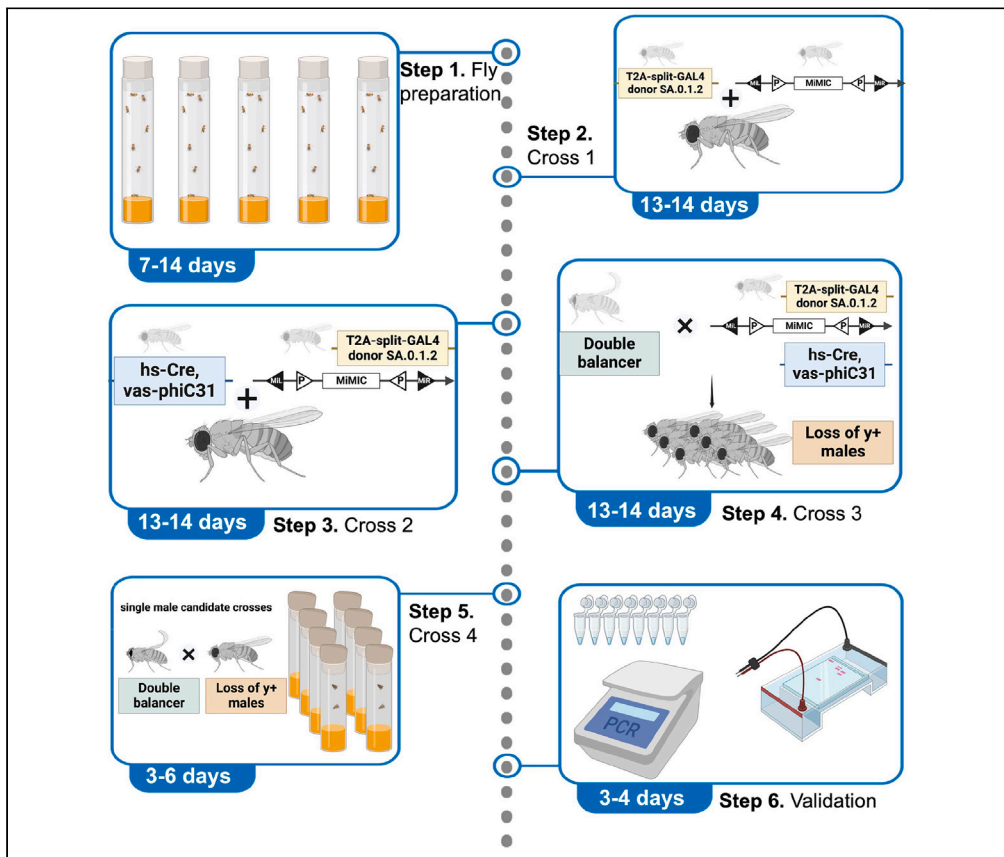


## Protocol

# Protocol for replacing coding intronic MiMIC and CRIMIC lines with T2A-split-GAL4 lines in *Drosophila* using genetic crosses



Here, we present a protocol for generating gene-specific split-GAL4 drivers from coding intronic Minos-mediated integration cassette/CRISPR-mediated integration cassette (MiMIC/CRIMIC) lines in *Drosophila*. We describe steps for four rounds of *in vivo* genetic crosses, PCR genotyping, and fluorescence imaging to ensure correct orientation of split-GAL4 integration before establishing stable fly stocks. This protocol offers a cost-effective alternative to traditional microinjection techniques for converting coding intronic MiMIC/CRIMIC lines into gene-specific split-GAL4 lines that are adaptable for fly researchers working on different tissues.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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### Highlights

Step-by-step guide to  
create gene-specific  
split-GAL4 lines by  
genetic crosses

Detailed steps for  
PCR genotyping to  
ensure correct split-  
GAL4 integration

Cost-saving  
alternative to  
microinjection to  
generate gene-  
specific split-GAL4  
lines

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## Protocol

Protocol for replacing coding intronic MiMIC and CRIMIC lines with T2A-split-GAL4 lines in *Drosophila* using genetic crosses

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## SUMMARY

Here, we present a protocol for generating gene-specific split-GAL4 drivers from coding intronic Minos-mediated integration cassette/CRISPR-mediated integration cassette (MiMIC/CRIMIC) lines in *Drosophila*. We describe steps for four rounds of *in vivo* genetic crosses, PCR genotyping, and fluorescence imaging to ensure correct orientation of split-GAL4 integration before establishing stable fly stocks. This protocol offers a cost-effective alternative to traditional microinjection techniques for converting coding intronic MiMIC/CRIMIC lines into gene-specific split-GAL4 lines that are adaptable for fly researchers working on different tissues.

For complete details on the use and execution of this protocol, please refer to Chen et al.<sup>1</sup>

## BEFORE YOU BEGIN

This protocol below describes the specific steps for generating split-GAL4 drivers using coding intronic MiMIC insertion on the 3<sup>rd</sup> chromosome. However, this protocol also applies to fly lines that have MiMIC insertion on the 2<sup>nd</sup> chromosome or CRIMIC insertions on either 2<sup>nd</sup> or 3<sup>rd</sup> chromosome. The balancer lines and phenotypic markers are slightly different for different MiMIC/CRIMIC insertions,<sup>2–4</sup> which will be detailed in a separate paragraph at the end of this protocol.

## Fly husbandry: Amplify fly lines of interest

⌚ Timing: 1–2 weeks

The fly stocks required for this protocol include the split-GAL4 donor, MiMIC/CRIMIC insertion, Cre recombinase & phiC31 integrase, double balancers, and the UAS-GFP strains (details can be found in the [key resources table](#)). Amplify these lines and collect enough virgin female flies to start the crosses. All the fly lines are reared at 25°C unless otherwise specified.

1. Order fly stocks from Bloomington Drosophila Stock Center (<https://bdsc.indiana.edu>) and verify that the flies show correct phenotypic markers as described in the genotypes.



△ **CRITICAL:** Phenotypic markers are essential for tracking transgenes for desired genotypes in *Drosophila* crosses. Incorrect phenotypic markers may indicate stock contamination.

2. Flip (transfer adults to vials containing fresh food) and amplify the fly lines.
  - a. For lines that need virgin females, we recommend flipping the flies three times a week (e.g., Mon, Wed and Fri) to maximize the number of progenies.
  - b. For lines that only need males, we recommend flipping once or twice a week (e.g., Tue and Fri). Keeping a regular flipping frequency avoids larval overcrowding and improves culturing conditions.

### Buffer preparations (gDNA extraction buffer for genotyping PCR and TAE buffer for gel electrophoresis)

⌚ Timing: 20 min

3. Prepare 250 mM NaCl (10× stock solution) by adding 50 mL ddH<sub>2</sub>O with 0.73 g NaCl. Dilute the 100× TE buffer and 10× NaCl stock solution to make 1× for gDNA extraction buffer. See the [materials and equipment](#) section for details.

△ **CRITICAL:** Do not add proteinase K (Thermo Fisher Scientific, Cat#EO0492) in this step. Proteinase K should be added freshly right before use.

4. Dilute the 50× TAE buffer to 1× for gel electrophoresis.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Agar	LabScientific	Cat#FLY-8020
Yeast	LabScientific	Cat#FLY-8040
Yellow cornmeal	Indian Head	N/A
Tegosept	LabScientific	Cat#FLY-5501
Molasses	Grandma's	N/A
Propionic acid	Fisher Chemical	Cat#A258-500
Ethanol (200 proof)	Decon Labs	Cat#2701G
100× TE buffer	EMD Millipore	Cat#574793-1L
Sodium chloride	Fisher Chemical	Cat#S671-3
Proteinase K	Thermo Fisher Scientific	Cat#EO0492
Nuclease-free water	Invitrogen	Cat#AM9937
Agarose	Fisher BioReagents	Cat#BP1356-500
50× TAE electrophoresis buffer	Thermo Scientific	Cat#B49
Ethidium bromide (1% solution)	Fisher BioReagents	Cat#BP1302-10
Critical commercial assays		
AccuPower Taq PCR PreMix	Bioneer	Cat#K-2601
GeneRuler 1 kb Plus DNA Ladder	Thermo Scientific	Cat#SM1331
Experimental models: Organisms/strains		
<i>D. melanogaster</i> : yw; lf/CyO; P{pC-(lox2-attB-T2A-GAL4DBD.0)}/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw; P{pC-(lox2-attB-T2A-GAL4DBD.0)}/CyO; TM2/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw; lf/CyO; P{pC-(lox2-attB-T2A-GAL4DBD.1)}/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw; P{pC-(lox2-attB-T2A-GAL4DBD.1)}/CyO; TM2/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw; lf/CyO; P{pC-(lox2-attB-T2A-GAL4DBD.2)}/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw; P{pC-(lox2-attB-T2A-GAL4DBD.2)}/CyO; TM2/TM6B	Desplan lab	N/A

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>D. melanogaster</i> : yw; If/CyO; P{pC-(lox2-attB-T2A-VP16.0)}/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw; P{pC-(lox2-attB-T2A-VP16.0)}/CyO; TM2/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw; If/CyO; P{pC-(lox2-attB-T2A-VP16.1)}/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw; P{pC-(lox2-attB-T2A-VP16.1)}/CyO; TM2/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw; If/CyO; P{pC-(lox2-attB-T2A-VP16.2)}/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw; P{pC-(lox2-attB-T2A-VP16.2)}/CyO; TM2/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw, {y <sup>+</sup> , hs-Cre}, {vas-phiC31}; +; TM2/TM6B	Desplan lab	N/A
<i>D. melanogaster</i> : yw, {y <sup>+</sup> , hs-Cre}, {vas-phiC31}; Sp/CyO; +	Desplan lab	N/A
<i>D. melanogaster</i> : yw; Sp/CyO; Dr/TM3	Desplan lab	N/A
<i>D. melanogaster</i> : yw; Tub-VP16AD, UAS-2xEGFP; Dr/TM3	BDSC	BDSC #60295
<i>D. melanogaster</i> : yw; Tub-GAL4DBD; UAS-2xEGFP	BDSC	BDSC #60298
<i>D. melanogaster</i> : yw; +; Mi{MIC, y <sup>+</sup> }XXX <sup>M12345</sup>	N/A	N/A
<i>D. melanogaster</i> : yw; Mi{MIC, y <sup>+</sup> }XXX <sup>M12345</sup> ; +	N/A	N/A
<b>Oligonucleotides</b>		
PCR primer 1 (Mi-L): 5'->3' GCGTAAGCTACCTTAATCTCAAGAAGAG	IDT	N/A
PCR primer 2 (GAL4DBD): 5'->3' ACTGGCTCACCTCGTTCTCC	IDT	N/A
PCR primer 2 (VP16): 5'->3' CATCCAGGTGCAGCTCATCG	IDT	N/A
PCR primer 3 (Mi-R): 5'->3' CGCGGCGTAATGTGATTTACTATCATAC	IDT	N/A
<b>Other</b>		
Dissecting stereo microscope	Leica Microsystems	Leica M80
Fluorescence stereo microscope	Leica Microsystems	Leica MZFLIII
Tabletop centrifuge	Stratagene	PicoFuge microcentrifuge 400550
Agarose gel imager	Bio-Rad	ChemIDoc XRS+ Imager Cat#170-8265
Thermocycler	Eppendorf	Mastercycler Pro
Electrophoresis system	Thermo Scientific	Owl EasyCast
25°C incubator	Thermo Scientific	Heratherm IGS60

**MATERIALS AND EQUIPMENT**

**gDNA extraction buffer**

Reagent	Final concentration	Amount
100x TE buffer (Tris-HCl/EDTA)	10 mM (Tris-HCl)/1 mM (EDTA)	1 mL
NaCl (10x, 250 mM)	25 mM	10 mL
ddH <sub>2</sub> O	N/A	89 mL
<b>Total</b>	<b>N/A</b>	<b>100 mL</b>

The buffer can be stored at room temperature (around 20°C–23°C) for up to 1 year.

**Fly food recipe**

Reagent	Final concentration	Amount
Agar	0.48%	5.4 g
Yeast	1.51%	17.1 g
Yellow cornmeal	3.59%	40.8 g
30% Tegosept solution in ethanol	0.54%	6.1 mL
Molasses	5.29%	42.1 mL
Propionic acid	0.49%	5.6 mL
Distilled H <sub>2</sub> O	N/A	1000 mL
<b>Total</b>	<b>N/A</b>	<b>1135 g</b>

The fly food can be stored at 25°C for up to 2 weeks, or at 4°C for up to 1 month.

**Note:** 30% Tegosept solution can be made freshly by adding 23.7 g Tegosept into 100 mL ethanol.

## STEP-BY-STEP METHOD DETAILS

### Cross 1: Building a fly strain that contains both MiMIC insertion (on the 3<sup>rd</sup> chromosome) and split-GAL4 donor (on the 2<sup>nd</sup> chromosome)

⌚ **Timing:** 15–30 min to set up genetic crossing, followed by 10–13 days for incubation. Once the F1 progeny flies come out, it takes around 30–45 min in 2–3 consecutive days for collecting the male progeny with appropriate genotypes.

To build a fly strain that contains both MiMIC insertion and split-GAL4 donor transgenes for the *in vivo* swapping, male flies with MiMIC insertion on the 3<sup>rd</sup> chromosome are crossed with virgin female flies with split-GAL4 donor (VP16/DBD) on the 2<sup>nd</sup> chromosome. Since many of the existing gene-specific split-GAL4 lines are either DBD or VP16 for a given gene,<sup>1</sup> we therefore recommend setting up crosses for both VP16 and DBD split-GAL4 swapping at the same time for each gene to maximize the versatility of pairing with other existing split-GAL4 hemi-drivers.

1. Collect approximately 10 virgin female flies from the donor stock *yw*; *pC-(lox2-attB-T2A-split-GAL4)/(CyO)*; *TM2/TM6B*.
  - a. Select lines with appropriate split-GAL4 splicing phase (DBD.0/1/2 or VP16.0/1/2) based on the target gene MiMIC insertion.
  - b. Verify the split-GAL4 donor stock has *white*<sup>+</sup> (from split-GAL4 donor transgene) with the 3<sup>rd</sup> chromosome balanced (e.g., *TM2/TM6B* in this case). The presence of *CyO* balancer is optional, depending on whether the split-GAL4 donor transgene is homozygous or not.
    - i. Check for the phenotypes corresponding to the markers in the split-GAL4 donor stock, namely: *white*<sup>+</sup> (red/orange eye color), *Curly* (curly wings), *Humeral* (extra macrochaetes), and *ebony* (darker cuticle pigmentation).

⚠ **CRITICAL:** Females must be virgin, otherwise the F1 progenies will be mixed with flies of the original stock genotypes. We recommend collecting virgin females, storing them for 4–5 days in a separate food vial, and only setting up the crosses when there are no larvae in the vial.

⚠ **CRITICAL:** The splice acceptor nomenclature in the split-GAL4 donor genotype is indicated by the number followed by DBD or VP16. For example, DBD.0 indicated the GAL4DBD donor with a splicing phase 0. It is important to note that a correct splicing phase of the split-GAL4 donor (DBD.0/1/2 or VP16.0/1/2) should be chosen so that the split-GAL4 donor can be spliced in-frame with the transcript of the target gene.

**Note:** Readers are encouraged to refer phenotypes associated with genetic markers and balancers to this book: *Atlas of Drosophila Morphology: Wild-type and Classical Mutants*.<sup>5</sup>

**Note:** The information about the coding intronic MiMIC/CRIMIC insertion phase can be found in the GDP Screen Database (<https://flypush.research.bcm.edu/pscreen/about.html>). For example, MiMIC insertion MI11085 is inserted in the *dpr5* coding intronic region and captures both *dpr5*-RA and *dpr5*-RB RNA transcripts with splicing phase 1 (*dpr5*-RA:1, *dpr5*-RB:1).

**Note:** In some cases, the MiMIC-inserted gene has multiple splicing isoforms which require different splicing phases of the split-GAL4 donor (e.g., *Dscam1*<sup>MI07658</sup>).

2. Collect approximately 5 male flies from the MiMIC stock *yw/Y*; *+*; *Mi{MIC, y<sup>+</sup>}XXX*<sup>MI12345</sup>

**Note:** Mi{MIC, y<sup>+</sup>}XXX<sup>MI12345</sup> transgene refers to a hypothetical example of MiMIC insertion (MI12345) in the hypothetical target gene (XXX) throughout the protocol.

**Alternatives:** This protocol is designed for generating a large batch of split-GAL4 hemi-drivers. If only a few target genes are needed, collect virgin females from the MiMIC stock and collect males from the donor stock. The reciprocal cross simply gives users a flexibility to perform the crosses and has no impact on the results.

3. Place the virgin female flies from step 1 and the male flies from step 2 in a vial with fresh fly food.
4. Incubate at 25°C.
  - a. Flip the parent flies into fresh fly food every 3–4 days for two weeks. Keep both the old vials (containing eggs and larvae) and the new vials (containing the adult parent flies).

⚠ **CRITICAL:** Remember to pass the crosses regularly (every 3–4 days) so that no F1 progenies are mixed with parent flies.

**Note:** Adult flies emerge within about 10 days after the initial cross set up at 25°C. This is generally applicable to all the genetic crosses in this protocol.

5. From the F1 progenies of the first cross, select approximately 5–10 male flies with split-GAL4 donor on the 2<sup>nd</sup> chromosome and MiMIC insertion on the 3<sup>rd</sup> chromosome.
  - a. Discard female flies, select males for *Humeral* (extra humeral macrochaetes) and against *Curly* (curly wings) genetic phenotypic markers.
  - b. All the selected males should carry *white*<sup>+</sup> (red/orange eye, from split-GAL4 donor) and *yellow*<sup>+</sup> (non-yellow body color, from the MiMIC insertion).

**Note:** Selecting against a genetic marker means we select flies without certain genetic markers. In this example, we select for straight wing flies that do not carry CyO balancer.

**Note:** *Humeral* is the genetic dominant phenotypic marker for TM6B balancer.

**Note:** Practically, TM6B (*Humeral*) is preferred over TM2, because the genetic dominant phenotypic marker in TM2 (*Ultrabithorax*) is harder to identify. However, in some cases, TM2 might be preferred over TM6B, depending on the location of the MiMIC insertion.

### Cross 2: Adding additional transgenes that allow for Cre-mediated excision of the split-GAL4 donor and recombinase-mediated cassette exchange (RMCE) *in vivo*

⌚ **Timing:** 15–30 min to set up genetic crossing, followed by 10–13 days for incubation, then 30–45 min in 2–3 consecutive days for progeny collection

The goal of this step is to introduce heat shock inducible Cre recombinase (hs-Cre) and germline expressing phiC31 integrase (vas-phiC31) to the system for the *in vivo* swapping.

6. Collect approximately 10–15 virgin female flies from the stock yw, {y<sup>+</sup>, hs-Cre}, {vas-phiC31}; +; TM2/TM6B.

⚠ **CRITICAL:** To facilitate balancing the converted split-GAL4 transgenes, the stock line (hs-Cre, vas-phiC31) needs to be balanced on the chromosome of targeted MiMIC/CRIMIC insertion (in this example case, the 3<sup>rd</sup> chromosome needs to be balanced with TM2/TM6B).

△ **CRITICAL:** The *hs-Cre*, *vas-phiC31* dual-recombinase transgene must be carried on the X chromosome of the female fly, as the X chromosome of the male progenies (collected in step 9) is from the female parent. The *hs-Cre* transgene is thought to be constitutively and ubiquitously expressed even in the absence of heat shock.

7. Place the virgin female flies from step 6 and the male flies from step 5 in a vial with fresh fly food.
8. Incubate at 25°C.
  - a. Flip the parent flies into fresh fly food every 3–4 days for two weeks. Keep both the old vials (containing eggs and larvae) and the new vials (containing the flies).
9. Select approximately 10–15 male flies with *hs-Cre*, *vas-phiC31/Y* on the 1<sup>st</sup> chromosome, donor construct on the 2<sup>nd</sup> chromosome, and MiMIC insertion on the 3<sup>rd</sup> chromosome from the F1 progenies of the cross 2.
  - a. Discard female flies, select males for *white*<sup>+</sup> (red/orange eye color), *Humeral* (extra macrochaetes) and against *ebony* (darker cuticle pigmentation).

**Note:** TM2 and TM6B balancers carry the recessive phenotypic marker *ebony*, so darker cuticle pigmentation indicates that the fly carries TM2/TM6B on the 3<sup>rd</sup> chromosome.

### **Cross 3: Excise and circularize the split-GAL4 donor for recombinase-mediated cassette exchange (RMCE) *in vivo***

⌚ **Timing:** 15–30 min to set up genetic crosses, followed by 10–13 days for incubation, then 1–2 h in 2–5 consecutive days for progeny collection

The selected male flies from step 9 will be crossed with double balancer virgin females. The constitutively active expression of Cre recombinase will excise and circularize the split-GAL4 donor cassette from its original locus and the RMCE can be further mediated by phiC31 integrase driven by vasa promoter in the germline to replace the targeted MiMIC/CRIMIC insertion with split-GAL4 cassette.

10. Select approximately 20 virgin female flies from the double balancer stock *yw; Sp/CyO; Dr/TM3*.
11. Place the virgin females from step 10 and the male flies from step 9 in a vial with fresh fly food.
12. Incubate at 25°C.
  - a. Flip the parent flies into fresh fly food every 3–4 days for two weeks.
  - b. Keep the new vials (containing the adult flies) and move the old vials (containing the eggs and larvae) at 25°C.

△ **CRITICAL:** Check adult fly number after each flipping and replenish the adult flies (virgin females from step 10 and the male flies from step 9) as needed. Therefore, it is crucial to keep Cross 2 (step 7) ongoing until successful split-GAL4 candidates are verified.

13. Select all the F1 male progenies that have split-GAL4 donor successfully inserted to the target gene (regardless of the integration orientation).
  - a. Select the male flies against *Humeral* (extra macrochaetes), discard all the female flies and other male flies.
  - b. Within the male flies selected in sub-step a), select flies with loss of *yellow*<sup>+</sup> (*yellow*<sup>-</sup>, paler cuticle pigmentation).

△ **CRITICAL:** Sub-step a) must be done before b), as the *TM3/TM6B* flies containing *ebony* marker will confound the identification of *yellow* marker.

**Note:** The yellow phenotype marks for the loss of *yellow*<sup>+</sup> on MiMIC insertion, indicating a successful RMCE event between MiMIC and split-GAL4 donor.

**Note:** Collect as many male flies as possible to maximize the success rate of identifying flies with the correct insertion orientation of the split-GAL4 donor. Selecting flies with the loss of *white*<sup>+</sup> is not necessary since the split-GAL4 donor can be removed in the cross 4.

### Cross 4: Set up single male candidate male crosses for genotyping PCR

⌚ **Timing:** 30–45 min to set up genetic crosses, followed by 3–6 days for incubation

Individual male flies collected in the step 13 will be crossed with double balancer virgin females. The goal is to pass down the transgene of MiMIC/CRIMIC swapped with split-GAL4 in each selected male fly. This step is a prerequisite for genotyping PCR to verify the orientation of the swapped split-GAL4 cassette (same or opposite with respect to the orientation of the target gene).

14. Select approximately 30 virgin female flies from the double balancer stock *yw; Sp/CyO; Dr/TM3*.
15. Set up single-male fly crossing.
  - a. Place individual male flies from step 13 in separate vials with fresh fly food.
  - b. Place 2–3 virgin female flies from step 14 to every vial in a).

⚠ **CRITICAL:** Make sure there is only one male fly in each vial. The candidate split-GAL4 insertion in the progenies must be identical among the F1 generation and to the parent, so that we can infer the orientation of donor in the progeny flies by sacrificing and testing the parent.

16. Incubate at 25°C.

**Note:** Larvae will be visible within 3–6 days after the initial crossing.

17. When larvae are visible, anesthetize adult flies by CO<sub>2</sub> and isolate the single male fly into a 0.2 mL PCR microtube. The female flies can be either transferred into the new fresh fly food vial or remained in the old fly food vial. The original food vials with larvae can be moved to room temperature (around 20°C–23°C) to slow down the growth while waiting for the genotyping PCR result (steps 21–26).

⏸ **Pause point:** The male flies can be stored at -20°C. gDNA extraction can be done on another day when more samples of male flies are collected. It is recommended to extract gDNA within a week of collecting the male flies at -20°C. The extracted DNA can be stored at 4°C for at least two weeks.

**Note:** If no larvae came out from the single fly crosses, discard the cross and screen other candidates. If the male fly is dead in the food vial, extracting genomic DNA from the dead male will work as long as the larvae in the single fly crosses are visible.

### Adjustment for targeting MiMIC insertion on the 2<sup>nd</sup> chromosome

**Note:** *yw; lf/CyO; pC-(lox2-attB-T2A-split-GAL4)/TM6B* flies are used in Step 1.

**Note:** *yw, {y<sup>+</sup>, hs-Cre}, {vas-phiC31}; Sp/CyO; +* flies are used in Step 6.

**Note:** Select the male flies for *yellow* (paler cuticle pigmentation) and either *Sternopleural* (extra number of bristles on the sternopleurite) or *Curly* (curly wings) in Step 13, discard all flies that have both *Sternopleural* and *Curly*,



### Adjustment for targeting CRIMIC insertion

**Note:** CRIMIC insertion has 3XP3-GFP marker (eye/ocelli-specific expression of GFP). In step 13, select the male flies for loss of 3XP3-GFP under the fluorescence microscope. The loss of 3XP3-GFP marker indicates a successful RMCE event between CRIMIC and split-GAL4 donor.

### Single fly gDNA extraction

⌚ **Timing:** 1–1.5 h

In this step, genomic DNA will be extracted from single adult male fly.

18. Prepare gDNA squishing buffer (gDNA extraction buffer + Proteinase K).
  - a. Add 10  $\mu$ L of Proteinase K to every 1 mL of squishing buffer (1:100 ratio).
  - b. Each fly requires 50  $\mu$ L of gDNA squishing buffer (e.g., prepare 1000  $\mu$ L of gDNA extraction buffer for 20 flies)
  - c. Vortex the tube to mix well.

**⚠ CRITICAL:** Proteinase K should be stored at  $-20^{\circ}\text{C}$  and must be added to the gDNA extraction buffer freshly right before using.

19. Homogenize the adult flies in the gDNA squishing buffer.
  - a. Draw up 50  $\mu$ L of gDNA squishing buffer using P200 pipette tip.
  - b. Homogenize the fly tissue by crushing the adult male using the pipette tip without expelling any liquid. It is normal that a small amount of liquid will still leak from the pipette tip.
  - c. Dispense all 50  $\mu$ L gDNA squishing buffer to the 0.2 mL PCR microtube.
  - d. Spin down the tubes in a microcentrifuge.

**Note:** To completely crush the flies, smash the fly before it floats around in the liquid. Usually, it takes 30 seconds to effectively homogenize the fly.

20. Perform gDNA extraction.
  - a. Incubate at  $37^{\circ}\text{C}$  for 30 min followed by  $95^{\circ}\text{C}$  for 3 min to inactivate the Proteinase K reaction.
  - b. Spin down the fly debris in a microcentrifuge and use the supernatant containing gDNA for PCR reaction.

**Note:** This reaction can be performed in a thermocycler or any heat block/water bath that fit PCR microtubes.

**⏸ Pause point:** The gDNA can be stored at  $4^{\circ}\text{C}$  for at least two weeks. Note that the gDNA solution should be re-spun down right before the PCR step. We recommended to proceed genotyping PCR in a week so that users can know which candidate crosses should be maintained.

### Genotyping PCR

⌚ **Timing:** 2–2.5 h

This section describes steps to validate the successful insertion of split-GAL4 donor cassette and to determine the orientation of the cassette integration. The orientation is critical because in-frame translation of the split-GAL4 cassette with the gene-of-interest's preceding exon can only be achieved when the orientation of the split-GAL4 is the same as that of the gene-of-interest. PCR will be performed on the gDNA extracted in Steps 18–20.

**Table 1. PCR reaction 1 (Total volume 20  $\mu$ L/reaction)**

Reagent	Amount
gDNA template	1 $\mu$ L
Lyophilized PCR premix	N/A
Primer 1: MiL (10 $\mu$ M)	1 $\mu$ L
Primer 2: GAL4DBD/VP16 (10 $\mu$ M)	1 $\mu$ L
Nuclease-free water	17 $\mu$ L

21. Set up the PCR reaction.

- Add reagents to a PCR tube as described in [Table 1](#).
- Flick the tube to mix all contents, then spin down in a microcentrifuge to make sure all the liquid remains at the bottom.

**Note:** Two PCR reactions (reaction 1 & 2, as detailed in [Tables 1](#) and [2](#)) with two different primer sets are required for each gDNA sample to determine the split-GAL4 insertion orientation.

**Note:** Flies with GAL4DBD transgenes (e.g., BDSC# 82987, wg[Sp-1]/CyO; Mi{Trojan-GAL4DBD.2}Gad1[Mi09277-TG4DBD.2]/TM6B, Tb[1]) or with VP16 transgene (e.g. BDSC# 60326 y[1] w[\*]; Mi{Trojan-dVP16AD.1}Shaw[Mi01735-TdVP16AD.1]) can be used as a positive controls. A negative control without adding gDNA should be included in PCR reaction.

**Optional:** Master mix solutions can be prepared for large number of gDNA samples that require the same primer sets. Combine forward primers: reverse primers: nuclease-free water in 1:1:17 ratio for the amount needed. Set up PCR reaction by adding 19  $\mu$ L of master mix solutions and 1  $\mu$ L of gDNA template to a PCR tube containing lyophilized PCR premix.

**Alternatives:** The lyophilized PCR premix is used in this protocol because it has dNTPs, enzymes, buffer, and tracking dye premixed. Other PCR reagents or kits can also be used according to manufacturer's instructions.

22. Run the PCR reaction in the thermocycler.

- Refer to the program shown in [Table 3](#).
- After the reaction, spin down the tubes in a microcentrifuge.

**Pause point:** The PCR product can be stored at 4°C for at least a week, and gel electrophoresis can be done on another day.

### Gel electrophoresis and imaging

⌚ Timing: 1.5–2 h

This step will enable the visualization of the PCR results and thus help to determine the orientation of the split-GAL4 insertion into coding intronic MiMIC lines. 1% agarose gel will be used for electrophoresis.

23. Prepare the 1% agarose gel.

- Calculate the desired amount of 1 $\times$  TAE buffer and agarose. The amount of 1 $\times$  TAE buffer and agarose used is in 10 mL: 0.1 g ratio. For example, a 2  $\times$  24 wells gel needs 150 mL 1 $\times$  TAE buffer and 1.5 g agarose.
- Add the desired amount of agarose and 1 $\times$  TAE buffer into the Erlenmeyer flask.
- Heat in microwave until boiling.

**Table 2. PCR reaction 2 (Total volume 20  $\mu$ L/reaction)**

Reagent	Amount
gDNA template	1 $\mu$ L
Lyophilized PCR premix	N/A
Primer 3: MiR (10 $\mu$ M)	1 $\mu$ L
Primer 2: GAL4DBD/VP16 (10 $\mu$ M)	1 $\mu$ L
Nuclease-free water	17 $\mu$ L

- d. Once it boils, swirl it to help dissolve the remaining agarose.
- e. Repeat steps c) and d) 2 more times.
- f. Wait until the liquid is warm, add the desired volume of 1% ethidium bromide to the agarose solution in the Erlenmeyer flask (1.5  $\mu$ L of 1% ethidium bromide per 50 mL gel). Swirl the flask to distribute the ethidium bromide into the solution.
- g. Pour the mixture into the desired-size gel mold and add combs.
- h. Let the gel cool for more than 30 min.

**Note:** Ethidium bromide is carcinogenic, and the gel should be discarded according to each institution's regulations. Avoid touching the ethidium bromide-contaminated area without personal protective equipment.

**Note:** Use a rubber hand cover to swirl the hot flask in step d. Pay special attention to the liquid as it might undergo superheating in the microwave and boils over violently when swirling.

**Alternatives:** Several alternatives to ethidium bromide such as SYBR Safe DNA Gel Stain can be used according to the manufacturer's instructions.

**△ CRITICAL:** Make sure there are no air bubbles in the gel after adding combs to the gel mold. Air bubbles will distort the structure of the well and hinder the interpretation of the imaging result. If the air bubbles are present in the well, use a pipette tip to remove them.

24. Agarose gel electrophoresis.
  - a. Correctly orient the gel so that the lanes align with the electric field. The wells should be placed near the negative post.
  - b. Add 1 $\times$  TAE buffer to cover the gel and the wells.
  - c. Pipette 10  $\mu$ L of the 1 kb ladder (with loading dye added) into the leftmost well of each row.
  - d. Pipette 15–20  $\mu$ L of PCR sample into other wells of the gel.
  - e. Run the electrophoresis. Maintain the voltage between 90–120 V. The runtime depends on the size of the gel. Make sure the bands take up most of the lane.
25. Gel imaging.
  - a. Transfer the gel and the gel tray to the gel imager.
  - b. Slide gel onto the sample stage and center the gel on the monitor.
  - c. Configure the imager to ethidium bromide mode and image under UV transilluminator.
  - d. Save or print out the image as needed.

**Table 3. PCR cycling conditions**

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	1 min	1
Denaturation	95°C	30 s	30 cycles
Annealing	60°C	30 s	
Extension	72°C	45 s	
Final extension	72°C	5 min	1
Hold	4°C	forever	

**Note:** The gel containing ethidium bromide should be disposed to the designated area after imaging according to each institution's regulations.

### 26. Gel annotation

- a. Compare with positive controls of the different insertion orientation and determine the split-GAL4 insertion orientation of the samples.

## EXPECTED OUTCOMES

Once the single male cross is set up for three days, we can proceed to genotyping PCR on the male fly to test for the orientation of the split-GAL4 (VP16/DBD) insertion. Genes and transgenic insertions are denoted to be in the "plus" (+) or "minus" (-) orientation according to FlyBase nomenclature. A "plus" orientation indicates that the 5' to 3' DNA strand (from left to right) displayed in the FlyBase database; conversely, a "minus" orientation means that the 5' to 3' DNA strand is from right to left displayed in the FlyBase database. The expected outcome is to have the split-GAL4 inserted in the same orientation as the gene. For instance, if the gene follows + orientation, we then want the split-GAL4 insertion also to be in + orientation.

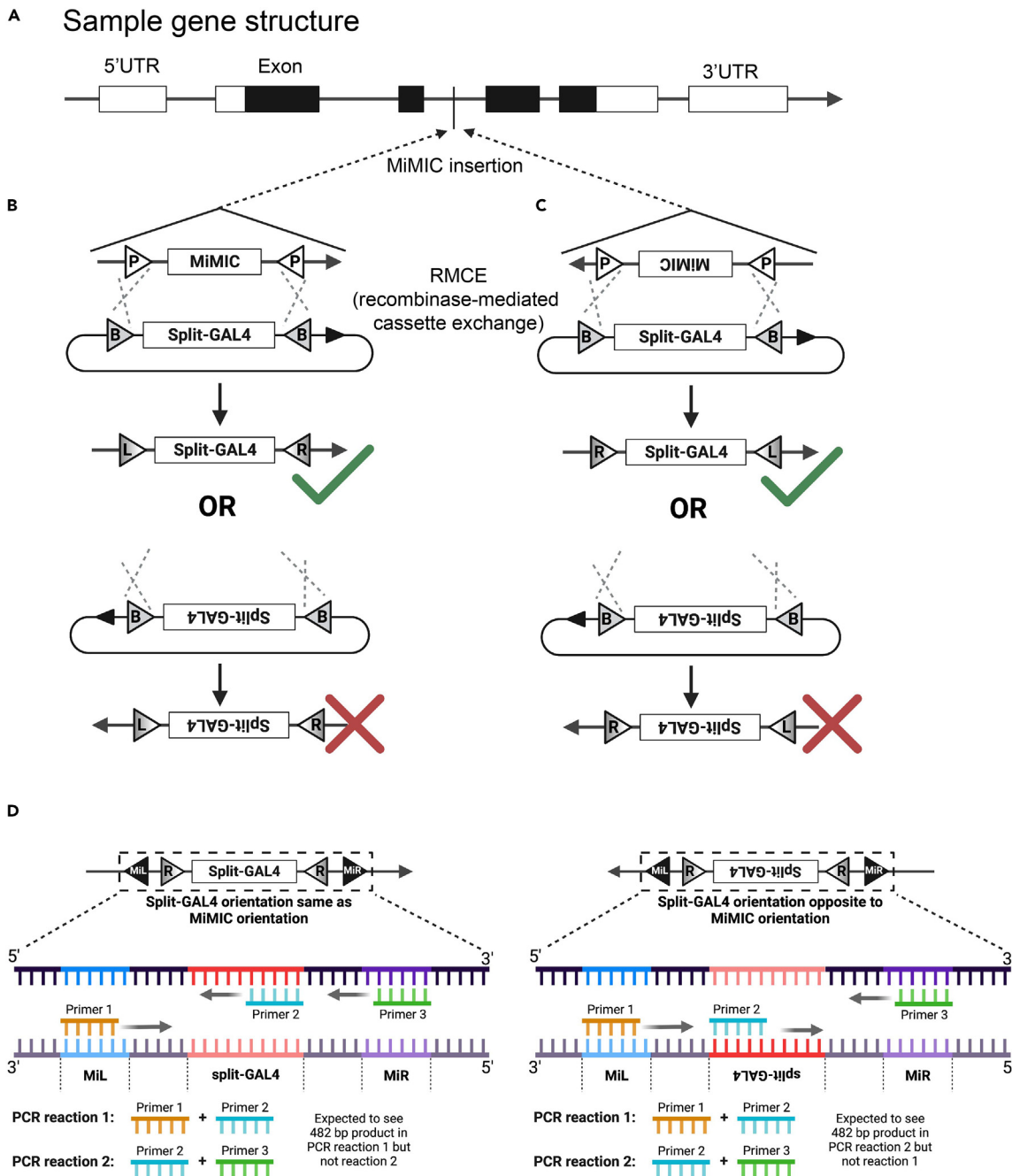
It should be noted that the PCR reactions in this protocol target the Minos inverted repeat (MiL/MiR) and the split-GAL4 donor (GAL4DBD/VP16); therefore, the PCR step only distinguishes whether the split-GAL4 orientation is the same (obtained 482 bp PCR products from reaction 1 only) or opposite (obtained 482 bp PCR products from reaction 2 only) with respect to the orientation of the MiMIC insertion (Figure 1). With the information of the MiMIC orientation, we can infer the orientation of the split-GAL4 insertion and compare it with the gene orientation.

Here, we use the CG14322<sup>[MI11688]</sup> MiMIC line as an example to show the expected PCR genotyping result. The CG14322 gene is encoded in the "+" orientation while the MiMIC<sup>[MI11688]</sup> insertion is in the "-" orientation. Therefore, the correct CG14322-split-GAL4 line should have the opposite orientation as the MiMIC insertion (i.e., obtaining PCR products from reaction 2, not reaction 1). In Figure 2C, we showed two male DBD split-GAL4 candidates (lanes 2–5) and two male VP16 split-GAL4 candidates (lanes 7–10) with loss of *yellow*<sup>+</sup> marker. PCR reactions 1 & 2 were run for each gDNA. The candidates #1 and #3 showed 482 bp band only in reaction 2, therefore, the split-GAL4 insertion is in the opposite orientation as the MiMIC insertion, i.e., in "+" orientation. The candidates #2 and #4 showed 482 bp band only in reaction 1, therefore, the split-GAL4 insertion is in the same orientation as the MiMIC insertion, i.e., in "-" orientation. In this case, the candidate #1 is the correct line for CG14322-T2A-GAL4DBD and the candidate #3 is the correct line for CG14322-T2A-VP16 (Figure 2C).

An additional step in the future is to test the validity of the gene-specific VP16/DBD hemi drivers by crossing it with flies carrying UAS-EGFP and constitutively expressing DBD/VP16 hemi driver<sup>6</sup> (e.g., BDSC# 60295, y[1] w[\*]; P{w[+mC] = Tub-dVP16AD.D}2; P{w[+mC] = UAS-2xEGFP}AH2; Dr [1]/TM3, Sb[+] and #60298, y[1] w[\*]; P{w[+mC] = Tub-GAL4DBD.D}2; P{w[+mC] = UAS-2xEGFP}AH3). We would expect to observe the progenies to have the expression of GFP for most of the genes under fluorescence microscopy. It takes 5–10 days for native GFP expression to be observed in the L3 larvae or adult flies, and an additional 5 days if immunofluorescence staining is required.

## LIMITATIONS

This protocol provides a step-by-step guide for generating gene-specific split-GAL4 hemi drivers via 4 rounds of genetic crossing (Figure 3). It provides an economical alternative to the microinjection method and enables the generation of split-GAL4 hemi drivers in batches from off-the-shelf fly lines. However, the recombinant transgenes (hs-Cre integrase and vas-phiC31 recombinase) are currently

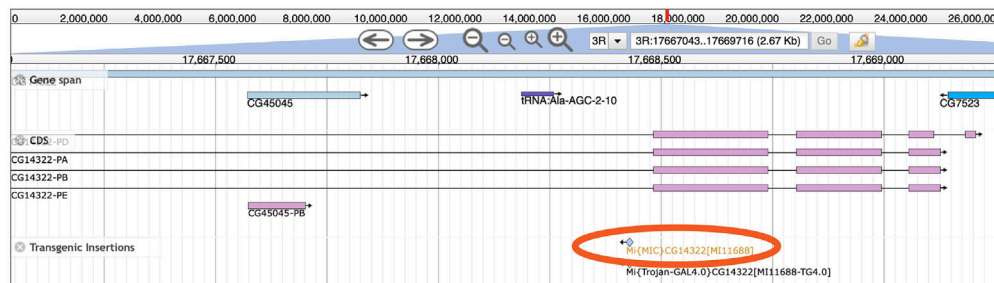


**Figure 1. Schematic representations of the MiMIC insertion and diagnostic primer design**

(A–C) Different scenarios of MiMIC insertion in relationship to the coding orientation of the targeted gene. (D) Locations of diagnostic primer sets and the expected PCR outcomes.

only available on the X chromosome, restricting the application of this protocol for MiMIC/CRIMIC insertions on the X chromosome. This limitation can be addressed by using fly lines that have *hs-Cre*, *vas-phiC31* recombinases on the 2<sup>nd</sup> or 3<sup>rd</sup> chromosome. To the best of our knowledge, the fly line is not directly available from Bloomington Drosophila Stock Center and should be generated beforehand. Furthermore, the crossing scheme and progeny selection criteria should be modified for the X-chromosome scenario.

**A** CG14322 gene region



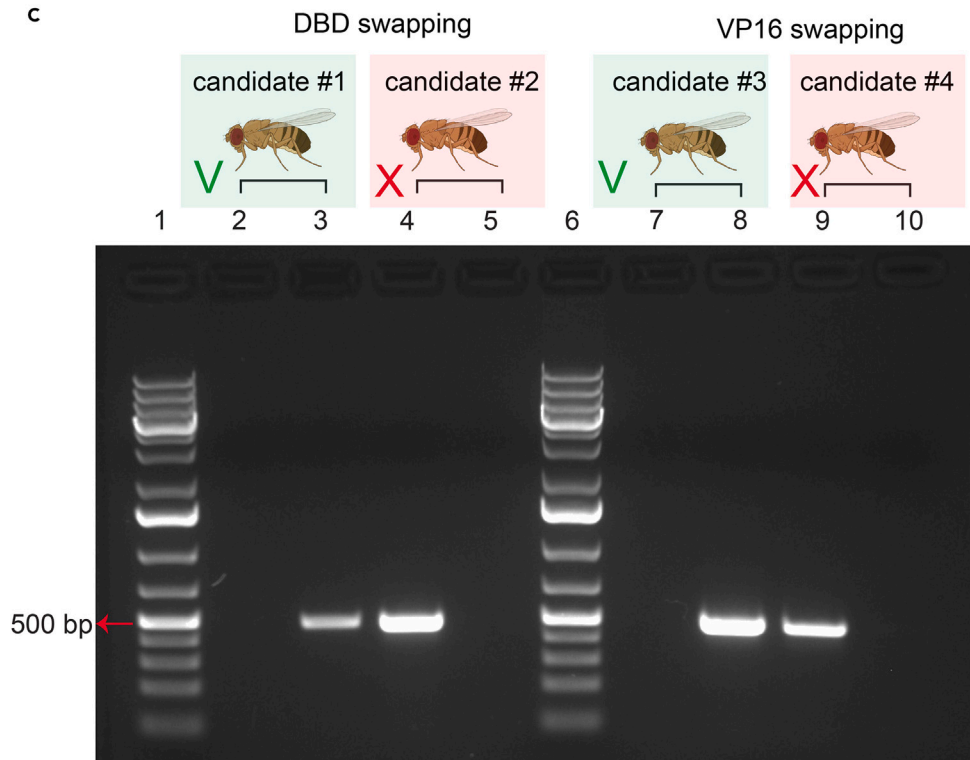
**B**

CG14322 coding sequence →

MiMIC[Mi11688] insertion ←

Desired split-GAL4 PCR result **Primer Set 2:** opposite as MiMIC

**C**



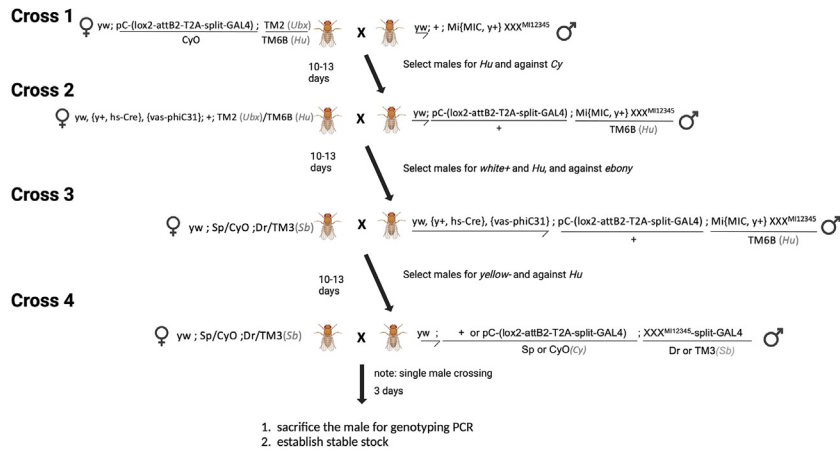
Correct lines: #1 for CG14322-T2A-GAL4DBD and #3 for CG14322-T2A-VP16

DNA Ladder: lanes 1, 6  
Primer set 1: lanes 2, 4, 7, 9  
Primer set 2: lanes 3, 5, 8, 10

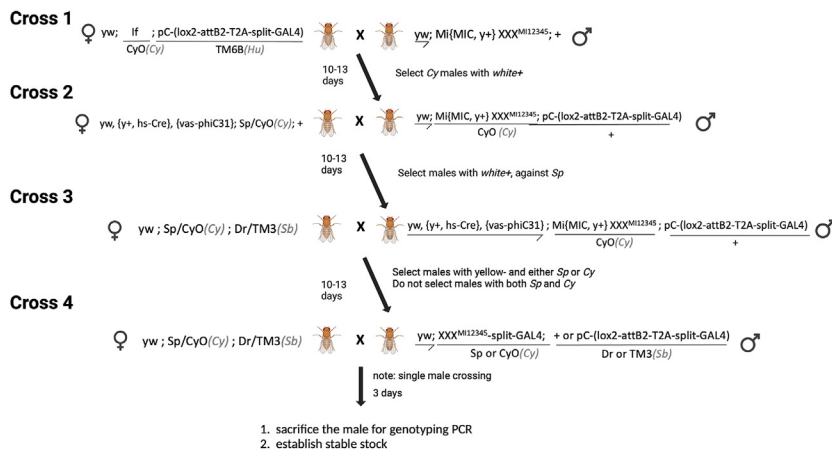
**Figure 2. Sample genotyping PCR result for CG14322 gene**

(A and B) Schematic representations of CG14322 coding sequence and the MiMIC[Mi11688] insertion.  
(C) Genotyping PCR result for CG14322-T2A-GAL4DBD and CG14322-T2A-VP16.

**A** Crossing scheme for MiMIC on third chromosome



**B** Crossing scheme for MiMIC on second chromosome



**Figure 3. Overview of the *in vivo* crossing steps**

Schematic representations of the crossing schemes for targeted MiMIC insertion on the third chromosome (A) or the second chromosome (B) to generate gene-specific split-GAL4 lines.

Another limitation is the expressivity of the MiMIC/CRIMIC transgene in some lines. Flies with MiMIC/CRIMIC insertion carry specific genetic markers ( $y^+$  for MiMIC lines and 3xP3-GFP for CRIMIC line) that can be identified. The selection of successful *in vivo* swapping between split-GAL4 donor and MiMIC/CRIMIC insertion is based on the loss of these markers. However, some MiMIC/CRIMIC insertion lines display very weak expression of the markers, making it impossible to screen for the lost-of-marker flies. In this case, screening all the male flies will increase the workload of the PCR significantly and delay the entire workflow. Lastly, MiMIC lines inserting at the 5'UTR, coding exon, and 3'UTR may not be used in this strategy.

**TROUBLESHOOTING**

**Problem 1**

No bands can be observed in either of the two genotyping PCR reactions. This might be due to failure in selecting the loss-of-markers flies that have successful *in vivo* swapping (step 13) or inadequate gDNA extraction (step 19).



## Potential solution

- Perform PCR with a primer pair targeting house-keeping genes (e.g., Act5C or Gapdh2) to test whether sufficient gDNA yields are obtained. The primers can be found in the <http://www.flyrnai.org/flyprimerbank>.<sup>7</sup>
- For MiMIC insertion flies, only select for flies with loss of *yellow*<sup>+</sup> (yellow body color) under the dissection microscope. For CRIMIC insertion flies, only select for flies with loss of 3xP3-GFP (eyes and ocelli have no green fluorescence) under the fluorescence microscope. Compare the candidate flies with others to make sure they really meet the selection criteria.
- Make sure the adult fly is completely crushed with the pipette tip in step 19 b.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yu-Chieh David Chen ([ycc4@nyu.edu](mailto:ycc4@nyu.edu)).

### Materials availability

The split-GAL4 donor fly lines are in the process of depositing at the Bloomington Drosophila Stock Center.

### Data and code availability

This study did not generate/analyze datasets/code.

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## AUTHOR CONTRIBUTIONS

Conceptualization, Y.-C.D.C.; methodology, Y.-C.D.C., S.A.L., H.G.L., and N.S.; investigation, Y.-C.D.C., S.A.L., H.G.L., and N.S.; validation, Y.-C.D.C.; formal analysis, Y.-C.D.C.; writing – original draft, S.A.L. and H.G.L.; writing – review and editing, Y.-C.D.C., S.A.L., and H.G.L.; visualization, Y.-C.D.C., S.A.L., and H.G.L.; supervision, Y.-C.D.C.; funding acquisition, C.D. and Y.-C.D.C.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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