Detection of *piggyBac*-mediated Transposition by Splinkerette PCR in Transgenic Lines of *Strongyloides ratti*

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[Abstract] Splinkerette PCR (spPCR) is a newly developed and efficient method to ascertain and characterize genomic insertion sites of transgenes. The method described in this protocol was successfully applied to confirm *piggyBac* transposon-mediated integration of transgenes into chromosomes of the parasitic nematode *Strongyloides ratti*. This work is described in detail in Shao *et al*. (2012) and presented here in a simplified diagram (Figure 1). Using this method, chromosomal loci of integration were determined based on target site and 5'- and 3' flanking sequences. Therefore, spPCR can be a useful method to confirm integrative transgenesis in functional genomic studies of parasitic nematodes. Potter and Luo (2010) contains a protocol for use of spPCR to detect and map *piggyBac* transposon-mediated chromosomal integrations in *Drosophila*, and was the source of our method for *Strongyloides*. The splinkerette- and *piggyBac*-specific oligos described in that reference could be used without modification in *Strongyloides*. For interested readers, a general review of the biology of parasitic nematodes in the genus *Strongyloides* may be found in Viney and Lok (2007), and a methods-based article on *S. stercoralis* as an experimental model, with information on transgenesis, may be found in Lok (2007).

Materials and Reagents

1. Free-living adult worms
2. Genomic DNA extraction
   Gentra Puregene Tissue Kit (QIAGEN), including:
   a. Cell lysis solution (catalog number: 8304295)
   b. Protein precipitation solution (catalog number: 8273807)
   c. DNA hydration solution (catalog number: 8274043)
3. Enzymes for digestion and treatment
   Restriction enzymes include BstY I, BamH I and Bgl II (New England Biolabs)
   Others include Shrimp Alkaline Phosphatase (United State Biological, catalog number: P4071-05) and Exonuclease I (New England Biolabs, catalog number: M0293S)

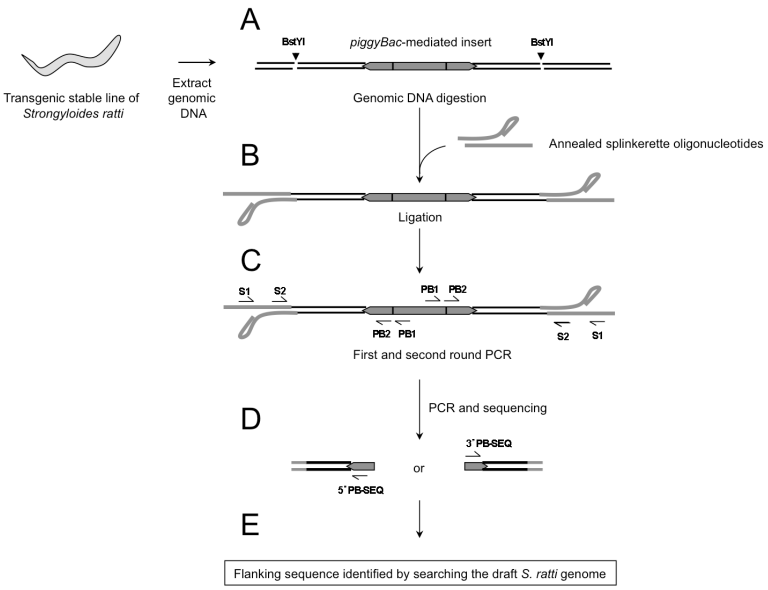

Figure 1. Diagrammatic representation of protocol for mapping transgene integrations in *Strongyloides* by splinkerette PCR (adapted from Potter and Luo, 2010)

4. Ligation reagents

   10x Ligase buffer and T4 Ligase (New England Biolabs, catalog number: M0202S)
5. PCR reagents

   5x Phusion High-Fidelity Buffer, Phusion HF DNA Polymerase (New England Biolabs, catalog number: M0530S)
6. Oligonucleotides and primers

   The oligonucleotides detailed in Table 1 must be synthesized

   All sequences are from Potter and Luo (2010), Table S13
7. TE buffer (New England Biolabs, catalog number: E6293)
8. 10x NEB Buffer 2
9. 1% agarose gel

Table 1. Oligonucleotides and primers for splinkerette PCR mapping of piggyBac transposon-mediated transgene integrations

Oligo or Primer	Sequence
SPLNK-BOT	5'-CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTG AATGAGACTGGTGTCGACACTAGTGG-3'
SPLNK-GATC-TOP	5'-GATCCCACTAGTGTCGACACCAGTCTCTAATTTTTTTTTT CAAAAAAA-3'
SPLNK#1	5'-CGAAGAGTAACCGTTGCTAGGAGAGACC-3'
SPLNK#2	5'-GTGGCTGAATGAGACTGGTGTCGAC-3'
3'SPLNK-PB#1	5'-GTTTGTTGAATTTATTATTAGTATGTAAG-3'
5'SPLNK-PB#1	5'-ACCGCATTGACAAGCACG-3'
3'SPLNK-PB#2	5'-CGATAAAACACATGCGTC-3'
5'SPLNK-PB#2	5'-CTCCAAGCGGCGACTGAG-3'
3'SPLNK-PB-SEQ	5'-ACGCATGATTATCTTTAAC-3'
5'SPLNK-PB-SEQ	5'-CGACTGAGATGTCCTAAATGC-3'

Equipment

1. PCR Thermal Cyclers (Mastercycler Personal, Eppendorf)
2. Gel electrophoresis
3. Gel documentation (FOTODYNE, model: FOTO/Analyst FX)

Procedure

1. Collect stably transformed free-living adult *Strongyloides* from charcoal cultures of fresh host feces incubated at 22 °C for 48 h (Lok, 2007). Extract genomic DNA (gDNA) from ~500 free-living adult worms using the Gentra Puregene Tissue Kit. Parasites do not need to be homogenized. Isolated worms are pelleted at 14,000 *x g* and then substituted for the "ground tissue" in step 2, followed by step 2b of the manufacturer's protocol accompanying the Gentra Puregene Tissue Kit, using reagent volumes recommended for tissue samples of 5-10 mg. Resuspend extracted gDNA in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to yield ~ 25 ng/µl.
2. Digest Genomic DNA (~100 ng) with BstYI, BamHI, and Bgl II for 2 h in a total volume of 15 µl for each reaction (Figure 1A). Follow manufacturer's instructions for reaction conditions and inactivation steps for each restriction enzyme.
3. Add 5 µl of 100 µM SPLINK-BOT oligonucleotide, 5 µl 100 µM SPLINK-GATC-TOP oligonucleotide and 10x NEB Buffer 2 in a total reaction volume of 100 µl. Heat to 95 °C for 3 min

then cool on bench to room temperature to anneal splinkerette oligonucleotides. Ligate digested gDNA with GATC sticky ends to annealed splinkerette oligonucleotides using T4 ligase for 8 h at 16 °C in a total volume of 30 µl (Figure 1B).

4. Two rounds of spPCR are conducted following ligation of genomic DNA restriction fragments to splinkerette oligo nucleotides.
   a. For the first round of spPCR, combine primer SPLINK#1, which targets the ligated splinkerette oligonucleotides, with either primer 3'SPLNK-PB#1 or primer 5'SPLNK-PB#1. 3'SPLNK-PB#1 and 5'SPLNK-PB#1 target the 3' and 5' ends of the transgene sequences, respectively. Carry out the amplification with Phusion Polymerase and 5-20 ng of splinkerette-ligated genomic DNA as template. Thermal cycling conditions are:
      i. 98 °C for 1 min
      ii. 98 °C for 20 sec
      iii. 55 °C for 15 sec
      iv. 72 °C for 2 min
      v. 29 more cycles from ii to iv
      vi. A final extension at 72 °C for 10 min.
   b. The second round of spPCR is nested PCR carried out with primer SPLNK#2 paired with either primer 3'SPLNK-PB#2 or primer 5'SPLNK-PB#2 and a 1:20 dilution of spPCR products from the first-round spPCR as template. Carry out the second-round amplification with Phusion High-Fidelity DNA Polymerase using the same thermal cycling conditions as specified for first-round spPCR. Analyze all PCR products by 1% agarose gel electrophoresis.
   c. Incubate the products of second-round spPCR with 1 µl Shrimp Alkaline Phosphatase (1 U/ml) and 1 µl Exonuclease I (20,000 U/ml) at 37 °C for 2 h, and then at 80 °C for 15 min (Figure 1C).
   d. Sequence the treated PCR products directly using primers 3'SPLNK-PB-SEQ and 5'SPLNK-PB-SEQ, targeting the 3' and 5' ends of the transgene sequence, respectively. Identify chromosomal locations of integrations based on generated sequences and matching fragments in the genome database (Figure 1D, 1E).

Acknowledgments

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