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Creating a library of random transposon mutants in *Leptospira*

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Abstract

Generation of a random transposon mutant library is advantageous in *Leptospira* as site directed mutagenesis remains a challenge, especially in pathogenic species. This procedure is typically completed by transformation of *Leptospira* with a *Himar1* containing plasmid via conjugation with *Escherichia coli* as a donor cell. Here we describe the methodology to generate random transposon mutants in the saprophyte *Leptospira biflexa* via conjugation of plasmid pSW29T-TKS2 harbored in *E. coli* β 2163. Determination of transposon insertion site by semi-random nested PCR will also be described. A similar methodology may be employed to generate Tn mutants of pathogenic *Leptospira* species.

Keywords

Spirochete; *Leptospira*; *Himar1*; Transposon Library; Mutagenesis; Conjugation; Nested PCR

1. Introduction

The genus *Leptospira* belongs to the phylum Spirochaetes, which consists of saprophytic species (e.g.- *L. biflexa*), intermediate species (e.g.- *L. licerasiae*) and pathogenic species (e.g.- *L. interrogans*), of which the latter are primarily responsible for the disease leptospirosis [1-3]. Leptospirosis is an emerging zoonotic disease of global importance, with over 1.7 million cases of severe disease occurring annually [4]. To facilitate our understanding of the biology of *Leptospira*, such as virulence factors, host adaptation mechanisms or its physiology, effective genetic manipulation tools are required [1, 5-9].

Studies of *Leptospira* genes completed in the 1990s were primarily focused on functional complementation of *Escherichia coli* mutants. This led to the identification and characterization of *Leptospira* genes *recA*, *rfb*, *asd*, and *trpE* [10-13]. Later, electroporation and conjugation were both successfully applied to introduce DNA (by suicide or replicative plasmids) into saprophytic and pathogenic *Leptospira* species with selective antibiotic markers of kanamycin, spectinomycin or gentamicin [14-21]. However, site directed mutagenesis studies of *Leptospira* have remained very limited, such as to *flaB*, *recA* and

hemH genes in *L. biflexa* [22-24], *metY*, *metX*, *metW* and *trpE* in *L. meyeri* [15, 25], and to a limited number of genes in pathogenic *L. interrogans* species [26].

Alternatively, transposon-based mutagenesis is a powerful tool for the study of bacterial physiology and pathogenesis through random DNA insertions into a chromosome [27]. *HimarI* transposon of the *mariner* family, which does not require host co-factors to randomly insert into a TA dinucleotide within a chromosome, has been used in a variety of prokaryotes, including the random Tn library generation of *Borrelia burgdorferi*, the spirochete that is the etiologic agent of Lyme disease [28-34]. Recently, *HimarI* has been adapted for use with *Leptospira* to create a large number of Tn mutants within saprophytic as well as pathogenic species, in which the latter has proven more challenging to generate [16-18]. Previous research has developed *HimarI* insertion into *Leptospira* by conjugation [17]. As an example, conjugative plasmid pCjTKS1 can deliver *HimarI* more efficiently than electroporation, with a DNA transfer frequency ranging from 1×10^{-6} to 8.5×10^{-8} transconjugants/recipient cell in *L. biflexa* and *L. interrogans*, respectively [17].

Here, we describe the methodology to generate random transposon mutants in the saprophyte *L. biflexa* via conjugation through *E. coli* β 2163 as a donor cell harboring pCjTKS1-derived conjugative plasmid pSW29T-TKS2. Extensive libraries of saprophytic and pathogenic *Leptospira* Tn mutants created by this method could be used for studying the phenotypes of diverse Tn gene mutations including but not limited to amino acid biosynthesis, motility, iron acquisition [35, 36], virulence [37, 38], and sensitivity to ethidium bromide [39], among others. Additionally, the generation of Tn libraries could foster and facilitate collaborations between laboratories investigating different aspects of *Leptospira* physiology through sharing the Tn mutant libraries. For example, collaborations can valuably reduce the substantial resource and labor expenditure associated with the generation, sequencing, and upkeep of the library. Further, such collaborations foster a positive research and learning environment between laboratories, ultimately supporting research to transcend institutional borders.

2. Materials

Use only sterilized/ autoclaved glassware for all procedures. Set aside specific glassware for EMJH preparation and *Leptospira* work. Do not use general glassware supplies as these may be contaminated with other chemicals to which *Leptospira* are sensitive.

2.1 Preparation for Conjugation

1. EMJH liquid medium: Prepare 100 mL of filter-sterilized Bovine Serum Albumin (BSA) supplement which consists of pre-autoclaved ultra-pure (18 M Ω at 25°C) water supplemented with 10% fraction V BSA, 0.01% calcium chloride, 0.01% magnesium sulfate, 0.004% zinc sulfate, 0.0003% copper sulfate, 0.005% ferrous sulfate, 0.00002% vitamin B12, 1.25% Tween-80, and pH adjust to 7.4 with 2N NaOH. Add 100 mL of BSA supplement to 890 mL of autoclaved EMJH base, which consists of 886.6 mL of ultra-pure water supplemented with 2.3 g of Difco *Leptospira* Medium Base EMJH (Becton Dickinson), 3.4 mL of

100% glycerol stock, and 10 mL of heat inactivated rabbit serum [40, 41] (Notes 1-3).

2. EMJH solid medium: 100 mL BSA supplement pre-warmed to 50°C is added to 890 mL of warm EMJH base (886.6 mL of ultra-pure water supplemented with 2.3 g of Difco Leptospira Medium Base EMJH (Becton Dickinson), 3.4 mL of 100% glycerol stock, and 1% noble agar prior to autoclaving), and 10 mL of heat inactivated rabbit serum. 20-30 mL of solution is poured into 100 mm x 15 mm Petri plates (Note 4).
3. Luria-Bertani (LB) liquid medium: 10% tryptone, 5% yeast extract, 10% sodium chloride in distilled water. Autoclave prior to use.
4. Diaminopimelic acid (DAP) solution: 100mM DAP in autoclaved ultra-pure water, filter sterilized.
5. Kanamycin solution: 50 mg/ mL of kanamycin in autoclaved ultra-pure water, filter sterilized.
6. *Leptospira biflexa* serovar Patoc strain Patoc1 (ATCC 23582/Paris) as the recipient cell (Notes 5 & 6).
7. *E. coli* strain β 2163 as the donor cell.
8. Plasmid pSW29T-TKS2 or other (Note 7).
9. Biosafety cabinet type 2A (minimum) with UV disinfection cycle.
10. Dark-field microscope.
11. Spectrophotometer with detection available at 420 nm.

2.2 Conjugation

1. Glass filter apparatus consisting of 125 mL filter flask, filter holder base with frit and stopper 25 mm diameter, 15 mL glass funnel 25 mm diameter, clamp for 25 mm diameter filter holder (Note 8).
2. Filter forceps, blunt end.
3. Membrane filter, hydrophilic PVDF type size 0.1 μ m pore size, 25 mm diameter (Note 9).
4. Aspiration system: in-line or bioreagent vacuum pump and tubing (Notes 10 & 11).
5. Glass beads for cell culture plating, cleaned and autoclaved.
6. 15 mL conical tubes.

2.3 Isolating clones in solid media and long term storage

1. Sterile mixed cellulose ester syringe filters, 0.22 μ m pore size, 33 mm diameter (Note 12).
2. 5 mL syringes without needle attached.

3. Glycerol solution: 60% glycerol in ultra-pure water, autoclaved.
4. -80°C and/ or -160°C Ultra-low temperature freezer(s).
5. 15 mL conical tubes.

2.4 Nested PCR using a semi-random primer to determine Transposon insertion site

1. Genomic DNA purification kit.
2. UV/ vis spectrophotometer
3. Standard PCR reagents.
4. Primers (Table 1).
5. Thermal cycler.
6. 10× Tris/Borate/EDTA (TBE) running buffer solution: 0.89 M Tris base, 0.89 M boric acid, 20 mM EDTA in distilled water, pH 8.3.
7. Agarose gel: 0.8% agarose in 1× TBE solution.
8. 6× DNA loading dye solution: 40% sucrose, 0.25% xylene cyanol FF, 0.25% bromophenol blue in distilled water.
9. Nucleic acid staining solution.
10. Electrophoresis power supply.
11. DNA molecular weight marker.
12. Nucleic acid gel documentation system.
13. Capillary DNA sequencer, or DNA sequencing outsourced to a commercial sequencing company.

3. Methods

All procedures should be performed within a biosafety cabinet type 2A to reduce risk of contamination of transconjugants and for biosafety. The random transposon mutagenesis protocol as well as conjugation plasmids were originally described and generously donated by Dr. Bourhy and Dr. Picardeau, respectively [17, 35, 42]

3.1 Preparation for Conjugation

1. 1-2 weeks prior to conjugation, aliquot *L. biflexa* into 25 mL of fresh EMJH liquid media in a 50 mL conical and incubate at 30°C with or without agitation until bacteria reaches mid-late exponential growth ($2-3 \times 10^8$ cells/ mL) (Note 13).
2. One day prior to conjugation, aliquot *E. coli* $\beta 2163$ harboring pSW29T-TKS2 from freezer culture into 5 mL of fresh LB liquid medium adulterated with 0.3 mM DAP and 50 $\mu\text{g}/\text{mL}$ of kanamycin. Incubate overnight at 30°C (Note 14).

3. The morning of conjugation, transfer 0.5 mL of the *E. coli* incubated overnight into 4.5 mL of fresh EMJH liquid medium adulterated with 0.3 mM DAP (without kanamycin) (Notes 15-17).
4. Incubate *E. coli* at 37°C with or without agitation until it reaches OD₄₂₀ of 0.2-0.5, which correlates to mid-exponential growth (typically 2-4 hours) (Note 18).
5. Check *E. coli* and *Leptospira* by dark-field microscopy to ensure both samples demonstrate good motility, are not contaminated, and are in mid-exponential growth (e.g.- approximately $2-3 \times 10^8$ cells/ mL) before proceeding to conjugation.

3.2 Conjugation- Day One

1. Setup filtration apparatus within the biosafety cabinet (Figure 1A). Apply a vacuum to the apparatus by using an in-line or vacuum pump aspiration unit (Notes 19 & 20).
2. Sandwich membrane filter between filter holder base with frit and stopper and glass funnel. Hold this system in place using the clamp to create a temporary seal between the glass pieces (Figure 1B).
3. Add 1:10 ratio of *E. coli* containing EMJH media to *Leptospira* containing EMJH media by placing both into the filtration apparatus via the 15 mL funnel (e.g.- 0.5 mL *E. coli* to 4.5 mL of *Leptospira*) (Notes 21 & 22).
4. Allow all media to flow through membrane filter into filtration flask, depositing *E. coli* and *Leptospira* on the upper facing surface of the membrane filter (Note 23).
5. Once flow-through is complete and no bubbles/ liquid remains on membrane filter surface, remove clamp and funnel.
6. Remove membrane filter with deposited bacteria from glass frit filter using forceps (Figure 2) (Note 24).
7. Place membrane filter on an EMJH media plate with 0.3 mM DAP (without kanamycin) with the bacteria-containing side facing up/ away from the media (Figure 3) (Notes 25 & 26).
8. Repeat this filtration procedure with additional media and membrane filters until *Leptospira*-containing EMJH media is exhausted (Note 27).
9. Once replete with membrane filters, Parafilm Petri plate and incubate for approximately 16-20 hours (overnight) at 30°C to allow conjugation to occur (Figure 4) (Note 28).

3.3 Conjugation- Day Two

1. The next day, pre-warm to 30°C a sufficient supply of EMJH liquid media (without kanamycin, without DAP) and EMJH plates (with 40 µg/ mL of kanamycin) for plating (Note 29).
2. Transfer a pair of membrane filters deposited with bacteria into a 15 mL conical tube with forceps (Note 30).
3. Add 0.8 mL of pre-warmed EMJH (without kanamycin, without DAP) to the 15 mL conical tube. Using a hand-held 1 mL serological pipette as a tool, move each membrane filter one at a time into the media.
4. Using the 1 mL serological pipette, create a gentle plunging/ up-and-down movement of the membrane filter into the media to transfer the bacteria from the membrane filter to the EMJH liquid media followed by briefly vortexing (Figure 6) (Note 31).
5. Repeat this procedure with additional pairs of membrane filters into different 15 mL conical tubes until membrane filter supply is exhausted (e.g.- if you started with ten membrane filters, you should now have five 15 mL conical tubes with membrane filters).
6. Incubate 15 mL conical tubes for 1 hour at 30°C with agitation.
7. After 1 hour, transfer liquid EMJH media from one tube equally between at least 3 EMJH plates with 40 µg/ mL of kanamycin (i.e.- approximately 250-300 µl liquid media per plate). Agitate to uniformly spread bacteria across plate using glass beads.
8. Remove beads from plates.
9. To avoid desiccation, wrap plates in aluminum foil and place at 30°C for at least 7-10 days (Figure 4). After this time period, periodically check plates for colonies under aseptic conditions for up to for 50 days. *Leptospira* colonies may appear on the plates as small, medium or large translucent circles within the solid media (Figure 8) (Note 32).

3.4 Isolating clones from solid media and long term storage

1. Once colonies began to appear on plates, isolate them from solid media by using a 1 mL filter pipette tip attached to a micropipettor by piercing the media where the colony is present (i.e.- a stab). The stab will collect a cylindrical column of agar with the transconjugant *Leptospira* within it (Figure 8) (Notes 33-35).
2. Transfer this isolated colony/ stab into 3-5 mL of pre-warmed EMJH liquid media containing 40 µg/ mL of kanamycin within 15 mL conical tubes.
3. Incubate the tubes for 5-28 days (growth rate is dependent upon which gene has been disrupted) at 30°C with or without agitation until *Leptospira* growth is observed as turbid media.

4. Once samples become turbid, distribute a 10 µl aliquot onto a microscope slide to confirm *Leptospira* culture is not contaminated by dark-field microscopy (Note 36).
5. If not contaminated and growth is robust and at mid- to late-exponential growth, sample can be labeled (e.g.- Tn1, Tn2, etc.) and used for downstream applications and long term storage at –80°C or –160°C (Notes 37-39).

3.5 Nested PCR using a semi-random primer to determine transposon insertion site

To determine transposon inserted site, nested PCR using a semi-random primer is completed as previously described [18, 43]

1. Purify genomic DNA from 1-2 mL of non-contaminated Tn *Leptospira* sample (Note 40).
2. *Optional:* Use purified genomic DNA to confirm the sample is the correct *Leptospira* species and/ or harboring the transposon by running PCR reactions for a species- specific gene (e.g.- LEPBla0024F & R for *L. biflexa*) or PCR for a transposon-specific primer set (e.g.- VTK1-TKS2 & VTK2-TKS2) (Table 1).
3. Set up a mix for round 1 of nested PCR by using semi-random primers with the following reagents and quantities per reaction tube (total volume = 24.55 µL): 2.5 µL of 10× Taq polymerase buffer without MgCl₂, 1.5 µL of 25 mM MgCl₂, 0.5 µL of 25 mM dNTPs, 0.6 µL of 10 µM oligo 1 (TnK1 or TnK2), 0.2 µL of 100 µM oligo 2 (Deg1 or Deg2), 0.25 µL of Taq DNA polymerase, 17 µL of nuclease-free water, 2 µL of template DNA (40 ng total) (Note 41).
4. Run round 1 with the following reaction times and temperatures: Step 1: Denature at 95°C for 5 minutes, Step 2: Denature at 95°C for 15 seconds, anneal at 45°C for 1 minute, extend at 72°C for 2 minutes, repeat step 2 thirty-nine additional times, Step 3: Extend at 72°C for 10 minutes (Note 42).
5. Set up a mix for round 2 of nested PCR by using semi-random primers with the following reagents and quantities per reaction tube (total volume = 25 µL): 2.5 µL of 10× Taq polymerase buffer without MgCl₂, 1.5 µL of 25 mM MgCl₂, 0.5 µL of 25 mM dNTPs, 0.25 µL of 100 µM oligo 3 (TnKN1 or TnKN2), 0.25 µL of 100 µM Oligo 4 (Tag), 0.2 µL of Taq DNA polymerase, 19 µL of nuclease-free water, 0.8 µL of PCR round 1 DNA (Note 43).
6. Run round 2 with the following reaction times and temperatures: Step 1: Denature at 95°C for 5 minutes, Step 2: Denature at 95°C for 15 seconds, anneal at 53°C for 30 seconds, extend at 72°C for 2 minutes, repeat step 2 thirty-four additional times, Step 3: Extend at 72°C for 10 minutes
7. Run nested PCR products on a 1× TBE gel which will visualize multiple bands typically ranging from 100-1000 bp in length (Figure 9) (Notes 44 & 45).
8. Send round 2 nested PCR product for Sanger sequencing analysis using oligo 3 as the sequencing primer (i.e.- either TnKN1 or TnKN2) (Note 46).

9. Use sequencing results to run a BLAST analysis to determine transposon insertion site (Notes 47 & 48).
10. Confirmation of transposon insertion site is conducted by running standard PCR with primers which flank approximately 100 bp proximal and distal to insertion site, which should increase PCR product corresponding to size of transposon insertion.

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Appendix: Notes

1. Ensure EMJH base has cooled to at least 50°C before adding BSA supplement or rabbit serum.
2. Can also add 0.0001% superoxide dismutase (SOD), 1% lactalbumin hydrolysate, and 0.004% sodium pyruvate to further enrich media and improve growth of more sensitive Tn mutants.
3. Saprophytic *Leptospira* which may be present in municipal water are thin enough to fit through a 0.22 µm filter membrane. Therefore, we autoclave our ultra-pure water before addition of BSA and other growth factors to ensure no microorganisms contaminate the medium.
4. Please bear in mind BSA enrichment supplement should not be warmed above 50°C –55°C as BSA can denature.
5. A low passage, verified or sequenced strain should be used for generation of the transposon mutant library. This helps to avoid a secondary phenotype as a result of SNPs that may be present in the parental strain [44].
6. Preliminary studies in our lab of transformation efficiency of *Himar1* via conjugation with the saprophytic strains *L. meyeri* and *L. yanagawae* did not result in a high yield of transconjugants. Therefore, these strains are not recommended until expertise of this protocol is completed first with the highly transformable strain *L. biflexa*.
7. pSW29T-TKS2 contains the *Himar1* random transposon system which confers kanamycin resistance for transconjugant selection along with a c9 hyperactive transposase, Ori R6K and OriT RP4. OriT and c9 are located outside of the transposable element. Other plasmids carrying the *Himar1* system are also useful for random transposon mutagenesis, such as pAL-614 [45], which confers spectinomycin resistance. However, kanamycin resistance-conferring transposons are preferred for Tn library generation as spectinomycin resistance can occur spontaneously in *Leptospira* [19].
8. We recommend the Millipore filter apparatus system as it works well with 25 mm diameter filter membranes. These items are: 125 mL filter flask item no.

1002505, filter holder base with frit and stopper 25 mm size item no. 1002502, 15 mL glass funnel 25 mm size item no. 1002514, clamp for 25 mm filter holder item no. 1002503.

9. We recommend Millipore Durapore membrane filter item no. VVLP02500. Be certain to use a 0.1 μm pore size filter, as *Leptospira* may pass through 0.22 μm pores.
10. We use a Welsh 2546B pump (which features an inlet water trap) which is attached to an intermediate 0.2 μm Whatman polydisc in-line filter between flask and vacuum pump
11. We recommend buying silicone tubing that can be autoclaved, since this decreases risk of contamination outside of the filtration system as well as decreases risk of equipment contamination.
12. We have found that some filters are less effective for filtering *Leptospira*. We recommend Millipore Millex-GS filters.
13. This growth can be from resurrection or from low passage *L. biflexa*. *L. biflexa* can be grown at 30°C or room temperature (18°C) to allow flexibility of conjugation experiment to your work schedule. If grown at room temperature, we recommend to move *L. biflexa* to 30°C at least one day prior to conjugation to increase growth rate.
14. Some laboratories prefer to aliquot *E. coli* β 2163 harboring pSW29T-TKS2 from freezer culture the morning of conjugation directly into EMJH media, bypassing the LB liquid medium growth step. This approach also works, however typically has a delayed growth (4-6 hours for exponential growth vs. 2-4 hours when grown from LB liquid medium). Neither approach noticeably reduces transformation efficiency.
15. Dilution of *E. coli* can range from 1:10 to 1:100, with the 1:100 conferring less salts into EMJH media used for conjugation, which may be advantageous. In our lab, either dilution has yielded no notable difference in transformation efficiency.
16. This EMJH media should not contain kanamycin, since *Leptospira* will still be sensitive to kanamycin until transconjugation occurs.
17. If you are planning on completing many conjugation rounds, scale up with several conicals of *E. coli* in EMJH.
18. Do not allow *E. coli* (or *Leptospira*) to reach stationary phase as this will decrease transformation efficiency.
19. To prevent tipping of filtration apparatus which would spill media, we recommend attaching a clamp system to the filter flask.
20. For a pump system, we find a vacuum pressure of 20-40 cm Hg works well. With a lower vacuum pressure the filtration step runs slowly. With higher vacuum pressure the system may create bubbles in the flow-through which can transfer into the vacuum tubing.

21. Vary amounts since transformation efficiency will vary by density of *E. coli* and *Leptospira* (err on the side of more *Leptospira*). We typically vary between 3-8 mL of *Leptospira* and vary the dilution ratio between 1:2 and 1:10. It is recommended to write down the volume of each added on the back of the EMJH plate (e.g.- Figure 5) where membrane filters are placed, since on conjugation day 2, two membrane filters will be paired for the plating step. By doing this, it will help optimize volume vs. cell density for your lab.
22. Biosafety: While *L. biflexa* is considered non-infectious, if this procedure is applied to pathogenic *Leptospira* it is essential to use prudent practices to avoid aspiration and contamination of *Leptospira*, especially if using a vacuum pump. Risk of infection in the laboratory by *Leptospira* is considered higher than average [46]. At least 67 laboratory acquired infections and 10 deaths have been reported for pathogenic *Leptospira* [47]. This is typically by direct or indirect contact with cultures or from the urine of an infected animal. Therefore, it is imperative to use an in-line filter when using this filtration technique with pathogenic *Leptospira*, to clean all tubing by chemical or autoclave sterilization methods regularly, and to wear relevant personal protective equipment.
23. Depending on concentration of bacteria and volume, this can take between 2-25 minutes to complete. Patience is key since disturbing the setup can result in bacteria bypassing the membrane filter if funnel is removed before all media has completed flow-through.
24. The membrane filter can carry static cling, and as a result may jump as it is being removed from glass frit filter. To avoid the membrane filter from becoming contaminated by falling to the cabinet surface, we place the funnel at a 45-degree angle on the glass frit filter surface (Figure 2). This gives purchase to the membrane filter as it is removed with forceps and reduces risk of static jump.
25. Ensure no bubbles remain between membrane filter and EMJH plate to optimize transfer of nutrients to bacteria.
26. If spaced properly up to 7 membrane filters can fit on one EMJH plate (Figure 3).
27. We usually prepare at least 50 mL of *Leptospira*-containing EMJH media disbursed between two 50 mL conicals. This gives us sufficient volume to complete between 7-9 conjugations (i.e.- create 7-9 membrane filters containing *E. coli* and *Leptospira*) on average.
28. Clean glassware and tubing used for conjugation- day 1 by chemical disinfection, rinse thoroughly with ultrapure water, and allow to dry. Place in autoclave sleeves and autoclave for future use.
29. For every 2 membrane filters (pairs), 0.8 mL of EMJH liquid and 3 EMJH solid media plates are required.
30. When transferring, you may notice a cloudy circular patch on the EMJH plate where the membrane filter was located. This is *Leptospira* that has migrated

through the membrane filter overnight. While there will still be sufficient *Leptospira* on the membrane, this is one reason why using a 0.1 µm filter pore and not exceeding a 20-hour incubation period for conjugation to occur on EMJH solid media are best practices (Figure 5).

31. Place the residual liquid media (i.e.- ~10 µL) in the serological pipette onto a microscope slide and visualize by dark field microscopy. *E. coli* and *Leptospira* should be visualized in close proximity and in certain instances appearing attached (i.e.- undergoing conjugation). This will allow you to verify amount of bacteria present and their motility. This will aid you in determining the likelihood that generation of transconjugants was successful (Figure 7).
32. Since *Leptospira* transconjugants may be motile, it is important to check periodically to ensure colonies remain clonal.
33. Only a small fraction of the colony is required for growth in EMJH (approx. 10-50 µl of volume of the agar containing the colony, or one stab into solid media).
34. Some colonies will appear faintly translucent as they spread in the media. To aid in removal we typically use a black sheet of paper placed below the Petri plate to more easily visualize the colonies during removal.
35. Continue to monitor plates for up to 50 days, since some mutants may grow slowly, or be motility-deficient which will delay the appearance of a colony. If a plate develops mold, discard the plate according to your standard biohazard disposal protocol.
36. If *Leptospira* culture is contaminated with *E. coli* or another microbe which may occasionally occur, the contaminating bacteria can be removed by syringe filtration. Take a clean 15 mL conical and place 1-3 mL of fresh EMJH liquid media into it. Filter into this fresh media the contaminated sample by using a 5 mL syringe outfitted with 0.22 µm syringe filter. *Leptospira* should transit through this filter while other microbes will not. Allow *Leptospira* to grow for a minimum of 5-7 days at 30°C with or without agitation. Likelihood of recovery is best if *Leptospira* concentration was at least 10⁷ cells/ mL prior to filtration.
37. Once transposon is *cis*-inserted into genome, it is not spontaneously lost. Therefore, kanamycin selection is not necessary after initial growth in EMJH liquid media with 40 µg/ mL of kanamycin.
38. It is typically considered that the concentration of Tween-80 and glycerol present in EMJH liquid media is sufficient for long term storage in cryogenic vials without adding additional cryoprotectant. Some laboratories use 5% DMSO as a cryoprotectant [48]. Our laboratory typically makes up 2 freezer tubes for each Tn mutant for long term storage at -80°C; one with 30% glycerol added and one without. We have not noticed any difference in growth between those stored with and without glycerol during resurrection.

39. It is highly recommended to share the Tn library with a collaborator. This will provide an off-site backup of your Tn library in case of catastrophic equipment failure.
40. Spot PCR is less reliable for this procedure- we recommend using a genomic DNA purification kit or an automated genomic DNA purification system, such as Purelink or Maxwell 16, respectively.
41. We typically run at least 2 different reactions at once to save on time (e.g.- TnK1 with Deg1, TnK2 and Deg1). One will typically amplify. If nested PCR does not yield multiple bands after round 2, we will try the other combinations.
42. Round 1 does not typically generate visible bands by gel electrophoresis. Therefore, it is not recommended to attempt visualization by electrophoresis from round 1 product
43. Match TnK1 to TnKN1, and TnK2 to TnKN2
44. We use RedSafe in lieu of ethidium bromide as an intercalating fluorescent dye and get excellent and well-resolved visualization of bands
45. The multiple bands visualized by electrophoresis does not infer multiple insertion sites. Instead, since oligo 2 is a semi-random/ degenerate primer [42], it is annealing to several locations upstream of the transposon insertion site, resulting in the appearance of multiple bands (Figure 9). Previous research confirmed multiple banding is due to single site transposon insertion [16]. Therefore, the PCR product can be sent for sequencing without single-band gel extraction.
46. Since some companies use enzymatic purification of PCR products which may impact multi-band PCR products, we occasionally complete PCR cleanup by column purification before sending products for sequencing. With Genewiz, we have found this is unnecessary as we still get good sequencing data, even if quality becomes low from enzymatic purification.
47. We typically use either NCBI Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) or Genoscope (<http://www.genoscope.cns.fr/agc/microscope/home/index.php>). Either website yields similar results. One advantage of using Genoscope is that many bioinformatics programs are also available within the program for initial characterization of the disrupted gene that is identified by BLAST analysis.
48. Transposon may have inserted in same or reverse orientation as disrupted gene since the FRT sites in pSW29T-TKS2 are not in tandem. This allows the transposon to become inverted.

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Figure 1:

A: Complete filtration apparatus with membrane between frit and funnel. B: Demonstration of applying a membrane filter to the frit and stopper of the filtration apparatus. Ensure aspiration is applied to system to hold membrane in place and secure funnel to top with clamp.

Figure 2: Removing membrane from glass frit filter with stopper. Note how 15 mL funnel is placed at 45-degree angle to frit for purchase while removing membrane filter so that membrane filter doesn't jump due to static or vacuum pressure (*Photo by Natalia Lizardo, Manhattanville College*).

Figure 3:

How to position 7 membrane filters on one EMJH solid media plate. Bacteria deposited on membrane filter are visible as a circular sheen in the middle of each membrane filter. No bubbles are present between membrane filters and solid media to allow transfer of constituents to bacteria during conjugation.

Figure 4:
Right: Three conjugation plates wrapped in Parafilm (end of conjugation day 1). Left: aluminum foil wrapped conjugation plates to be incubated for 10-50 days (end of conjugation day 2). Incubator is set to 30°C.

Figure 5:
Plate showing 4 membrane filters, as well as 2 circular cloudy patches (arrows) where membrane filters were removed. These cloudy patches are *Leptospira* that have traversed through the membrane filter during the 18-hour incubation period into the EMJH solid media.

Figure 6:
15 mL conical with two membrane filters in 0.8 mL of EMJH liquid medium (without kanamycin, without DAP). This will be incubated for 1 hour at 30°C with agitation.

Figure 7:

Microscopy of *E. coli* β 2163 and *L. biflexa* potentially undergoing conjugation. In instances where *E. coli* and *Leptospira* are overlapped (e.g.- arrows), cells are monitored to determine if that move together. If so, *E. coli* will appear bound to cell membrane of *Leptospira* (visualized as *E. coli* traversing longitudinally along the length of the leptospire). Image captured with Zeiss Axiolab A.1 (EC PLAN-Neofluar 20X/ 0.5 NA) with attached AxioCam 105 color CMOS camera.

Figure 8:

EMJH Solid Medium Plates with Transconjugants. Left: Plate without 1% rabbit serum showing transconjugants with 8 colony stabs (e.g.- arrows). Right: Plate enriched with 1% rabbit serum showing transconjugants of various motility. Care must be taken to ensure colony stabs are clonal; plating on more than 3 EMJH plates is recommended when high transformation efficiency/ high density is anticipated. Noble Agar concentration for both plates is 1%.

Figure 9:

Example of Semi-Random Primer Nested Results after Round 2. Lanes 1-5: 5 *L. biflexa* Tn mutants. Lane 6: negative control. For this experiment, round 1 primers used were TnK1 and Deg1. Round 2 primers used were TnKN1 and Tag. Electrophoresis completed using a 0.8 × TBE gel adulterated with RedSafe, visualized with a Bio-Rad Gel Doc EZ System.

Table 1:

Primers used for Tn insertion site determination

Primer Name	Sequence (5'→3')	Purpose	Expected size
VTk1-TKS2	GATCATGATATCGATTACAAGG	Transposon Specific Sequence	258 bp
VTk2-TKS2	GTTAAATATTTTGTATAAAATTCGCG		
LEPBIa0024F	AATATAAAACTCTCGATTTCGCTTAACA	Amplification of LepBIa0024 in <i>L. biflexa</i>	500 bp
LEPBIa0024R	TCACAAITGCTTGTGTCAACC		
Deg1	GGCCACGCGTCGACTAGTACNNNNNNNNNGATAT	Semi-random degenerate primer for first round of nested PCR with 5' Tag	N/A
Deg2	GGCCACGCGTCGACTAGTACNNNNNNNNNNNTCTT		
TnK1	CTTGTCATCGTCATCCTTG	Transposon specific primer for first round of nested PCR	N/A
TnK2	GTGGCTTTATTGATCTTGGG	Transposon specific primer for first round of nested PCR	N/A
TnKN1	CGTCATGGTCTTTGTAGTCTATGG	Transposon specific primer for second round of nested PCR	varies
TnKN2	TGGGGATCAAGCCTGATTGGG	Transposon specific primer for second round of nested PCR	varies
Tag	GGCCACGCGTCGACTAGTAC	Tag sequence of semi-random degenerate primers for second round of nested PCR	varies