

Splinkerette PCR for Mapping Transposable Elements in *Drosophila*

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Abstract

Transposable elements (such as the P-element and piggyBac) have been used to introduce thousands of transgenic constructs into the *Drosophila* genome. These transgenic constructs serve many roles, from assaying gene/cell function, to controlling chromosome arm rearrangement. Knowing the precise genomic insertion site for the transposable element is often desired. This enables identification of genomic enhancer regions trapped by an enhancer trap, identification of the gene mutated by a transposon insertion, or simplifying recombination experiments. The most commonly used transgene mapping method is inverse PCR (iPCR). Although usually effective, limitations with iPCR hinder its ability to isolate flanking genomic DNA in complex genomic loci, such as those that contain natural transposons. Here we report the adaptation of the splinkerette PCR (spPCR) method for the isolation of flanking genomic DNA of any P-element or piggyBac. We report a simple and detailed protocol for spPCR. We use spPCR to 1) map a GAL4 enhancer trap located inside a natural transposon, pinpointing a master regulatory region for olfactory neuron expression in the brain; and 2) map all commonly used centromeric FRT insertion sites. The ease, efficiency, and efficacy of spPCR could make it a favored choice for the mapping of transposable element in *Drosophila*.

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Introduction

The ability to introduce transgenes into an organism has revolutionized biological investigations. This is particularly true for the *Drosophila* model organism. In *Drosophila*, the most commonly used methods for introducing a transgene into the genome is mediated by the P-element transposon [1,2] or the piggyBac transposon [3]. In these approaches, the transgene to be integrated is flanked by P-element or piggyBac transposable element ends, which can integrate the transgene into the germline in the presence of a transposase enzyme. The result is a transgene inserted into the genome flanked by transposable element ends.

It is often useful to determine the exact genomic insertion site for the transgene. Most commonly, this is used for determining 1) which gene is mutated by the insertion; 2) the enhancer regions captured by a particular enhancer trap; 3) which chromosomal segments might be rearranged by a particular insertion such as an FRT site; 4) which gene might be overexpressed by an inserted regulatory element; or 5) the location of a transgene for recombination with other genetic elements, such as a mutation or other transgenes. Since transposon integrated transgenes are derived from cloned sequences, they contain known sequences, which can be utilized to molecularly determine their insertion sites. The most commonly used method for mapping transgene insertion sites is iPCR [4,5] and plasmid rescue [6]. In the iPCR method (Figure 1A), genomic DNA containing the inserted transposable element is digested with a restriction enzyme that must also cut

within the cloned transposable element. This generates a restriction site within the transposon transgene as well as within the neighboring genomic DNA. This transposon-genomic DNA fragment is then ligated back to itself to form a circular DNA structure. By using carefully selected PCR primers which align to the transposon, the genomic fragment is amplified (Figure 1A) and then sequenced. Plasmid rescue is a similar strategy in which the circularized transposon-genomic DNA must contain an origin of replication and a drug resistance marker, which is then isolated after transforming into bacteria for sequence analysis.

The iPCR method is sufficient to map the insertion site for most transposable elements, and has been used in high-throughput screens to isolate the insertion sites for thousands of P-element and piggyBac transposable elements (e.g. Ref. [7]). A major limitation is that the transposable element must be digestible by the restriction enzyme used for iPCR. If not, the transgenic insert, which is often greater than 10 kb, will also be included in the iPCR reaction along with additional flanking genomic DNA. Such large fragments are difficult to PCR amplify. This usually limits the choice of restriction enzymes to four-base pair cutters (BfuCI, HinPI, MspI) that cut close to the transposon end, and in turn limits the size of the genomic DNA that can be isolated. This is problematic when the transgenic transposon insertion is inside a natural transposon, immobilized transposable elements that are present at multiple copies within the genome. In fact, 3.8% of the *Drosophila* genome consists of natural transposons [8] with a 4.7 fold increase in natural transposon density near centromeric regions. The restriction sites

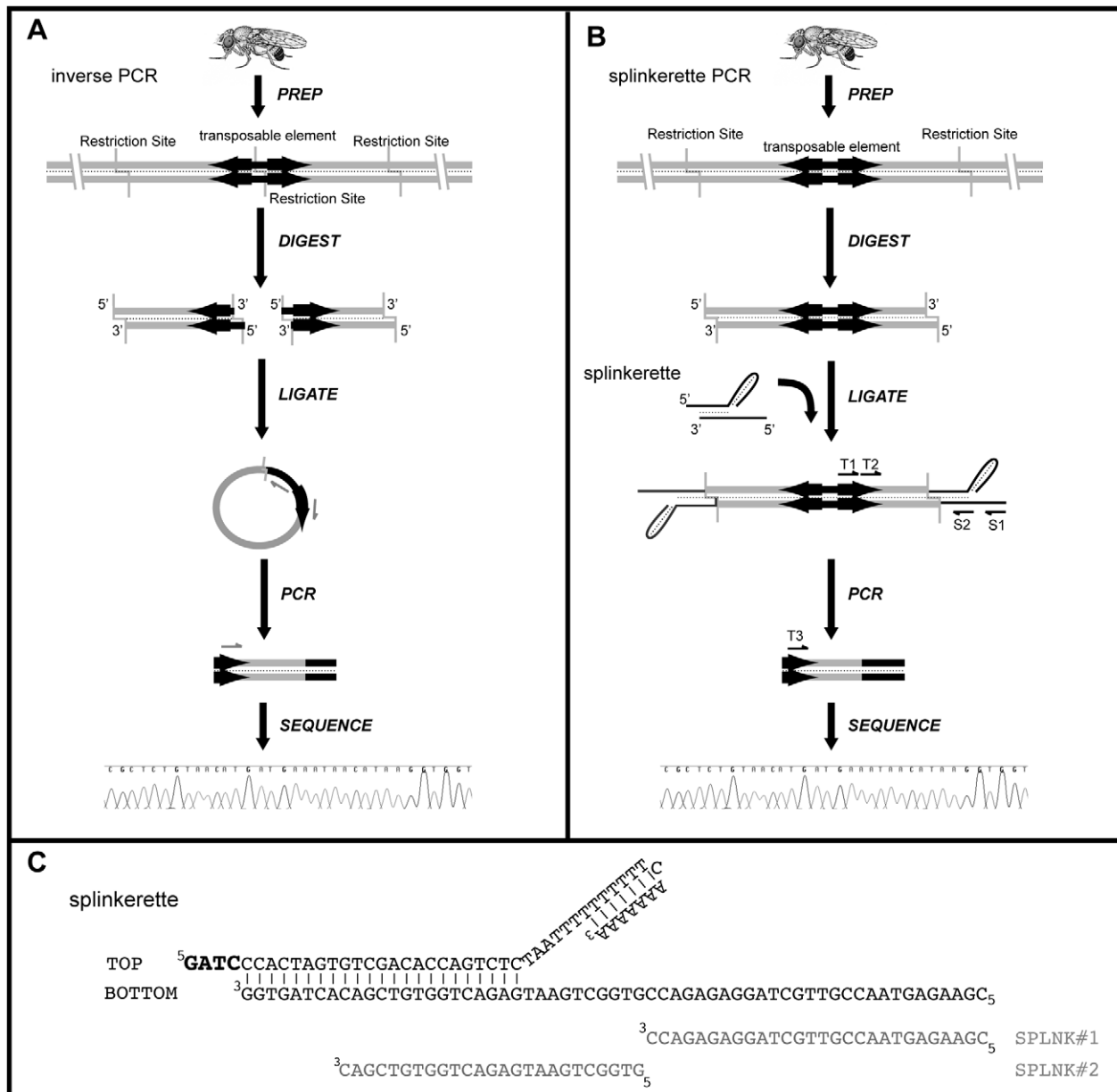


Figure 1. Schematic of PCR methods for mapping transposable elements. **A**) Schematic for the inverse PCR method. Genomic DNA isolated from a fly strain containing a transposable element is digested with an enzyme that cuts within the transposon. These fragments are circularized by a ligation reaction. A PCR reaction with primers designed to the transposon end and an internal sequence amplifies the flanking genomic region. This PCR product is sequenced by a nested primer. **B**) Schematic for the splinkerette PCR method. Genomic DNA is isolated from the fly line containing the transposable element to be mapped. The genomic DNA is digested by an appropriate enzyme that produces sticky ends. The enzyme could cut within the transposable element (similar to scheme A for iPCR) but such digestion is not necessary for the splinkerette PCR reaction. A double stranded splinkerette oligonucleotide with a stable hairpin loop and compatible sticky ends is ligated to the digested genomic DNA. This is followed by two rounds of nested PCR ('S1' and 'T1' indicate the primer pairs for the first round from splinkerette and transposon, and 'S2' and 'T2' indicate the primer pairs for the second round of PCR). This generates a PCR fragment that contains the flanking genomic DNA between the transposable element insertion site and the genomic digestion site. A third nested primer directed against the transposon (T3) is then used for a standard Sanger sequencing reaction. In this schematic, only one end of the transposable element is targeted for isolation of flanking genomic DNA. The other end can also be targeted by using different 'T' primer pairs specific to this other end. **C**) The annealed splinkerette oligonucleotide sequence is shown along with alignment of the PCR primers SPLNK#1 (S1) and SPLNK#2 (S2). The GATC sticky end is bolded. doi:10.1371/journal.pone.0010168.g001

commonly used for iPCR often cut within these natural transposons, and so the iPCR method may not be able to isolate genomic DNA that is beyond these natural transposons.

An alternative approach for mapping insertion sites is spPCR (Figure 1B). This technique was originally developed to amplify

the genomic DNA between a known restriction site and a target gene [9], and then adapted to map the insertion sites of viral integrating gene traps in the mouse genome [10]. In this technique, genomic DNA is digested to yield overhanging sticky ends (Figure 1B). The restriction enzyme is not required to cut

within the transgene. Onto this sticky end is ligated a double stranded oligonucleotide (the splinkerette) that 1) contains a compatible sticky end, 2) contains a stable hairpin loop, and 3) is unphosphorylated (Figure 1C). Two rounds of nested PCR are then performed to amplify the genomic sequence between the transposon insertion and the annealed splinkerette. This is followed by a sequencing reaction with another nested primer. The spPCR reaction remains highly efficient and specific due to the splinkerette design. Since the splinkerette oligonucleotide is not phosphorylated at its 5' sticky end, only the bottom 3' recessed strand of the splinkerette sticky end is ligated to the 5' phosphorylated sticky end of digested genomic DNA. In addition, the PCR primer ('S1' in Figure 1B) which anneals to the splinkerette only amplifies DNA that has been generated as a result of a successful first strand synthesis. As a result, the PCR reaction occurs preferentially between genomic DNA that has ligated to a splinkerette oligonucleotide. In addition, background products are reduced due to the stable hairpin loop on the splinkerette: 1) it will not ligate to genomic DNA to generate non-specific priming and 2) it reduces end-repair priming [9]. Since the enzyme does not need to cut within the transgene, any restriction enzyme that produces sticky ends can be used with the appropriate splinkerette oligonucleotide. This suggests that larger genomic fragments flanking the transgene insertion site can be isolated.

We have adapted spPCR for the mapping of transposable elements (both P-elements and piggyBacs) in *Drosophila*. The spPCR protocol we present is simple, efficient, and highly effective. To date, every transgene we have attempted to map ($n > 250$) could be mapped by spPCR. Splinkerette PCR could be applied to the mapping of transgenes which were impossible using iPCR or plasmid rescue. To demonstrate the utility of spPCR, we have mapped the insertion sites for enhancer traps located within natural transposons, one of which highlights a master regulatory region for expression in a population of olfactory neurons. We have also mapped all the commonly used centromeric FRT insertion sites, some of which were within natural transposons. The spPCR protocol can be further extended for the mapping of any transgene in the *Drosophila* genome.

Materials and Methods

Splinkerette PCR

Details for performing spPCR for P-element and piggyBac elements can be found in the spPCR protocol, Splinkerette Protocol S1. For PCR amplifications, Phusion Taq polymerase (Finnzymes) was used. In a spPCR reaction, the size of non-genomic DNA (*i.e.*, P-element specific DNA) amplified when mapping GAL4 enhancer traps is 279 bp for the 5' P end and 43 bp for the 3' P end. For all other P-elements, the size of non-genomic DNA in a PCR reaction is 111 bp for the 5' P end and 43 bp for the 3' P end. Subtracting these numbers from the PCR fragment sizes indicate the extent of the isolated flanking genomic DNA.

Inverse PCR

Purified genomic DNA (~1 µg; QIAGEN DNeasy kit) was digested by BfuCI (NEB) for 8 h. Digested DNA (~0.5 µg) was self ligated (T4 DNA Ligase, NEB) for 2 h at 25°C in a total volume of 50 µl. For isolating 5' P-element insertion sequence, primer pairs PGAW2 (CAGATAGATTGGCTTCAGTGGAGACTG) and PGAW3 (CGCATGCTTGTTCGATAGAAGAC) were used. For isolating 3' P-element insertion sequence, primer pairs PRY4 (ACTGTGCGTTAGGTCCTGTTTCGTT) and PRY1 (CCTTAGCATGTCCGTGGGGTTTGAAT) were used. iPCR products were sequenced with Sp1 (ACACAACCTTTTCCTCTCAACAA; 5'

insertion sites) or SpeI (GACACTCAGAATACTATTC; 3' insertion sites). The PCR protocol for 5' iPCR was 98°C 75 sec, 35 cycles of 98°C 30 sec, 65.5°C 30 sec, 72°C 2 min, followed by 72°C 7 min. For 3' iPCR the PCR protocol was 98°C 75 sec, 35 cycles of 98°C 30 sec, 62.5°C 30 sec, 72°C 2 min followed by 72°C 7 min. Phusion Taq (NEB) was used for all PCR reactions.

In an iPCR reaction, the size of non-genomic DNA (*i.e.*, P-element specific DNA) amplified when mapping GAL4 enhancer traps is 553 bp for the 5' P end and 243 bp for the 3' P end. For all other P-elements, the size of non-genomic DNA in a PCR reaction is 1218 bp for the 5' P end and 243 bp for the 3' P end. Subtracting these numbers from the PCR fragment sizes indicate the extent of the isolated flanking genomic DNA.

Transgenic animal construction

GH146-GAL4 transgene. The cloning of the GH146-GAL4 enhancer region and generation of GH146-GAL4 transgenic animals were described in [11].

NP225-GAL4 transgene. NP225-GAL4 is located within a previously unmapped *mdg3* natural transposon in the genomic region 5' to the *Lobe* gene (Figure 2C). The presence (6.4 kb band) or absence (876 bp band) of this *mdg3* transposon in different fly strains was determined using PCR primer pairs P1 (TCGAGCGTGTATTATGCTTTG) and P2 (TTGTACAC-TCTGAGGCCAG) (see Figure 2C_i). This *mdg3* natural transposon was also found in NP line NP80-GAL4, but it is not in GH146-GAL4 or *white*¹¹⁸. FlyBase (GenomeBrowser, R5.19) indicates that there is a *roo* natural transposon in the 3' region of the *Oaz* gene. However, this *roo* natural transposon is not present in NP225-GAL4 or NP80-GAL4 genomic DNA, as determined by genomic PCR analysis (data not shown).

The genomic region corresponding with the NP225-GAL4 insert, including the *mdg3* natural transposon (see Figure 2B), was PCR amplified from NP80-GAL4 genomic DNA (which also contains the *mdg3* element in this location) using NP225regionFOR-CACC(-CACCCTGATAGTTTTTCAAAGATTCGACTTCGCTG) and NP225regionREV(CGGAGACAGTCGACAAAAAATTTGAAC-G) primers. This gives a band of approximately 8 kb which was cloned into the pENTR-TOPO cloning vector (Invitrogen). This NP225 genomic region was then shuttled into the Phi-C31 attB pBPGUw vector [12] using the Gateway LR Clonase II Enzyme kit (Invitrogen). This places the enhancer region 5' to the *Drosophila* synthetic core promoter and GAL4 coding region. The construct was integrated into two attP landing sites, attP2 [13,14] and attP86Fb [15] by Phi-C31 integrase-mediated transformation [13].

The NP225-GAL4 insertion site was confirmed (Figure 2C_i) using primer pairs T2 (5'SPLNK-GAWB#2, GAGCTTTT-TAAGTCGGCAAATATCG) and P2.

Immunohistochemistry. Confocal images were taken on a LSM 510 Confocal Microscope (Zeiss). The procedures for fixation, immunohistochemistry and imaging were as described previously [16]. Primary antibodies used were Rat anti-CD8 (Caltag Laboratories, 1:200), Mouse anti-nc82 (DSHB, 1:25), and Rabbit anti-β-galactosidase (1:100).

Results

General strategy

The spPCR method requires a sticky end generated by a Class II restriction endonuclease with 5' overhanging nucleotides [9]. To allow for a wide range of fragment sizes, we chose a GATC overhang for our splinkerette oligonucleotide design (Figure 1C; Splinkerette Protocol S1). This allows for the use of 4 restriction enzymes that will yield compatible GATC sticky ends- BfuCI

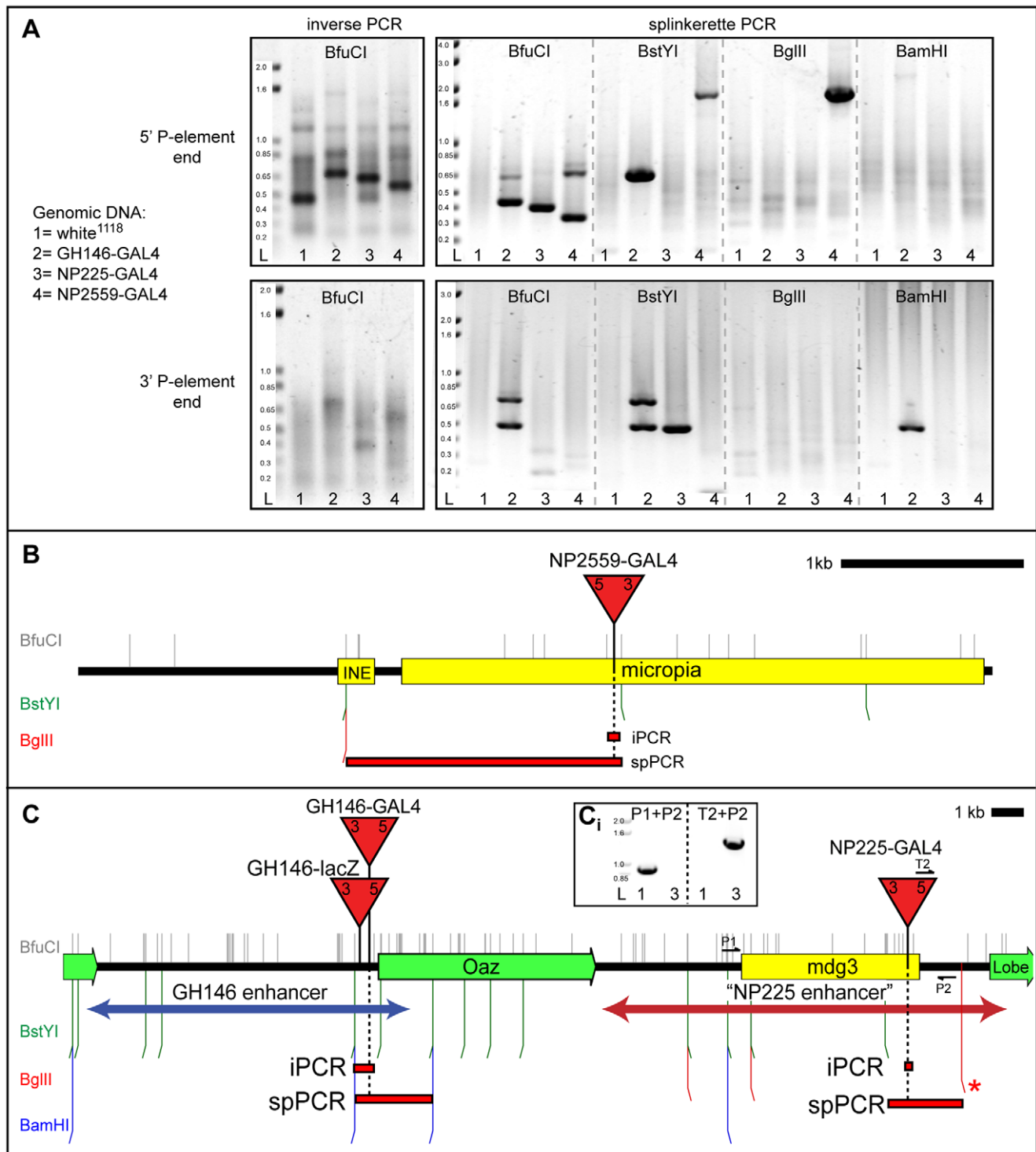


Figure 2. Comparison of spPCR and iPCR for mapping of P-elements in *Drosophila*. **A)** Representative agarose gels showing 5 μ l of PCR products for inverse PCR and spPCR reactions. Genomic DNA from four fly strains (*white*¹¹¹⁸ serves as a negative control) were subjected to iPCR or spPCR to isolate the 5' or 3' flanking genomic DNA of the P-element insertion site. For spPCR, genomic DNA was digested separately with four restriction enzymes (BfuCI, BstYI, BglIII, BamHI) which produce GATC sticky ends compatible with the spPCR protocol. BfuCI iPCR products are larger than BfuCI spPCR products since iPCR amplifies more P-element specific (non-flanking genomic) DNA. DNA ladder (L) units are in kb. **B)** Schematic of the genomic locus containing the mapped NP2559-GAL4 enhancer trap element within the *microopia* natural transposon. **C)** Schematic of the genomic loci for the mapped GH146-GAL4 and NP225-GAL4 enhancer trap elements. The cloned GH146 and NP225 enhancer regions are shown as double-headed arrows. The PCR products for the NP225-GAL4 5' P-element BstYI and BglIII spPCR fragments could not be seen on an agarose gel, but reliable sequence was obtained after phosphatase/exonuclease I treatment of the PCR product (see Splinkerette Protocol S1 for details). The flanking BglIII site (marked by a *) is predicted based on the largest size (~800 bp) of sequenced spPCR products. **C_i)** Agarose gel showing PCR products from the diagrammed primer pairs. The P-element specific T2 primer is also diagrammed in Figure 1. The lanes are labeled as in A. The *mdg3* transposon at this location is not in the *white*¹¹¹⁸ strain. Red triangles represent P-elements (not drawn to scale). Location of restriction sites are diagrammed as vertical

lines color coded according to the restriction enzymes. Restriction sites within the P-element are not shown. Black bars represent genomic DNA, green bars represent genes, yellow bars represent natural transposons, and red bars represent the extent of the longest amplified iPCR or spPCR genomic DNA fragment flanking the P-element insertion site.
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(↓ GATC), BstYI (R ↓ GATCY), BglII (A ↓ GATCT), and BamHI (G ↓ GATCC) which cut with decreasing frequency in the *Drosophila* genome (see Splinkerette Protocol S1). We reasoned that this degree of flexibility in restriction rates should allow for essentially any size fragment to be isolated. For example, if small fragments are required, then digesting with BfuCI or BstYI could be performed. If larger flanking genomic DNA were required (for example, to extend beyond a natural transposon), then BglII or BamHI could be used. We have validated and optimized a set of oligonucleotide primers for the isolation of both 5' and 3' flanking genomic DNA for P-element and piggyBac transposons (Splinkerette Protocol S1 and Table 1). Most significantly, since the 3' P-element primer set was designed to match the P-element's 3P transposon end, the same primer set can be used to isolate the 3' flanking genomic DNA of any P-element, regardless of internal transgenic components. As such, mapping by spPCR is much simpler compared to iPCR since one set of conditions can be used to map any P-element. To date, we have successfully used spPCR to map all transposable elements (P-element and piggyBac) we have attempted (n>250; Figure 2 and data not shown). This high success rate is possible due to the ability of spPCR to isolate longer and longer flanking genomic regions (by using rarer and rarer genomic restriction sites) until a unique BLAST score is achieved. To facilitate the use of spPCR for the mapping of transposable elements in *Drosophila*, we have generated a simple and effective protocol for use by the *Drosophila* community (Splinkerette Protocol S1).

Mapping enhancer traps inside natural transposons—identification of a master regulatory region of olfactory neuronal expression

Enhancer traps are among the most useful transgenic lines in *Drosophila* [17,18,19]. Besides being a useful tool to drive effector

transgenes in tissue specific patterns, enhancer traps also highlight important regulatory elements in the *Drosophila* genome. We are interested in GAL4 enhancer traps (and their regulatory elements) that label a particular neuronal population in the brain, the olfactory projection neurons (PNs). There are ~60 different types of PNs that target dendrites to ~54 discrete foci called glomeruli in the main olfactory organizing center, the antennal lobe. PNs send axons to higher olfactory processing centers in the brain (the mushroom bodies and lateral horn). Our initial attempts to map by iPCR many of our PN expressing GAL4 enhancer traps failed due to their locations in natural transposons (Figure 2 and data not shown). In contrast, we used spPCR to successfully map all of these GAL4 enhancer traps. Figure 2 provides an example for using spPCR to map two such enhancer trap insertions.

The NP2559-GAL4 enhancer trap is inserted into a *microphia* natural transposon, and could not be mapped by iPCR (Figure 2A, 2B). However, by using spPCR and digestion with BstYI or BglII, genomic DNA flanking the 5' P-element end could be isolated that extended beyond this natural transposon and into unique genomic sequences (Figure 2A, 2B, Table S1).

The mapping of enhancer traps within natural transposons led to a particularly interesting result while investigating the genomic regulatory region corresponding to the GH146-GAL4 enhancer trap. The GH146-GAL4 enhancer trap [20,21] labels most of the PN classes in the *Drosophila* brain (Figure 3A, 3B). The insertion site for GH146-GAL4 maps by iPCR and spPCR to the promoter region of the *oaz* gene on chromosome arm 2R (Figure 2A, 2C) [22]. This suggests that this genomic region contains the regulatory elements required to specify expression in this particular neuronal population. Indeed, a lacZ enhancer trap in this location (GH146-lacZ) also expresses in the same PN population (data not shown). To determine if this genomic region

Table 1. Oligonucleotides for Splinkerette PCR of *Drosophila* P-elements¹.

Oligonucleotide Name	Oligonucleotide Sequence	Purpose
SPLNK-GATC-TOP ²	GATCCACTAGTGTGCGACACAGTCTCT-AATTTTTTTTTTCAAAAAA	Top strand of splinkerette oligonucleotide with GATC sticky end
SPLNK-BOT ²	CGAAGAGTAACCGTTGCTAGGAGAGACCGT-GGCTGAATGAGACTGGTGTGACACTAGTGG	Bottom strand of splinkerette oligonucleotide
SPLNK#1 ²	CGAAGAGTAACCGTTGCTAGGAGAGACC	Splinkerette specific primer for Round 1 PCR
SPLNK#2 ²	GTGGCTGAATGAGACTGGTGTGCGAC	Splinkerette specific primer for Round 2 PCR
3'SPLNK#1	CACTCAGACTCAATACGACAC	Round 1 PCR primer for 3' end of all P-elements
3'SPLNK#2	GGATGTCTCTTCCGAC	Round 2 PCR primer for 3' end of all P-elements
3'SPLNK-SEQ	CGGGACCACCTTATG	Sequencing primer for all 3' end spPCR reactions
5'SPLNK#1-CASPR	ATAGCACACTTCGGCAGC	Round 1 PCR primer for 5' end of pCaSpeR based P-elements ³
5'SPLNK#2-CASPR	ATTCTCCGACACAACC	Round 2 PCR primer for 5' end of pCaSpeR based P-elements ³
5'SPLNK-CASPR-SEQ	CCTCTCAACAAGCAAACG	Sequencing primer for 5' end pCaSpeR PCR reactions
5'SPLNK#1-GAWB	TGGGAGAGTAGCGACACTCC	Round 1 PCR primer for 5' end of GAL4 enhancer trap P-elements
5'SPLNK#2-GAWB	GAGCTTTTAAAGTCGGCAATATCG	Round 2 PCR primer for 5' end of GAL4 enhancer trap P-elements
5'SPLNK-GAWB-SEQ	CTCAACAAGCAAACGTGC	Sequencing primer for 5' end of GAL4 enhancer trap PCR reactions

¹See Splinkerette Protocol S1 for spPCR conditions for piggyBac elements.

²Splinkerette oligonucleotide sequences from [10].

³See Splinkerette Protocol S1 for list of compatible pCaSpeR based P-elements.

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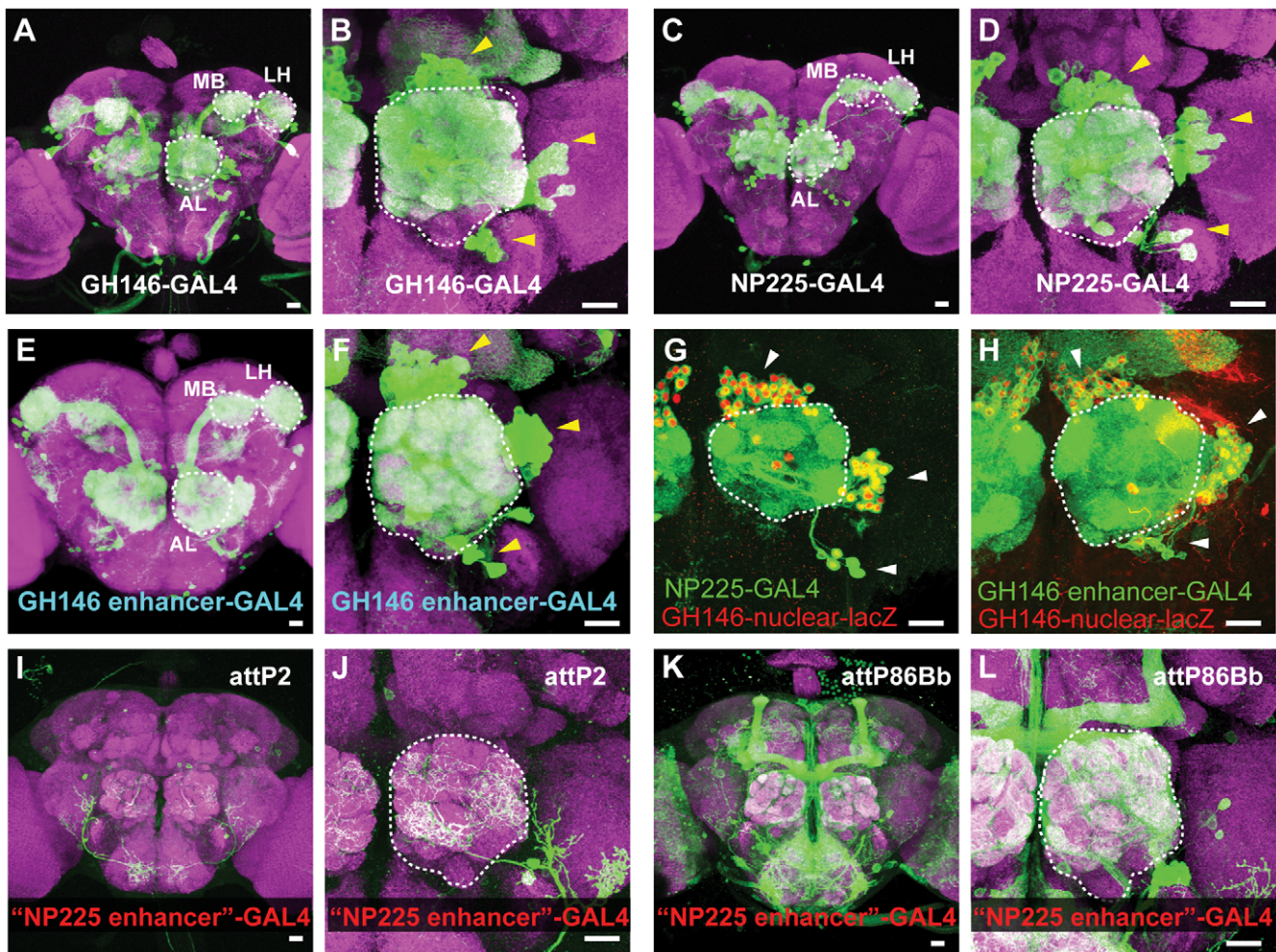


Figure 3. Splinkerette mapping of an enhancer trap within a natural transposon highlights a master regulatory region for PN expression. **A)** The expression pattern of the GH146-GAL4 enhancer trap in a representative confocal projection of a whole mount *Drosophila* brain immunostained for a general neuropil marker (monoclonal antibody nc82) in magenta, and for mCD8 in green (which detects GAL4-dependent UAS-mCD8-GFP expression). The antennal lobe (AL), mushroom body calyx (MB) and lateral horn (LH) regions are outlined. **B)** A higher magnification of the antennal lobe region for the GH146-GAL4 expression pattern. Arrowheads point to the three clusters (dorsal, lateral, ventral) of cell bodies of the projection neurons (PNs). The antennal lobe is outlined. **C)** The expression pattern of the NP225-GAL4 enhancer trap in whole mount brain confocal projection. **D)** A higher magnification of the antennal lobe region for the NP225-GAL4 expression pattern. **E–F)** The representative expression pattern of a transgenic construct that drives GAL4 expression from the cloned GH146 enhancer region diagrammed in Figure 2C. The expression pattern in PNs appears identical to the GH146-GAL4 enhancer trap line. **G)** Representative confocal projections of the antennal lobe of GH146-lacZ and NP225-GAL4 animals immunostained for β gal (in red) and for mCD8 (which reports GAL4-dependent UAS-mCD8-GFP expression) in green. **H)** Confocal projection of the antennal lobe of GH146-lacZ and transgenic GH146-GAL4 animals. **I–J)** The expression pattern of transgenic flies that contain the genomic DNA near the NP225 insertion site (“NP225 enhancer”) driving GAL4 integrated into the attP2 Φ -C31 genomic site. **K–L)** The expression pattern of transgenic flies that contain the “NP225 enhancer” region driving GAL4 integrated into the attP86Bb Φ -C31 genomic site. Expression in the antennal lobe is from innervation from olfactory receptor neurons, and not PNs. Scale bars: 20 μ m. doi:10.1371/journal.pone.0010168.g003

is sufficient to induce expression in PNs, and thus contains the regulatory DNA required for this expression, a transgenic construct was generated which used this genomic region to drive the GAL4 transcription factor (Figure 2C). Transgenic flies, in which this transgene was inserted at a different location in the genome, was sufficient to drive the same expression pattern as the GH146-GAL4 and GH146-lacZ enhancer traps (Figure 3E, 3F). This indicates that this genomic region contains all the regulatory elements sufficient to reproduce the expression pattern of the native GH146-GAL4 enhancer trap.

In a screen of thousands of GAL4 enhancer traps, additional enhancer traps were identified that gave rise to very similar GH146-GAL4 expression patterns [23]. All of these GAL4 enhancer traps were mapped by iPCR to the same genomic locus

as GH146-GAL4 (e.g. NP5288-GAL4, data not shown) except for one: NP225-GAL4. The expression pattern of this enhancer trap is essentially identical to GH146-GAL4 ([23], also Figure 3C, 3D) except that NP225-GAL4 does not label the anterior paired lateral neuron which innervates the mushroom body lobes [24]. Nonetheless, NP225-GAL4 labels the same set of PNs as GH146-GAL4 and GH146-lacZ (Figure 3G). The NP225-GAL4 insertion site could not be molecularly mapped by plasmid rescue (data not shown) or by iPCR because it is within a *mdg3* natural transposon (Figure 2A, 2C; Gal4 Enhancer Trap Insertion Database, Kyoto, Japan).

We were interested in determining the genomic locus responsible for the expression pattern of NP225-GAL4. Does it represent a new location in the *Drosophila* genome that can regulate

precise expression in this PN population? If so, how is this genomic region structurally related to the GH146-GAL4 enhancer region? To address these questions, we used spPCR to isolate flanking genomic sequences that extended beyond the *mdg3* natural transposon (Table S1). Surprisingly, NP225-GAL4 mapped to a region 16 kb away from the GH146-GAL4 insertion site, 3' to the *oaz* gene (Figure 2C). This genomic region was not predicted by the Berkeley *Drosophila* Genome Project (R5.19) to contain a *mdg3* natural transposon, and hence represents a divergence between the sequenced strain and the strain used for the GAL4 enhancer trap screen.

To determine if this genomic region was also sufficient to drive expression in PNs, we generated a GAL4 transgenic construct driven by the putative NP225-GAL4 enhancer region surrounding the transposon insertion site (Figure 2C), and integrated it into two different genomic locations (Figures 3I, 3J, 3K, 3L). Neither of these transgenic lines expressed in the PN population, but did show great variability in their expression patterns. This indicates that the genomic region near the insertion site of NP225-GAL4, when removed from its original genomic locus, cannot induce expression in PNs (the innervation in the antennal lobe in Figure 3K and 3L is from olfactory receptor neuron innervation). The great variability in the expression patterns between the two insertion sites further suggests that the NP225 genomic region is highly susceptible to position effects, perhaps by presence of a natural transposon [25], and can be influenced by the regulatory elements of neighboring genomic elements. As such, it is likely that the NP225-GAL4 enhancer trap has co-opted the regulatory elements in the genomic region defined by GH146-GAL4 to direct its expression pattern. Since every enhancer trap identified to date that expresses in this particular neuronal PN pattern localizes to this genomic locus suggests that this genomic region is a master regulatory region for PN expression. It will be interesting to further dissect how this genomic region can lead to expression in a diverse set of PNs.

Mapping difficult insertions sites—the centromeric FRT insertions

Since centromeric regions are more likely to contain natural transposons, the mapping of transgenes inserted into this region would be an ideal test for the efficacy of the spPCR method. A set of commonly used transgenes inserted into the centromeric region are P-elements that contain FRT recombination sites [26,27]. Chromosomes that contain FRT sites can be induced to undergo

mitotic recombination, and hence are widely used for performing mosaic analysis in *Drosophila*. These FRT containing P-elements were originally mapped by *in situ* hybridization to polytene chromosomes which can localize the insertion to a polytene band (on the order of approximately 150,000 bp). Molecularly mapping the exact FRT insertion site will also precisely define which genes are accessible for mosaic analysis.

All FRT insertion sites were successfully mapped by spPCR (Table 2, Table S1). As expected, some of the FRT containing P-elements were inserted into natural transposons, and likely could not have been mapped by iPCR or plasmid rescue. The P{neoFRT}80B insertion is located within a 1360 natural transposon. Mapping the P{neoFRT}19A insertion was a particularly good test for spPCR: it is located within a natural transposon (*jockey*) which itself is directly adjacent to another natural transposon (*Rtc1*). Nonetheless, by performing spPCR using BamHI as the restriction enzyme, flanking genomic DNA could be isolated that extended beyond these natural transposons. Also of note, P{neoFRT}42D is inserted into the first exon of the *coronin* gene. The FRT42D insertion might affect the function of this gene (which is predicted to be involved in regulating the actin cytoskeleton [28]), and caution should be used when performing mosaic analyses using this FRT insertion. As an alternative, P{FRT(w^{hs})}G13 could be used, which is inserted more centromeric (at 42B1) than FRT42D (at 42D6). A disadvantage of using FRTG13 is that it is marked by the *white+* transgene which leads to dark red eyes in a *white* mutant background. This dark red eye can be inconvenient when trying to identify FRTG13 recombinants with additional transgenes that are also marked by *white+*. To circumvent this problem, we have validated PCR primers that can be used to specifically test for the insertion of FRTG13 (as well as the other FRT insertions) (Table 3).

Discussion

Splinkerette PCR is a powerful method for isolating the genomic regions flanking known sequences. We have applied spPCR for mapping of P-elements and piggyBac elements, but it can be easily adapted for the mapping of viral integration sites or of additional transposable elements carrying transgenes, such as *minos* [29], *hobo* [30], *mariner* [31], or *Hermes* [32]. We have also shown that even the most difficult insertion sites can be mapped using spPCR. As such, transposon insertions for all GAL4 enhancer traps which are currently unmappable by iPCR could potentially be mapped by spPCR. In a large scale enhancer trap

Table 2. Splinkerette PCR mapping of commonly used FRT insertions.

FRT Name ¹	Previous Map Position ²	Splinkerette Map Position	Insertion Site ³	Inside natural transposon	Insertion Notes ⁴
P{neoFRT}19A	19A	19A2	X:19804903	<i>jockey</i> and <i>Rtc1</i>	Repetitive region
P{neoFRT}40A	40A	40A3	2L:21794705		1 st intron of <i>CG31612</i>
P{FRT(w ^{hs})}G13	42B	42B1	2R:2389386		5' region of <i>jing</i>
P{neoFRT}42D	42D	42D6	2R:2760212		Inside 1 st exon of <i>coro</i>
P{FRT(w ^{hs})}2A	79D-F	80B1	3L:22865175		5' region of <i>Arf79f</i>
P{neoFRT}80B	80B	80D1	3L:23096809	1360(3899)	3' region of <i>Ckll alpha</i>
P{neoFRT}82B	82B	82B2	3R:278974		5' region of <i>CG31522</i>

¹P{neoFRT} from [27]. P{FRT(w^{hs})} from [26,27,28].

²Based on polytene chromosome *in situ* hybridization [26,27,28].

³BDGP version R5.14.

⁴Sequence results of spPCR reactions provided in Table S1.

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Table 3. PCR conditions to test for FRT insertions.

For Checking:	Primer Pair:	Phusion Taq annealing Tm	Size
40A FRT	40A-Specific-FOR 3'SPLNK#1	59°C	539 bp
40A FRT	40A-Specific-REV 5'SPLNK#2-CASPR	62°C	275 bp
82B FRT	82B-REV#1 5'SPLNK#2-CASPR	62°C	700 bp
G13 FRT	G13-SPECIFIC-FOR 3'SPLNK#1	59°C	278 bp
G13 FRT	G13-SPECIFIC-REV 5'SPLNK#2-CASPR	62°C	319 bp
2A FRT	2A-SPECIFIC-FOR 3'SPLNK#1	59°C	564 bp
2A FRT	2A-SPECIFIC-REV 5'SPLNK#2-CASPR	62°C	384 bp

Primer Name	Primer Sequence
3'SPLNK#1	CACTCAGACTCAATACGACAC
5'SPLNK#2-CASPR	ATTCGTCGGCACACAACC
40A-Specific-FOR	GTGGGCTCGGCAACATTCTG
40A-Specific-REV	TCGAGACCTCATCCGAACG
82B-REV#1	AATGCCGTACCTACACACG
G13-SPECIFIC-FOR	CCGCTCTCGAAAACCTGCTG
G13-SPECIFIC-REV	AAACGCCATCGATTGGCAAG
2A-SPECIFIC-FOR	GCCAAACACACCACACCCG
2A-SPECIFIC-REV	CAACAGCGGAGCAAGTGCAG

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screen, 6966 insertions were analyzed by iPCR. Of those, 2536 (36%) could not be mapped by iPCR due to small flanking sequence or insertion into repetitive sequence or natural transposons (GETDB, Kyoto, Japan). Similarly, in a large scale piggyBac gene disruption screen, 11% of insertions could not be mapped by iPCR [33]. Splinkerette PCR could be applied for the mapping of these insertion sites.

In our spPCR protocol, we targeted GATC sticky ends for the isolation of flanking genomic DNA. This allows for use of a number of different restriction enzymes that cut genomic DNA with different frequencies, and hence generate a predictable range of flanking genomic DNA that could be isolated. Importantly, by modifying only the TOP strand of the splinkerette oligonucleotide, different sticky ends could be targeted (see Figure 1C). For example, replacing GATC with AATT will target the splinkerette to sites generated by EcoRI (G ↓ AATTC) digestion, and replacing GATC with GGCC will target the splinkerette to sites generated by NotI (GC ↓ GGCCGG) digestion. Few other changes to the spPCR protocol (besides enzyme choices) would be required. This would extend the flexibility of the spPCR method to isolate different flanking genomic segments not targeted by our current protocol.

Splinkerette PCR is also simpler to set up than iPCR. For example, mapping the 3' end of the P-element transgenes PZ, PlacW, PGAWB, and PEP by iPCR each require a specific set of primers for the PCR reaction, and another set of primers for the sequence reaction, which in turn depends on which enzyme was used for the restriction digest (<http://www.fruitfly.org/about/methods/inverse.pcr.html>). In contrast, the 3' end of all P-elements can be mapped by using the same splinkerette primers and conditions. Given the high success rate of spPCR and its ease of use, it could be applied for standard mapping of transgenes, or for high-throughput screens.

The spPCR conditions described here can be applied to most P-elements, even if the internal components of the P-element vector are unknown. This also applies to naturally occurring P-elements. While performing control experiments using the CASPR set of

spPCR primers, we made the startling discovery that our *white*¹¹¹⁸ stock contained a KP element inserted on the third chromosome (data not shown). KP elements are naturally occurring P-elements that contain the same 5' and 3' P-element ends as the pCaSpeR based constructs, but do not contain a visible marker or a transposase gene [34,35]. And although they cannot mobilize without the addition of exogenous transposase, if they are presented with transposase, their mobilization might cause mutations that could go unnoticed if no dramatic defects in viability or sterility result. As such, spPCR could be used to test for the presence of unexpected P-elements in one's lab stocks, especially if those stocks will be used for behavior or for transformation of transgenic constructs. Of note, the isogenized *white* stocks from Bloomington Stock Center (Stock Numbers 5905 and 6326) and the Canton-S strain (Stock number 1) do not contain P-elements as determined by spPCR (data not shown).

Splinkerette PCR is similar in design to adapter-ligation PCR used in *Arabidopsis* to map T-DNA insertions [36]. In this technique, an annealed double stranded oligonucleotide is also ligated to digested genomic DNA. The major difference between spPCR and adapter-ligation PCR is the design of the annealing oligonucleotide: spPCR uses unmodified oligonucleotides that form a hairpin loop to reduce unwanted PCR amplifications, whereas adapter-ligation PCR used phosphorylated and C7 amino modified oligonucleotides for such a purpose. The advantage of adapter-ligation PCR is that only a single round of PCR is required, whereas spPCR often requires two rounds of nested PCR. Given the success of spPCR in the *Drosophila* system, adapter-ligation PCR might also be adaptable for mapping of transposable elements. Also of note, thermal asymmetric inter-laced-PCR (TAIL-PCR) has been successfully used to map many P-element and piggyBac insertions [37]. However, this protocol requires three rounds of nested PCR using three different degenerate primers. Since spPCR uses unmodified oligonucleotides, it might be more cost effective when initially trying to map difficult transposon insertions.

The splinkerette protocol described here has also been streamlined and simplified from previously described mammalian protocols [9,10,38]. For example, we found that ligation of the splinkerette to digested genomic DNA can be shortened to two hours at room temperature (as opposed to overnight incubations at 4°C or 16°C), and that column purification of the splinkerette ligation reaction was not necessary. As a result, splinkerette mapping can easily be performed in one to two days with reduced expense. Such changes might also be applicable to the splinkerette protocols used in mammalian systems.

Supporting Information

Table S1 Splinkerette PCR sequences for FRT insertion sites, GH146-GAL4, NP225-GAL4, and NP2559-GAL4.

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Splinkerette Protocol S1 A detailed step-by-step protocol for performing spPCR to isolate the flanking genomic DNA of P-element and piggyBac insertions.

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Author Contributions

Conceived and designed the experiments: CJP. Performed the experiments: CJP. Analyzed the data: CJP. Wrote the paper: CJP LL.

Splinkerette PCR Protocol for Mapping Transposable Elements in *Drosophila*

Potter and Luo

Protocol Outline

- 1) Genomic Prep (30 mins)
- 2) Genomic Digest (2 hrs → O/N)
- 3) Ligation of digested genomic DNA to annealed splinkerette oligonucleotide (2 hrs)
- 4) Round 1 PCR (2 hrs)
- 5) Round 2 PCR (1.5 hrs)
- 6a) Ant Phos/ExoI treat Round 2 DNA (2hrs)
- 6b) or Run Round 2 PCR on gel and purify band (1 hr)
- 7) Sequence

Step 1. Genomic Prep

There are many established methods for genomic DNA preparations. When performing splinkerette PCR on a small number of lines, we recommend the QIAGEN DNeasy kit (Qiagen Inc., Valencia, CA). Elute in 200 μ l AE buffer. Typical DNA concentrations are ~ 20-50 ng/ μ l.

For many samples (e.g., 96 well plates) follow the genomic preparation protocol established in [1]. The splinkerette protocol also works with a simple genomic prep from single flies (<http://engels.genetics.wisc.edu/flyDNA.html>).

Step 2. Genomic Digest

Table 1. Genomic Digest Reaction.

Components	Volume
genomic DNA (~20-40 ng/ μ l)	25 μ l
H ₂ O	2 μ l
10X BSA	3.5 μ l
10X NEB BUFFER	3.5 μ l
ENZYME	1 μ l
Total	35 μl

Table 2. Restriction Enzyme Conditions (to generate **GATC sticky ends).**

Enzyme	Cut Site	Digest Temp	Heat inactivate?	Purify digest?
BamHI	G↓GATCC	37°C	No	yes
BglII	A↓GATCT	37°C	No	no
BstYI	R↓GATC Y	60°C	Yes, 20 min @80°C	no
BfuCI	↓GATC	37°C	Yes, 20 min @80°C	no

2.1 See Table 12 for details on the compatible restriction enzymes. Digest genomic DNA \geq 2 hrs. As listed in Table 2, heat inactivate or purify the enzymatic reaction if required. This is necessary in cases where the splinkerette oligonucleotide ligated to genomic DNA regenerates the restriction site.

Note: We recommend first using **BstYI**. This enzyme is often sufficient for mapping most insertions. Longer DNA fragments can be isolated by using BglII or BamHI as needed.

When doing splinkerette PCR on a few samples, we also recommend performing splinkerette PCR on both the 5' and 3' ends of the transposable element. Each reaction should indicate the same genomic insertion site. If not, this is a good indicator that the fly line has multiple transposon insertions. Also, it is highly recommended to perform splinkerette PCR on a negative control genomic sample that does not contain P-elements, such as *white*¹¹¹⁸ or Canton-S flies. This will verify that there are no contaminants in the reagents, as well as confirm that wild-type stocks are indeed free of P-elements.

Step 3. Ligation to Splinkerette Oligonucleotide

Table 3. Conditions for Ligating Digested Genomic DNA to Annealed Splinkerette Oligonucleotide

Components	Volume
Digested genomic DNA	35 μ l
H ₂ O	2.5 μ l
10X NEB Ligase Buffer	5 μ l
annealed splinkerette oligonucleotide (see Table 14)	6 μ l
NEB T4 DNA Ligase (400U/ μ l)	1.5 μ l
Total	50 μl

3.1 Incubate at room temperature \geq 2 hrs. Proceed directly to Round 1 PCR.

Note: ligation reactions can be incubated at 16°C for 16 hrs, but an increase in efficacy was not found.

Step 4. Round 1 Splinkerette PCR

Table 4. Round 1 PCR Reaction.

Components	Volume
Ligated genomic DNA	10 μ l
H ₂ O	8.25 μ l
5x Phusion HF Buffer	5 μ l
10 mM dNTP	0.5 μ l
SPLNK#1, 10 μ M	0.5 μ l
Primer #1, 10 μ M (see Table 5)	0.5 μ l
Phusion Taq (Finnzymes)	0.25 μ l
Total	25 μl

Table 5. Primer Conditions for Round 1 PCR.

Purpose	Primer #1	xx anneal temp
3' end of any P-element	3'SPLNK#1	61°C
5' end- CASPER	5'SPLNK#1-CASPR	61°C
5' end- GAWB (GAL4 enhancer trap)	5'SPLNK#1-GAWB	63.5°C
3'end – piggyBac	3'SPLNK-PB#1	58°C
5'end – piggyBac	5'SPLNK-PB#1	64°C

Table 6. PCR conditions for round 1 PCR.

98°C 75 sec →	98°C 20 sec 64°C 15 sec (x 2) →	98°C 20 sec xx °C 15sec (x 30) → 72°C 2 min	72°C 7 min →	4°C hold
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- 4.1 Perform Round 1 PCR as detailed in Tables 4-6. We recommend using NEB Phusion Taq (Finnzymes) as this gave us the longest PCR fragments. PCR conditions have been optimized for Phusion Taq.

Step 5. Round 2 Splinkerette PCR

Table 7. Round 2 PCR Reaction.

Components	Volume
Round 1 PCR	≤1 µl
H ₂ O	17.25 µl
5x Phusion HF Buffer	5µl
10 mM dNTP	0.5 µl
SPLNK#2, 10 µM	0.5 µl
Primer #2, 10 µM (see Table 8)	0.5 µl
Phusion Taq	0.25 µl
Total	25 µl

Table 8. Primer Conditions for Round 2 PCR.

Purpose	Primer #2	xx anneal temp
3'end of any P-element	3'SPLNK#2	58°C
5' end- CASPER	5'SPLNK#2-CASPR	62°C
5' end- GAWB (GAL4 enhancer trap)	5'SPLNK#2-GAWB	66°C
3'end - piggyBac	3'SPLNK-PB#2	59°C
5'end - piggyBac	5'SPLNK-PB#2	66°C

Table 9. PCR Conditions for Round 2 PCR.

98°C 75 sec →	98°C 20 sec xx °C 15 sec (x 30) → 72°C 90 sec	72°C 7 min →	4°C hold
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- 5.1 Perform Round 2 PCR as detailed in Tables 7-9. Best results are often obtained when using 0.5 µl round 1 PCR as template.
- 5.2 Analyze 5µl of Round 2 PCR on a 0.7% agarose gel. PCR band sizes will depend on which enzyme was used. Usually, BglII reactions yield ~1.5 kb bands, and BstYI reactions yield ~0.5 kb bands.
- 5.3 PCR bands can be gel extracted for sequence analysis. Alternatively, the remainder of the round 2 PCR reaction can be treated with Antarctic Phosphatase and Exonuclease and used directly for sequencing as detailed below. Good sequence runs can still be obtained after Step 6 even if only very weak bands are present on the gel.

Step 6. Antarctic Phosphatase/Exonuclease I treatment

Table 10. AntPho/ExoI Reaction Conditions.

Components	Volume
Round 2 splinkerette PCR	20 μ l
10X NEB AP Buffer	3.0 μ l
H ₂ O	3.0 μ l
NEB Antarctic Phosphatase	2.0 μ l
NEB Exonuclease I	2.0 μ l
Total	30 μl

- 6.1 Incubate reactions at 37°C for 2 hrs, followed by a 80°C incubation for 15 min. Use 15 μ l of the reaction for sequencing with the appropriate sequencing primer listed in Table 11.

Table 11. Sequencing Primers

Purpose	Primer
3'end of any P-element	3'SPLNK-SEQ
5'end-CASPER	5'SPLNK-CASPR-SEQ
5'end-GAWB	5'SPLNK-GAWB-SEQ
3'end –piggyBac	3'SPLNK-PB-SEQ
5'end –piggyBac	5'SPLNK-PB-SEQ

Note: The following splinkerette specific sequence might be at the end of a sequence reaction: GATCCCACTAGTGTGCGACACCAGTCTCATTAGCCACGGTCTCTCCTAGCAACGGTTACTCTTCG

Table 12. Restriction Enzymes with Compatible **GATC** Sticky Ends.

Name	Cut Site	Sites/Mb in <i>D. melanogaster</i> genome ¹	Average fragment length in base pairs ¹
BamH1	G↓GATCC	170.2	5874
BglII	A↓GATCT	183.3	5454
BstYI	R↓GATC Y	704.1	1420
BfuCI	↓GATC	2758.4	363

1. http://tools.neb.com/~vincze/gnsites/comp.php?genomes=Fruit_Fly

Table 13. Oligonucleotide Sequences

SPLNK-GATC-TOP	GATCCCACTAGTGTGCGACACCAGTCTCTAATTTTTTTTTTCAAAAAA
SPLNK-BOT	CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTGAATGAGACTGGT GTCGACACTAGTGG
SPLNK#1	CGAAGAGTAACCGTTGCTAGGAGAGACC
SPLNK#2	GTGGCTGAATGAGACTGGTGTGCGAC
3'SPLNK#1	CACTCAGACTCAATACGACAC
3'SPLNK#2	GGATGTCTCTTGCCGAC
3'SPLNK-SEQ	CGGGACCACCTTATG
5'SPLNK#1-CASPR	ATAGCACACTTCGGCAGC
5'SPLNK#2-CASPR	ATTCGTCCGCACACAACC
5'SPLNK-CASPR-SEQ	CCTCTCAACAAGCAAACG
5'SPLNK#1-GAWB	TGGGAGAGTAGCGACTCC

5'SPLNK#2-GAWB	GAGCTTTTTAAGTCGGCAAATATCG
5'SPLNK-GAWB-SEQ	CTCAACAAGCAAACGTGC
3'SPLNK-PB#1	GTTTGTTGAATTTATTATTAGTATGTAAG
3'SPLNK-PB#2	CGATAAAACACATGCGTC
3'SPLNK-PB-SEQ	ACGCATGATTATCTTTAAC
5'SPLNK-PB#1	ACCGCATTGACAAGCACG
5'SPLNK-PB#2	CTCCAAGCGGCGACTGAG
5'SPLNK-PB-SEQ	CGACTGAGATGTCCTAAATGC

Note: Splinkerette oligonucleotides SPLNK-GATC-TOP, SPLNK-BOT, SPLNK#1, SPLNK#2 are derived from [2].

Table 14. Reaction Conditions for Annealing Splinkerette Oligonucleotides

Component	Volume
SPLNK-BOT (150 ng/μl)	50 μl
SPLNK-GATC-TOP (150 ng/μl)	50 μl
10X NEB Buffer 2	100 μl
H ₂ O	800 μl
Total	1000 μl

14.1 Heat to 95°C for 3 minutes. Allow to cool on bench to room temp (~30 mins). Store 200 μl aliquots at -20°C.

Table 15. P-element Constructs Compatible with 3'SPLNK:

any pCaSpeR based construct	pGT1 (BG lines)	EPgy2 (EY lines)	P{SUPorP} (KG lines)
pGaWb (GAL4 enhancer traps)	pGatB/n	pPTGAL4	pPwl+hsGS (gene switch)
pUAST	pUASP	pUASdesFPc	EP
GS	Act5C>y+>GAL4 (Ay5C)	Flip-out constructs	ptubP-GAL80
UAS-CD8GFP	pWizDir	p[acman]	pRISE
pICOn	pTARG	p{Switch1}	p{Switch2}
pP{wHy}	pFRT	pP{wlo-inGS}	pP{wlo-hsinGS}
pPI25.1	<i>Drosophila</i> KP element	Dual-tagging gene trap vector pGT1	pUChsneo (neomycin)
pXP (Exelixis)	pP{neoFRT}	P{FRT(whs)}	Note: This list may not be complete.

Table 16. P-element Constructs Compatible with 5'SPLNK-CASPR:

pCaSpeR	P{SUPorP} (KG lines)	EPgy2 (EY lines)	pCa4B2G
pCa4B	Ganesh-Z1	pRISE	pUAST
pUASp	pXP (Exelixis)	piggyBac_PB (Exelixis)	pICOn
p{Switch1}	p{Switch2}	pP{wHy}	RNAi cloning and transformation vector pFRiPE
<i>Drosophila</i> KP elements	Dual-tagging gene trap vector pGT1	pUChsneo (neomycin)	pP{neoFRT}
P{FRT(whs)}-PerrimonFRT	Note: This list may not be complete.		

5' and 3' piggyBac splinkerette PCR:

Primers were based on the pXL-Bac II backbone [3]. Derivates of this vector (e.g., pXL-BacII-ECFP, pXL-Bac-DsRed, pXL-BacII-SAstopDsRed [1], pBAC-GH146 [4]) will work with these splinkerette primers. These primers are also compatible with the Exelixis piggyBac vector PB and its derivatives [5].

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