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Chapter 2

Screening Mutants by Single Fly Genomic PCR

Usha Nagarajan and Marios Georgiou

Abstract

Imprecise P-element excision or FRT-mediated recombination is routinely performed to mutagenize a gene of interest. It is, however, tedious to maintain all independent and individual excised mutant fly lines before the presence of a mutation is confirmed. Here, we provide a method to detect and confirm the presence of a mutation, as and when mutant flies are generated. By allowing for the maintenance and expansion of only the confirmed mutant lines, this protocol will help to save time, money, and space.

Key words Genetic screening, Mutagenesis, Genomic PCR, Single fly PCR, Mutant, Drosophila

1 Introduction

Drosophila melanogaster is one of the most commonly utilized and popular models to characterize genes of unknown function [1, 2]. Loss-of-function (LoF) alleles are important resources that help to characterize and understand unknown gene function. Loss of gene function can be studied through the generation of null or hypomorphic mutant alleles, or by knocking down gene expression by RNA interference [3]. Many gene functions are still uncharacterized for the want of loss-of-function alleles. Imprecise excision of P-elements or precise recombination of homologous regions in the genome can be employed to generate mutants; however, screening for mutants is a laborious and cumbersome process.

Our laboratory is interested in the study of the ligand-dependent Notch signaling pathway. We carried out a genetic screen to identify the molecular players that interact with and regulate this signaling pathway (unpublished). As many of the novel interactors are uncharacterized, we set out to generate mutants. We set out to train undergraduate students with the aim of saving both time and resources, and we standardized and devised a simple protocol, with this aim in mind. If a large number of mutants are generated, maintaining them is a huge burden (e.g.,

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preparing fly food and performing regular fly husbandry). Instead, this procedure gives quick results as and when the mutants are generated, and it helps to reduce the number of stocks that need to be maintained. As this method involves both fly genetics and molecular biology, students are highly motivated when using this method.

2 Materials

2.1 P-Element Excision to Generate Mutant Alleles

1. Control Stocks: W^{1118} (Bloomington Stock #3605/5905/6326)
2. Fly stocks with a P-element inserted in the gene of interest (e.g., Bloomington Stock #30151)
3. Balancer fly stocks.
   (a) Double Balancer—w[1118]; wg[Sp-1]/CyO; MKRS/TM6B, Tb[1] (Bloomington Stock #76357)
   (b) Linked Balancer—T(2;3)SM6a-TM6B, Tb[1] (Bloomington Stock #5687).
4. Transposase stocks:
   (a) w[*]; wg[Sp-1]/CyO; ry[506] Sb[1] P{ry[+t7.2]=Delta2–3}99B/TM6B, Tb[+] (Bloomington Stock #3629)
   (b) y[1] w[1]; Ki[1] P{ry[+t7.2]=Delta2–3}99B (Bloomington Stock #4368).
5. Recombining FRT-stocks flanking the gene of interest [4, 5].

2.2 Single Fly Genomic DNA Extraction and PCR

2.2.1 Materials Required for Method 1

1. Prepare 50 ml of 2× Cracking Buffer by adding 1 ml of 10 N NaOH, 2.5 ml of 10% SDS, and 10 g sucrose in 20 ml of sterile distilled water and make up the volume to 50 ml of sterile distilled water.
2. Optional: Proteinase K 10 mg/ml
3. Bromophenol Blue or 6× BPB dye.

2.2.2 Materials Required for Method 2

1. Prepare 50 ml of Fly Genomic DNA extraction Buffer containing (10 mM Tris–HCl [pH 8.2], 1 mM EDTA, and 25 mM NaCl) by adding 500 μl of 1 M Tris–HCl (pH 8.2), 100 μl of 0.5 M EDTA, and 250 μl of 5 M NaCl in 20 ml of sterile distilled water and make up the volume to 50 ml using sterile distilled water.
2. Proteinase K 10 mg/ml.
3. 4 M or 2.5 M KCl.

2.3 PCR and Agarose Gel Electrophoresis

1. Agarose.
2. Bromophenol Blue (BPB)/6× loading dye.
3. Forward and reverse primers.
4. DNA PCR buffer.
5. Taq polymerase.
6. dNTP mixture,
7. Distilled water.

2.4 Apparatus/ Equipment Required

1. Microcentrifuge tubes and PCR tubes.
2. Centrifuge.
3. Water bath.
4. Incubator.
5. PCR machine.
6. Agarose gel electrophoresis apparatus.

3 Methods

3.1 P-Element Excision to Generate Mutant Lines

1. Day 1: (1) Set up a cross between 20 virgin flies containing P-elements inserted within a gene of interest (Bloomington Stock #30151) For example, we have used the stock w^{1118}; P {EP}mgr^{G5308}/TM6C, Sb^1 (Bloomington Stock #30151) inserted in the gene *merry-go-round (mgr*, CG6719) and 10 male flies containing transposase (Bloomington Stock #4368) in a medium-sized bottle. (2) Expand double balancer lines: Sp/Cyo; MKRS/TM6B (Bloomington Stock #76357) or Sp/Cyo-TM6B (this can be generated by combining the linked balancer T(2;3)SM6a-TM6B [Bloomington Stock #76357] together with the double balancer line Sp/Cyo; MKRS/TM6B [Bloomington Stock #76357]) in preparation for **step 3**.
2. Day 3: Transfer the crossed adult flies to a fresh vial/bottle to generate a duplicate of the cross.
3. Day 14: (1) Collect all mosaic eyed males that eclose from the cross. (2) Collect virgins of the double balancer lines.
4. Day 15: In a vial, labeled (1), set up a cross with an individual male fly together with 2–3 virgins from the double balancer line. Repeat this process of single-fly male crosses with double balancer flies in clearly labeled vials (up to 100 crosses). We call these Set-A vials 1–100.
5. Day 18: Move the adult flies to a fresh vial labeled the same as the original cross (Set-B vials 1–100).

6. Day 19: (1) Separate the males to a 1.5 ml microcentrifuge tube labeled same as the original cross and allow it starve for 1-h.

   (2) For control samples, transfer a single male w^{1118} fly and a single male fly from the original P-element line into separate microcentrifuge tubes. Maintain 5 such tubes for both w^{1118} and P-element flies with clear labels. Ensure that all vials and microcentrifuge tubes are labeled correctly.
7. Day 20: Based on the PCR and gel electrophoresis analysis, retain only those vials (both Set A and Set B) that generate mutant stocks and discard all other vials that showed negative results (*see* **Notes 6** and 7).

3.2 Single Fly Genomic DNA Extraction

Day 19: (*see* **Note 1** for advice on whether to use Method 1 or Method 2).

3.2.1 Method 1

Steps to be followed

1. Prepare 50 ml of fresh 2×-cracking buffer (prepare fresh every time).
2. Set a water bath to 70 °C.
3. Leave the labeled microcentrifuge tubes with male flies and control flies on ice for 30 min.
4. Leave the tubes at room temperature for 5 min.
5. Add 100 μl of 2×-cracking buffer.
6. Crush and Grind the fly using a Teflon pestle.
7. Vortex and incubate the tubes in the water bath maintained at 70 °C for 5-min.
8. Allow to cool at room temperature for 5 min.
9. Centrifuge at maximum speed for 3 min.
10. Transfer the supernatant (take only up to 50–80 μl to avoid the fly debris) for further analysis.
11. Add 1.5 μl of 4 M KCl or 2.5 μl of 2.5 M KCl and mix well.
12. Divide the saved supernatant into two aliquots (Aliquot 1 and 2 with 10 μl of the supernatant).
13. Immediately subject the supernatant in Aliquot 1 for gel electrophoresis and Aliquot 2 for PCR analysis.
14. To 5 μl of Aliquot 1 add 0.5 μl of Bromophenol Blue or 6× BPB dye, mix thoroughly, and subject the samples to agarose gel electrophoresis.
15. To 5 μl of Aliquot 2 add PCR master mixture and perform PCR analysis.

3.2.2 Method 2

Steps to be followed.

Day 19

1. Prepare 50 ml of Fly genomic DNA extraction Buffer.
2. Set a water bath to 95 °C and an incubator to 37 °C. Leave the labeled microcentrifuge tubes with male flies and control flies on ice for 30 min.
3. Leave the tubes at room temperature for 5 min.
4. Add 100 μl of Fly Genomic DNA extraction Buffer.
5. And 1 μl of Proteinase K and mix well.
6. Crush and grind the fly using a Teflon pestle (Wheaton).
7. Centrifuge for 1-min at maximum speed.
8. Transfer the supernatant (take only up to 50–80 μl to avoid the fly debris) for further analysis.
9. Incubate the tubes at 37 °C for 30-min.
10. Incubate the tubes at 95 °C for 5-min.
11. Incubate the tubes on ice for 5-min and store at −20 °C.

    Day 20 (or within a week).
12. Take 10 μl of the genomic DNA samples and divide them into three aliquots (Aliquot 1, 2, and 3).
13. To 5 μl Aliquot 1 add 0.5 μl of Bromophenol blue or 6× BPB dye, mix thoroughly, and subject the samples to agarose gel electrophoresis.
14. To 2 μl of Aliquot 2 add PCR master mixture and perform PCR analysis.
15. To 3 μl of Aliquot 3, add 1 μl of primers for sequencing.

3.3 PCR and Agarose Gel Electrophoresis

(*see* **Note 2**)

Day 19

1. Cast either two (up to 4 if there are many samples) 1% agarose gels or one (up to 2) 0.8% agarose gel for running genomic DNA samples and one (up to 2) 1% agarose gel with sufficient number of wells depending on the number of samples [6] (*see* **Note 2**).
2. Set up a PCR reaction (*see* below; 25 μl final volume in each PCR tube).

    If possible two sets of primers, one flanking the P-element and another at a distant location in the genome should be designed for confirmation (*see* **Note 3**). The PCR reaction mixture should be scaled up based on the number of samples to be analyzed.

PCR reaction mix:

10× DNA Buffer: 0.5 μl.

dNTP mixture: 0.2 μl.

Forward Primer: 0.2 μl.

Reverse Primer: 0.2 μl.

Taq polymerase: 0.1 μl.

Distilled Water:22.8 μl.

Template DNA: 1.0 μl.

3. In a 1.5 ml microcentrifuge tube, make up a PCR master mix with all PCR components without template DNA. Volume will depend on the total number of samples to be analyzed (number of mutant samples + two control fly samples (*see* Subheading 3.1) + two negative controls for the PCR reaction; additionally, add two extra volumes of PCR reaction mix to allow for pipetting errors). *See* **Note 4**.
4. Label the PCR tubes clearly.
5. Aliquot 24 μl of the PCR master mix in the labeled PCR tubes.
6. Add 1 μl of corresponding template DNA (1 μl of water for the negative control PCR reactions).
7. Gently spin the reaction mix and carry out PCR using the following settings.

   Step 1—95 °C—2 min.

   Step 2—94 °C—30 s.

   Step 3—60 °C—2 min.

   Step 4—72 °C—2 min (Steps 2–4, 25 cycles).

   Step 5—72 °C—5 min.

   Step 6—4 °C—5 min.
8. In the meantime, run the genomic DNA samples on one of the 1% agarose gels and visualize.
9. Once the PCR is completed, run the samples on another 1% agarose gel. *See* **Note 5**.
10. Based on the analysis of the gels, retain the fly stocks. *See* **Note 6**.

4 Notes

1. If you plan to perform PCR on the same day for further mutant analysis, Method 1 is suggested. If you plan to store the genomic DNA to carry out PCR on another day (but within a

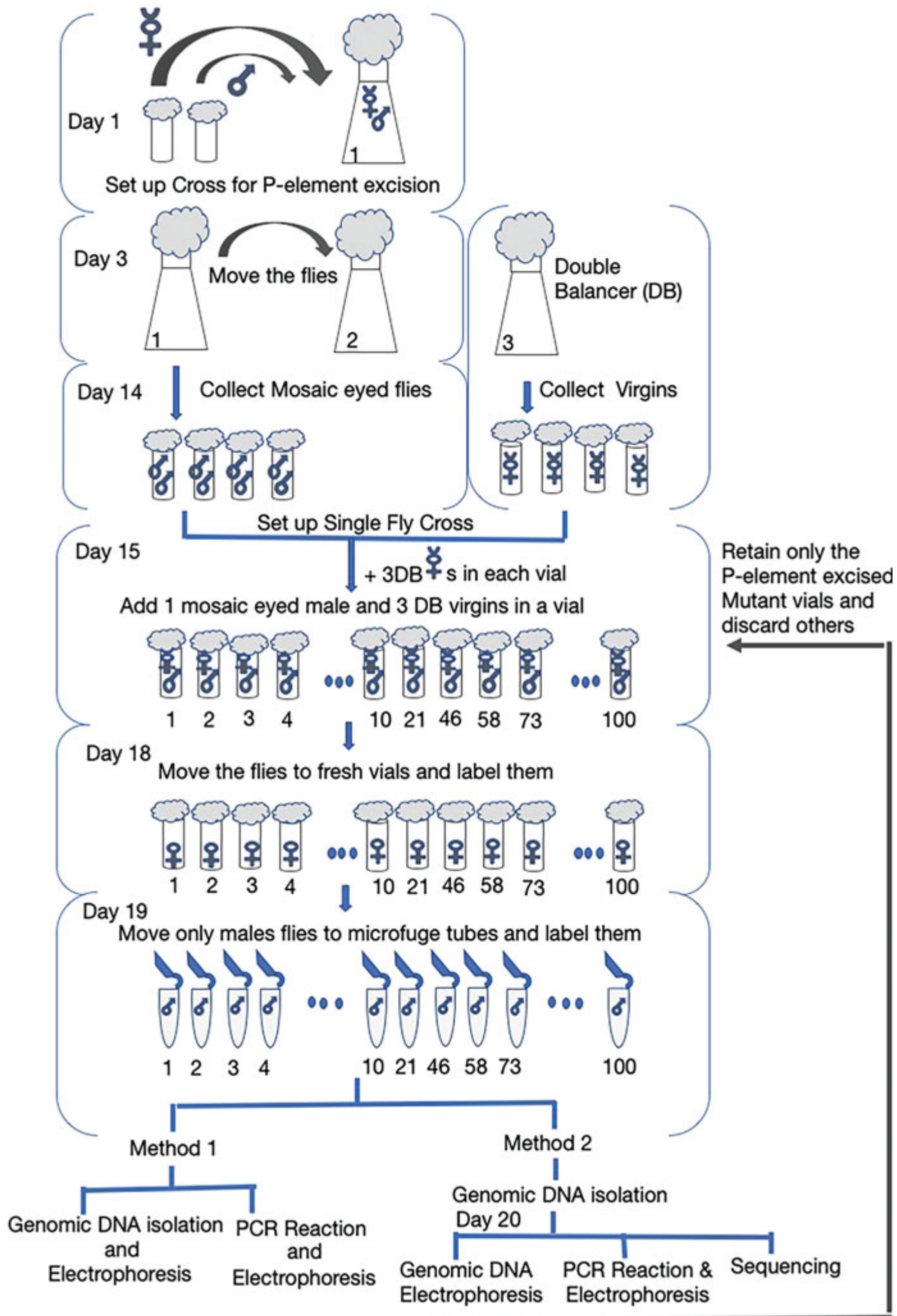

Fig. 1 Scheme for single fly genomic PCR. Genomic PCR and confirmation of mutation. See text for a detailed explanation of all protocol steps (*See* **Note 7**)

week's time) and simultaneously send the sample for sequencing before mutant analysis, Method 2 is suggested. The summary of the protocol and methods followed are shown in Fig. 1.

2. For electrophoresis of genomic DNA, 0.8–1% agarose gels are made. As genomic DNA is usually around 5–10 kb, using 0.8% gels give better separation or resolution. Similarly, 1–2% gels are preferred for smaller sized fragments of 0.2–1 kb (such as PCR products).
3. Primer sequences flanking the P-element regions are designed using standard software, such as Amplify 3.0.
4. Negative controls contain all the ingredients of the PCR mixture except the template. This is to ensure that the amplification of the PCR product is not due to contamination. As the number of samples increases, small volumes of reaction mixture stick to the pipettes and tubes during pipetting, and tend to reduce the total volume. Therefore it is always better to add two extra volumes of reaction mixture to overcome the loss due to pipetting errors.
5. Include a 1 kb DNA marker to run along with the samples. Run the samples in the same order in both the gels for easier comparison of samples between gels.
6. When genomic DNA samples from mutants are subjected to electrophoresis, as mutant flies have shorter DNA than the control WT/P-element containing flies, genomic DNA of mutant flies show faster mobility than the control flies. Figure 2 shows an example experiment. In Fig. 2, genomic DNA from mutants in lanes 6 and 7 (corresponding to mutants 21 and 73) showed faster mobility than control samples.

   For PCR products using primers flanking the P-element, the presence of a DNA fragment of expected size should be seen only in the control samples, but should be absent in mutant samples, and in negative controls; absence of the band would confirm the imprecise excision of the P-element to produce a mutation. At the same time, PCR products of expected size, amplified using the primers away from the P-element, should be seen in both mutants and control samples, other than the negative control.

   In the two PCR product gels in Fig. 2, lane 11 (the negative control) does not show any PCR product, as expected. Lanes 1 and 8 are control samples corresponding to wildtype and P-element flies. In both gels, PCR products of expected sizes of 583 and 185 bp are observed, thereby confirming that the PCR reaction has worked. Only mutant samples 21 and 73 show no band at 583 bp using primers flanking the P-element (upper PCR gel, Fig. 2) but show a band of the expected size (185 bp) using primers away from the P-element (lower PCR gel, Fig. 2). All other mutant samples (lanes 2–7) show bands in both the gels, indicating that the P-elements were not excised in these lines. This indicates that samples

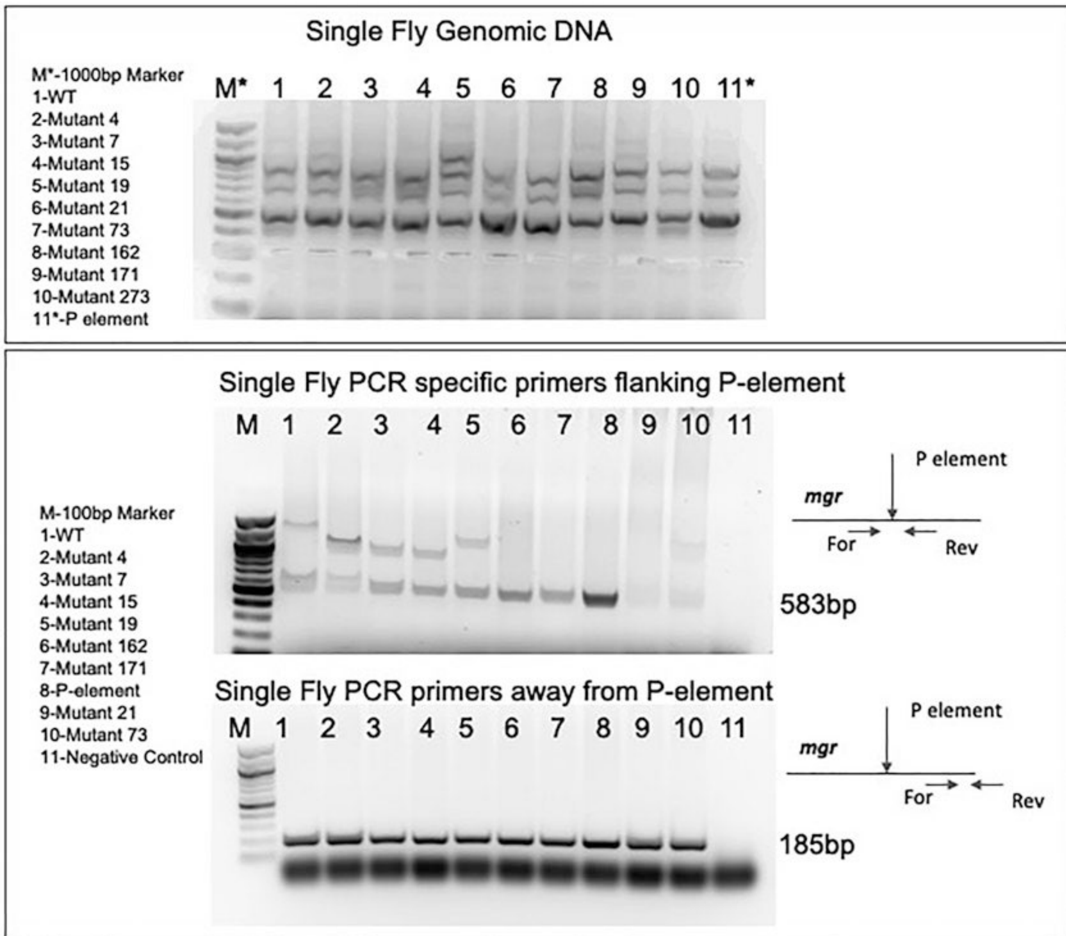

Fig. 2 Agarose gel electrophoresis of Single Fly Genomic DNA and PCR products. Genomic DNA from Mutants Lane 6 and 7 corresponding to Mutants 21 and 73 show faster mobility than other Mutants. Single Fly PCR was set up using the same samples using two sets of primers (one primer set flanking the P-element on gene of interest (mgr) and the other primer set away from the P-element) to confirm the presence of a mutation. In Lane 9 and 10 corresponding to Mutants 21 and 73, a PCR product of 583 bp is absent (upper PCR gel) but a PCR product of 185 bp is present in the lower PCR gel, confirming the excision of the P-element within these lines (*See* **Note 6**)

21 and 73 may be mutants generated due to P-element excision. This can be further confirmed by sequencing the genomic DNA and PCR products. Based on these results, all fly vials other than vials containing mutants 21 and 73 can be discarded and mutants 21 and 73 alone can be expanded for further analysis.

7. The above discussed protocol and method (summarized in Fig. 1) can be performed easily by an experienced researcher. However, for inexperienced researchers, such as undergraduate students, collecting virgins on a large scale and handling

100–200 mosaic-eyed male flies to set up single fly crosses will be difficult. Labeling all samples and duplicating them may be confusing. Instead, based on the experience we have gained during the tumor screening project [3], we revised and simplified the protocol. By setting up crosses on a smaller scale, in a staggered manner, using vials instead of bottles, we could perform a large-scale screen together with undergraduates, and generate novel genetic mutant lines, giving students valuable research experience.

For example, once virgin collection begins, we set up crosses every alternate day, on a small scale in a vial with only 6 females and 3 males. On the third day, the flies were transferred to a fresh vial and labeled correctly. This process of collecting virgins and setting crosses continued for a week to 10 days.

In this way, students are not overburdened and in case of mistakes, affected vials can be discarded.

In our laboratory, when cloning gene fragments into a vector, we employ a simple colony PCR method to screen for positive clones. We have modified and standardized a similar technique to use with fly stocks. This is a simple screening method to identify mutant fly lines. This method is easy to perform, saves time and resources, and can be modified to give undergraduate students valuable research experience. Another important advantage is that, as this method uses individual flies to identify independently excised events, mutant alleles of various strengths falling into different complementation groups are rapidly produced.

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