pool and must be determined by power analysis. For large-scale experiments, we recommend that the power analysis be performed in consultation with a biostatistician.

Bottlenecks can impact the recovery of random mutants from the output pool. Although severe bottlenecks were not observed in our pilot study[17] (**Figure 7**), large-scale experiments may be affected by random loss of mutants. Increasing the challenge dose by increasing the number of cells per mutant in the input pool will minimize the likelihood of bottlenecks[35]. The newly published Galaxy tool MAGenTA provides the necessary tool to assess bottlenecks and fitness[31].

Tn-Seq experiments planned for an alternative route of infection, other *Leptospira* strains, or other rodent models require additional considerations. If the kinetics of infection in the host species is not known or is not continuously exponential during the course of infection within each tissue to be examined, DNA should be obtained from culturing of the tissues rather than directly from the tissues. This additional step will minimize overestimates of mutant fitness due to detection of DNA from dead spirochetes. If the output pool is obtained from culturing the tissues, the input pool should be cultured to address effects of *in vitro* growth. We also recommend a pilot experiment with a small number of mutants to determine whether bottlenecks will be a concern and to aid the power analysis for large-scale experiments.

Confirmation of altered fitness as determined by Tn-Seq will require testing of individual mutants in the animal model. Currently, each mutant must be sequenced individually prior to Tn-Seq to identify the mutant carrying the insertion in the gene of interest. However, if allele replacement in pathogenic *Leptospira* becomes easy, sequencing of individual mutants will no longer be necessary. Alternatively, transcription activator-like effectors have been successfully used to diminish *lig* genes expression in *L. interrogans*[36]. This technique could be used to down-regulate genes disrupted in mutants with altered fitness identified by Tn-Seq.

When interpreting the Tn-Seq data, interactions between bacteria should to be taken into account. It is theoretically possible that a decrease in fitness could be due to competition between mutants rather than a direct effect of the transposon insertion. Additionally, an absence of change in *in vivo* fitness of a mutant in a pool could be due to cooperation between mutants. For example, a mutant that fails to produce an essential factor could be complemented intercellularly by production of the factor by other mutants in the pool. We observed an increase in fitness for several mutants, which may be a consequence of the reduced metabolic burden of no longer synthesizing proteins that are not essential for growth, as shown for *Salmonella enterica*[37].

This protocol can be used to identify genes involved in metabolism or survival under stressful conditions *in vitro* [38,39]. For example, growing *Leptospira* in different conditions like high sodium chloride concentration, limited iron, and acidic pH could identify genes responsible for acidic survival or stress resistance. These *in vitro* experiments can also be performed with saprophytic strains such as *L. biflexa* strain Patoc I because this Tn-Seq method can be applied to all sequenced *Leptospira* strains.

Disclosures

The authors have nothing to disclose.

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