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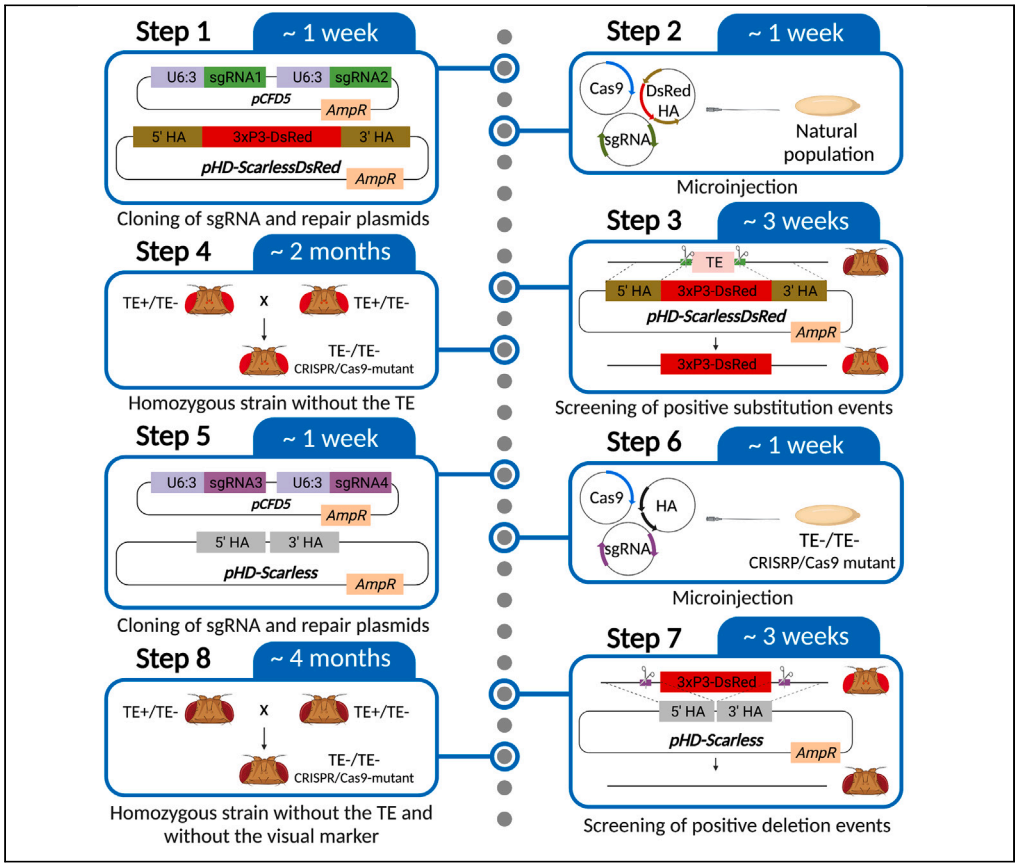
Two-step CRISPR-Cas9 protocol for transposable element deletion in *D. melanogaster* natural populations



We present a protocol for generating a precise deletion, without altering the genetic background of the strain, of a transposable element (TE) in a natural population of *Drosophila melanogaster* using two steps of CRISPR-Cas9 homology-directed repair. We describe steps for replacing the TE by a fluorescent marker and for subsequent marker removal using single-guide RNAs, repair plasmids, and microinjection. We also detail steps for screening the deletion of the TE and generating a homozygous mutant strain.

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

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Highlights

Generation of a precise deletion without altering the genetic background of the strain

The protocol allows for the visual screening of mutants in the two CRISPR editing steps

Deletion of a transposable element to study its functional impact in the genome

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Two-step CRISPR-Cas9 protocol for transposable element deletion in *D. melanogaster* natural populations

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SUMMARY

We present a protocol for generating a precise deletion, without altering the genetic background of the strain, of a transposable element (TE) in a natural population of *Drosophila melanogaster* using two steps of CRISPR-Cas9 homology-directed repair. We describe steps for replacing the TE by a fluorescent marker and for subsequent marker removal using single-guide RNAs, repair plasmids, and microinjection. We also detail steps for screening the deletion of the TE and generating a homozygous mutant strain.

For complete details on the use and execution of this protocol, please refer to Merenciano and Gonzalez.[1]

BEFORE YOU BEGIN

Natural population expansion

Timing: 2–4 weeks

This protocol has two microinjection steps. In the first one, a *Drosophila* natural population will be microinjected to perform CRISPR-Cas9 homology-directed repair to replace a TE by a fluorescent marker. Thus, we recommend expanding the *Drosophila* natural population before starting the protocol to have enough flies to lay eggs for the microinjection step.

Microinjection setup

Timing: 1–2 weeks

If in-house injection facilities are not available, contact a *Drosophila* microinjection company and send them the previously expanded flies to be microinjected.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
5-α competent *E. coli* cells	NEB	Cat # C2987H

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Agarose	Merck	Cat # 05066
Proteinase K	Thermo Fisher	Cat # EO0491
Tris-HCl	Merck	Cat # 10812846001
NaCl	Merck	Cat #S9888
EDTA	Merck	Cat #E9884
CutSmart buffer	NEB	Cat #B7204
BbsI-HF	NEB	Cat #R3539
Ampicillin	Merck	Cat # A0166
Yeast extract	Merck	Cat #Y1625
Tryptone	Merck	Cat # T7293
Agar powder	Merck	Cat # 05040
Glucose	Sudelab	Cat # 141341.0914
Fresh yeast	Comercial Flequera, S.L	N/A
Wheat flour	El Corte Inglés	Cat # EAN:8410069016454
Propionic acid	Merck	Cat # 81910-1L
Nipagin	Merck	Cat # H5501-500GR
Q5 Hot Start High-Fidelity 2× Master Mix	NEB	Cat # M0494S
DreamTaq Green PCR Master Mix (2×)	Thermo Fisher	Cat # K1081
Critical commercial assays		
NucleoSpin Gel and PCR Clean-up kit	Macherey-Nagel	Cat # 11992242
GenElute Plasmid Maxiprep kit	Merck	Cat # PLX15
NEBuilder HiFi DNA Assembly kit	NEB	Cat # E5520
Experimental models: Organisms/strains		
D. melanogaster: *FBti0019985* outbred population (adult male and female flies, 5–20 days old)	Merenciano et al.[2]	N/A
Oligonucleotides (5′-3′)		
sgRNA1 primer: gcggcccgggttcgattcccggccg atgcatatctcaaataagtctagctgttttagagctagaaatagcaag	N/A	N/A
sgRNA2 primer: attttaacttgctatttctagctctaaaacc agagaaacgtcgagctgcgtgcaccagccgggaatcgaaccc	N/A	N/A
5′HAa_fwd1 for TE deletion: ctgcgatcttaattgagactgtcacactataaac	N/A	N/A
5′HAa_rev1 for TE deletion (PAM mutated): gagataa**C**gctaaacaaaaaagcatttt	N/A	N/A
5′HAb_fwd1 for TE deletion (PAM mutated): aaaatgctttttttgtttagc**G**ttatctc	N/A	N/A
5′HAb_rev1 for TE deletion: atctttctagggcattctatttatgcgatctacg	N/A	N/A
DsRed_fwd1 for TE deletion: cataaatagaatgccctagaaagataatcatattgtg	N/A	N/A
DsRed_rev1 for TE deletion (PAM mutated): c**T**gactaaaagaagaaaaggcgatcgccctagaaagatagtctgc	N/A	N/A
3′HA_fwd1 for TE deletion (PAM mutated): tatctttctagggcgatcgccttttcttcttttagtc**A**g	N/A	N/A
3′HA_rev1 for TE deletion: gctcttccttaaccgtatgctgagcggtcatatac	N/A	N/A
pHD_fwd1 for TE deletion: gctcagcatacggttaaggaagagccgtcgc	N/A	N/A
pHD_rev1 for TE deletion: gtgacagtctcaattaagatcgcaggtgctg	N/A	N/A
U63seqfwd: acgttttataacttatgcccctaag	N/A	N/A
pCFDseqrev: gcacaattgtctagaatgcatac	N/A	N/A
pHD-BB2: tgatatcaaaattatacatgtcaacg	N/A	N/A
pHD-HSP70-R: cttatcgatttcgaaccctcgaccg	N/A	N/A
pHD-SV40-F: ggccgcgactctagatcataatc	N/A	N/A
pHD-BB1: ctttcgactgagcctttcgt	N/A	N/A
TE_verif_fwd: aacaatgcaagtccgtgctc	N/A	N/A
TE_verif_rev: gtggttcctccacccttgtg	N/A	N/A
sgRNA3: gcggcccgggttcgattcccggccgatg cttgaacactaatgacaatttgttttagagctagaaatagcaag	N/A	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
sgRNA4: attttaacttgctatttctagctctaaaa cagctcacaactgcgcagctctgcaccagccgggaatcgaaccc	N/A	N/A
5′HAa_fwd2 for DsRed deletion: ctgcgatcttaattgagactgtcacactataaac	N/A	N/A
5′HAa_rev2 for DsRed deletion (PAM mutated): gtcattagtgttcaacAgttttatgatgcccacttc	N/A	N/A
5′HAb_fwd2 for DsRed deletion (PAM mutated): ggcatcataaaacTgttgaacactaatgac	N/A	N/A
5′HAb_rev2 for DsRed deletion: aaaaggcgatcgcattctatttatgcgatctacg	N/A	N/A
3′HA_fwd2 for DsRed deletion (PAM mutated): cataaatagaatgcgatcgccttttcttctttttagtccgcagagaa acgtcgagctgcgcagttgtgagctgAgc	N/A	N/A
3′HA_rev2 for DsRed deletion: gctcttccttaaccgtatgctgagcggtcatatac	N/A	N/A
pHD_fwd2 for DsRed deletion: gctcagcatacggttaaggaagagccgtcgc	N/A	N/A
pHD_rev2 for DsRed deletion: gtgacagtctcaattaagatcgcaggtgctg	N/A	N/A
TE_verif2_rev: cgtaggatcagtgggtgaaaatg	N/A	N/A
Recombinant DNA		
pCFD5	Port and Bullock[3]	Addgene Plasmid #73914
pHD-ScarlessDsRed	N/A	Addgene Plasmid #64703
pnos-Cas9-nos	N/A	Addgene Plasmid #62208
Software and algorithms		
FlyBase	N/A	https://flybase.org
SnapGene viewer	N/A	https://www.snapgene.com/snapgene-viewer
Target Finder	Gratz et al.[4]	http://targetfinder.flycrispr.neuro.brown.edu/
NEBuilder Assembly Tool	N/A	https://nebuilder.neb.com/#!/

MATERIALS AND EQUIPMENT

Ampicillin LB plates

Reagent	Final concentration
Yeast extract	5 g/L
Tryptone	10 g/L
NaCl	10 g/L
Ampicillin	0.10 g/L
Agar powder	15 g/L
ddH_2O	N/A
Total	**N/A**

Autoclave the mix without the Ampicillin at 121°C for 15 min. Add Ampicillin after the solution is cooled to 55°C. Pour the buffer into petri dishes inside a fume hood and allow them to solidify. The Ampicillin LB plates can be stored at 4°C for up to 2 months.

Squishing buffer for genomic DNA extraction

Reagent	Final concentration
Tris-HCl (pH = 8.2)	10 mM
EDTA	1 mM
NaCl	25 mM
ddH_2O	N/A
Proteinase K	200 mg/mL
Total	**N/A**

Proteinase K should be added to the mix right before use. The squishing buffer can be stored at 20°C–25°C for up to one month.

Fly food medium		
Reagent	Final concentration	Amount
Glucose	43 g/L	86 g
Fresh yeast	57.50 g/L	115 g
Agar powder	6.75 g/L	13.5 g
Wheat flour (or cornmeal)	29 g/L	58 g
Propionic acid	4 mL/L	8 mL
Nipagin	1.15 g/L	2.3 g
ddH_2O	N/A	up to 2 L
Total	N/A	2 L

Mix the glucose, fresh yeast, and agar with 1.5 L ddH_2O and let the mix boil for 10 min. Add the flour and 0.5 mL of ddH_2O and mix it until no lumps are found and let the mix boil for 5 min more. Add ddH_2O up to 2 L. Wait until the temperature of the mix is 65°C to add the propionic acid and the Nipagin and mix thoroughly. Pour the fly food in vials for *Drosophila* rearing. Fly food can be stored at 4°C for up to 3 weeks.

STEP-BY-STEP METHOD DETAILS

sgRNA selection and primer design for transposable element deletion

Timing: 45 min

Select two sgRNAs and order primers for cloning them into the pCFD5 plasmid (Addgene: 73914). This plasmid allows the expression of multiple sgRNAs under the control of the strong ubiquitous RNA pol III promoter U6:3.[3] sgRNAs should be designed to target the 5′ and 3′ most proximal regions flanking the TE to be deleted. We will exemplify the procedure using the *FBti0019985* TE insertion of *D. melanogaster* (423 bp), which has been successfully deleted using this two-step protocol in Merenciano and Gonzalez, 2023.[1]

1. Get the nucleotide sequence of the TE insertion that you want to delete including 500 bp of each flanking region. For *D. melanogaster*, it can be found in FlyBase (http://flybase.org/).[5]
2. Copy and paste the nucleotide sequence into a CRISPR target finder tool such as http://targetfinder.flycrispr.neuro.brown.edu/.
   a. Select the reference genome of the species of interest. Following the example, select "*Drosophila melanogaster.*" Select a guide length of 20 nucleotides and find "*all CRISPR targets*". Click on "*Find CRISPR targets*".
   b. On the next page, select "*High Stringency*" and "*NGG only*" for PAM.
   c. Choose a pair of sgRNAs with no predicted off-target sites and located in the closest upstream and downstream flanking regions of the TE insertion, respectively. For *FBti0019985* deletion, we chose the following sgRNA pair: sgRNA1 5′-tatctcaaataagtctagct-3′ and sgRNA2 5′-cagag aaacgtcgagctgcg-3′.

⚠ CRITICAL: if the closest sgRNAs to the TE insertion have predicted off-target sites, choose other sgRNAs. If off-target sites are unavoidable, choose sgRNAs with off-targets in a different chromosome from the genomic region of interest.

Note: to ensure that no SNPs that could prevent an effective Cas9 cleavage are present in the chosen target regions, we strongly recommend to sequence the genomic region including the selected sgRNAs of the strain to be injected.

3. Design and order desalted primers for cloning the sgRNAs into the pCFD5 plasmid following the pCFD5 cloning protocol found in http://www.crisprflydesign.org/wp-content/uploads/2016/07/pCFD5cloningprotocol.pdf.

Note: primers should always include the same homology arms to the pCFD5 plasmid for their assembly and the sgRNA sequence in the middle. For *FBti0019985* deletion, the forward primer for cloning sgRNA1 is 5′-gcggcccgggttcgattcccggccgatgcatatctcaaataagtctagctgtttta gagctagaaatagcaag-3′ and the reverse primer for cloning the sgRNA2 is 5′-attttaacttgctatttct agctctaaaaccagagaaacgtcgagctgcgtgcaccagccgggaatcgaaccc-3′. These primers contain the sgRNA1 and sgRNA2 sequences (underlined), respectively.

Repair plasmid and primer design for transposable element deletion

Timing: 1 h

To generate a precise deletion of the TE insertion, and to facilitate the screening of positive CRISPR/Cas9 deletion events, a repair plasmid for homology-directed repair must be designed and microinjected together with the pCFD5 plasmid containing the sgRNAs. The repair plasmid will be derived from the pHD-ScarlessDsRed plasmid (Addgene: 64703). This plasmid contains a 3xP3-DsRed marker cassette flanked by piggy-PBac transposon ends and is usually used to generate targeted modifications with a minimal locus disruption due to the marker cassette removal through a single cross with a strain containing a piggy-PBac transposase. In this protocol, the introduction of this plasmid thus allows the substitution of the TE by a visual marker that facilitates the screening of positive CRISPR events. The DsRed marker allows the selection based on the DsRed fluorescent signal in the adult eye and in the ocelli. Two homology arms are needed to produce recombination between the repair plasmid and the genome. Each homology arm must be ~1000 bp. The boundaries of the right (5′) and left (3′) homology arms must be the right and left boundaries of the TE insertion, respectively. Crucially, the PAM sequence (NGG) corresponding to the two targeted regions must be modified in the homology arms to avoid Cas9 cutting the repair plasmid.

4. Design and order primers for the cloning of the 5′ and 3′ homology arms in the flanking regions of the 3xP3-DsRed marker cassette.
   a. Identify ~1000 bp of the nucleotide sequence of each flanking region of the TE insertion in the strain to be injected, which will correspond to the 5′ and 3′ homology arms, respectively. For *FBti0019985* deletion, 5′ homology arm contained the sequence in 2R:9870299-9871095 (FlyBase Release 6) while the 3′ homology arm contained the sequence in 2R:9871529-9872365 (FlyBase Release 6).

Note: DNA sequences can be visualized using the software SnapGene viewer or any other sequence viewer.

   b. Download the sequence of the pHD-ScarlessDsRed plasmid (https://flycrispr.org/scarless-gene-editing/) and identify the sequence of the 3xP3-DsRed marker cassette and the sequence of the pHD-ScarlessDsRed backbone.
   c. Use the NEBuilder Assembly Tool (https://nebuilder.neb.com/#!/) to design the primers for the assembly of the homology arms into the pHD-ScarlessDsRed plasmid (Figure 1A).
      i. Introduce the nucleotide sequence of all the different fragments to be assembled (5′ homology arm, 3xP3-DsRed marker cassette, 3′ homology arm and pHD-ScarlessDsRed plasmid backbone obtained in steps 4a-b).

Note: be sure that the fragments are in the correct order, being the 3xP3-DsRed marker cassette between the two homology arms.

      ii. Follow the NEBuilder Assembly Tool instructions to obtain the primer sequences that will allow the introduction of both homology arms into the repair plasmid. For *FBti0019985* deletion, primer sequences can be found in the "key resources table" (Figure 1A).

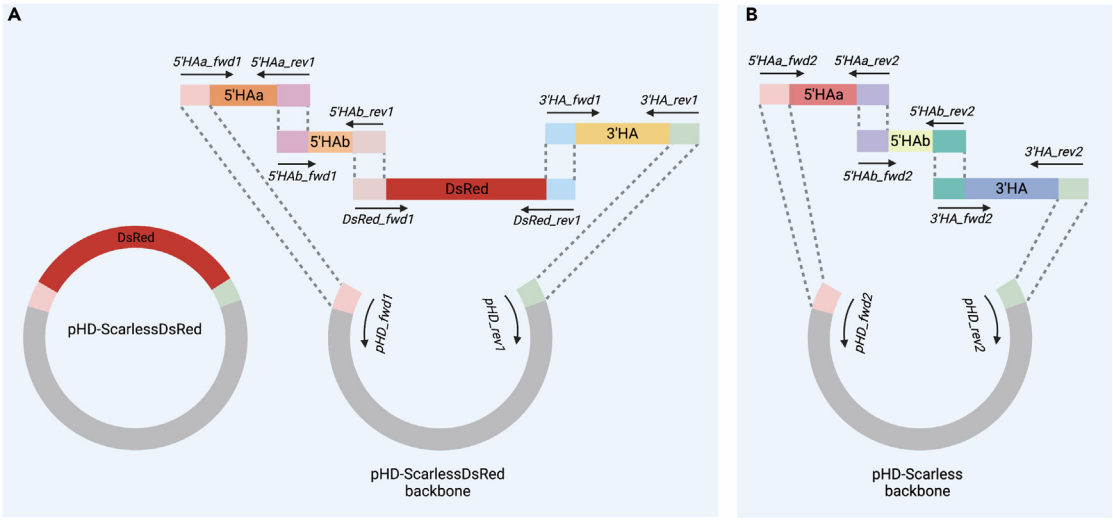

Figure 1. Schematic representation of the repair plasmids

(A) Schematic representation of the pHD-ScarlessDsRed repair plasmid for TE deletion (*FBti0019985*).

(B) Schematic representation of the pHD-Scarless repair plasmid for visual marker DsRed deletion. Primers are represented by arrows not in scale.

⚠ CRITICAL: use the designed primers to modify the two PAM sequences in the homology arms (corresponding to the two target regions) in order to avoid Cas9 cutting the repair plasmid. We recommend changing the second nucleotide of the PAM region (NGG) since Cas9 can sometimes recognize other PAMs like NGA. If the PAM to be mutated is in a genic region, try not to create a non-synonymous mutation that could in turn affect the coding capacity of the gene. To modify the PAM sequences, two strategies can be followed depending on the position of the PAM in the homology arms. The first is to extend one primer sequence until the closest PAM region and modify it to not contain the NGG sequence (the total primer sequence should not exceed 75 bp). In the example, we modified the PAM region of the 3′ homology arm (G > T) following this strategy. However, if the PAM region is not close enough to the primer sequence, we recommend splitting the homology arm in two fragments. In that case, go back to step 4c and introduce the sequences of all fragments to be assembled. After that, modify the sequence of the desired primers to not contain the NGG sequence of the PAM. In the example, we modified the PAM region of the 5′ homology arm (G > C) following this strategy, thus having two fragments for this homology arm: 5′HAa and 5′HAb (Figure 1A).

Optional: a single sgRNA can be design and cloned into pCFD5 if the nearby region of the insertion does not contain any gene that could be disrupted by the introduction of the marker. In this case, the visual marker will be introduced by homology directed repair nearby the TE insertion. This has the advantage of performing a single cut, rather than two in the TE flanking regions. Both the TE and the visual marker can be removed later in the second step of the protocol.

Optional: some extra nucleotides can be added to the DsRed_fwd1 and DsRed_rev1 primers to artificially generate both a PAM region (NGG) and a sgRNA target site in the flanking regions of the 3xP3-DsRed marker cassette. This will allow the removal of the 3xP3-DsRed marker cassette in the second CRISPR/Cas9 homology-directed repair step without introducing SNPs in the PAM regions needed to prevent an effective Cas9 cleavage of the repair plasmid.

sgRNA cloning for transposable element deletion

Timing: 3–4 days

In this step, sgRNAs for TE deletion are cloned into the pCFD5 plasmid following the protocol described in http://www.crisprflydesign.org/wp-content/uploads/2016/07/pCFD5cloningprotocol.pdf.

5. Digest the pCFD5 plasmid with BbsI-HF restriction enzyme at 37°C for 2–16 h.

Digestion reaction master mix	
Reagent	Amount
pCFD5 plasmid	8 μg
BbsI-HF enzyme	1 μL (10 μ)
CutSmart buffer, 10×	3 μL (10×)
ddH_2O	up to 30 μL

6. Run a PCR with the primer pair obtained in step 3 (sgRNA1 forward and sgRNA2 reverse primers) using the pCFD5 circularized plasmid as a template. Use a high-fidelity polymerase, such as the Q5 2× Hot Start High-Fidelity Master mix from NEB.
   a. Set up the following PCR reaction mix according to the protocol for Q5 2× Hot Start High-Fidelity Master Mix (https://international.neb.com/protocols/2012/12/07/protocol-for-q5-high-fidelity-2x-master-mix-m0492).

PCR reaction master mix	
Reagent	Amount
Q5 High-Fidelity 2× Master Mix	25 μL
sgRNA forward primer, 10 μM	2.5 μL
sgRNA reverse primer, 10 μM	2.5 μL
pCFD5 plasmid	up to 100 ng
ddH_2O	up to 50 μL

   b. Follow the PCR cycling conditions of the Q5 Hot Start High-Fidelity 2× Master Mix (https://international.neb.com/protocols/2012/12/07/protocol-for-q5-high-fidelity-2x-master-mix-m0492).

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	25-35 cycles
Annealing	50°C–72°C (see Note)	30 s	
Extension	72°C	30 s/kb	
Final extension	72°C	2 min	1
Hold	4°C–15°C	forever	

Note: recommended annealing temperature is 61°C.

7. Run both the digested pCFD5 plasmid from step 5 and the PCR product from step 6 in a 1% agarose gel.
8. Gel purify both the digested pCFD5 plasmid from step 5 and the PCR product from step 6 with a gel extraction kit such as the NucleoSpin Gel and PCR Clean-up kit following the manufacturer's instructions (http://bioke.com/blobs/manuals/MN/NS/UM_PCRcleanup_Gelex_NSExII.pdf).
9. Insert the PCR product into the linearized pCFD5 plasmid using the NEBuilder HiFi DNA Assembly kit following the manufacturer's instructions. We used a 1:2 vector:insert ratio with 0.05–0.1 pmol total amount of each fragment to be assembled. (https://international.neb.com/protocols/2014/11/26/nebuilder-hifi-dna-assembly-reaction-protocol).

10. Transform NEB 5-α *E. coli* cells with 2 μL of the chilled assembled product, following the manufacturer's transformation protocol (https://international.neb.com/protocols/0001/01/01/high-efficiency-transformation-protocol-c2987).
11. Validate by PCR the plasmid assembly.
    a. Pick 10–20 single colonies with a pipet tip or a plastic inoculation needle.
    b. Dissolve the individual picked colonies in the following PCR master mix.

    Note: for this diagnostic PCR, use an inexpensive PCR system such as DreamTaq Green PCR Master Mix (Thermo Fisher) and primers U63seqfwd and pCFDseqrev provided in the pCFD5 cloning protocol (primer sequences can be found in the "Key resources table").

    ⚠ CRITICAL: circle and label the picked colonies on the original plate and let them grow for 16 h again at 37°C to keep them for following procedures. Alternatively, after dissolving the individual picked colonies in the PCR master mix, they can be replated in a new LB plate and grown for 16 h at 37°C. After that, store the plate in the fridge at 4°C until further use.

PCR reaction master mix

Reagent	Amount
DreamTaq Green PCR Master Mix (2×)	12.5 μL
Forward primer, 10 μM (U63seqfwd)	1 μL
Reverse primer, 10 μM (pCFDseqrev)	1 μL
Picked colony	-
ddH_2O	up to 25 μL

    c. Follow the PCR cycling conditions of the Thermo Scientific DreamTaq Green PCR Master Mix (2×) (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0012704_DreamTaq_Green_PCR_MasterMix_K1081_UG.pdf).

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	30 s	25–35 cycles
Annealing	50°C–72°C (see Note)	30 s	
Extension	72°C	1 min/kb	
Final extension	72°C	5 min	1
Hold	4°C–15°C	forever	

    Note: recommended annealing temperature is 57°C.

    d. Run PCR products on a 1% agarose gel. Verify insertion by Sanger sequencing some of the PCR products with the U63seqfwd or the pCFDseqrev primers (primer sequences can be found in the "key resources table"), as the region to be sequenced is small (~200 bp).

    Note: it is also possible to skip step 11d and go to step 12 to perform a 16 h culture of all the selected colonies, purify the pCDF5 and finally, perform the PCR and verify the insertion by Sanger sequencing.

12. Do a 16 h culture of the selected verified colony and purify the pCFD5 plasmid with a Maxiprep kit (GenElute Plasmid Maxiprep kit) following the manufacturer's instructions (https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/586/617/00007361w-mk.pdf).

Note: make a bacterial glycerol stock of the selected colony and keep it at −70°C for long-term storage of plasmids.

13. Quantify the purified plasmid and check its quality.

Pause point: pCFD5 plasmid containing the sgRNAs can be stored at −20°C until injection.

Repair plasmid cloning for transposable element deletion

Timing: 1 week

In this step, homology arms for the homology-directed repair are cloned into the pHD-ScarlessDsRed plasmid using the NEBuilder HiFi DNA Assembly kit.

14. Run a PCR to obtain each one of the fragments (5 fragments for the *FBti0019985* deletion, Figure 1A) that need to be assembled using the primers designed in step 4.

Note: use a high-fidelity polymerase, such as the Q5 2× Hot Start High-Fidelity Master mix from NEB.

Note: homology arm fragments must be obtained using the genomic DNA of the strain to be injected as a template, while the 3xP3-DsRed marker cassette and pHD-ScarlessDsRed plasmid backbone must be obtained using the pHD-ScarlessDsRed plasmid as a template.

a. Set up the following PCR reaction mix according to the protocol for Q5 Hot Start High-Fidelity 2× Master Mix (https://international.neb.com/protocols/2012/12/07/protocol-for-q5-high-fidelity-2x-master-mix-m0492).

PCR reaction master mix

Reagent	Amount
Q5 High-Fidelity 2× Master Mix	25 µL
Forward primer, 10 µM	2.5 µL
Reverse primer, 10 µM	2.5 µL
gDNA or pHD-ScarlessDsRed plasmid	up to 100 ng
ddH_2O	up to 50 µL

b. Follow the PCR cycling conditions of the Q5 Hot Start High-Fidelity 2× Master Mix (https://international.neb.com/protocols/2012/12/07/protocol-for-q5-high-fidelity-2x-master-mix-m0492).

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1

(*Continued on next page*)

Continued			
Steps	Temperature	Time	Cycles
Denaturation	98°C	10 s	25–35 cycles
Annealing	50°C–72°C	30 s	
Extension	72°C	30 s/kb	
Final extension	72°C	2 min	1
Hold	4°C–15°C	forever	

15. Run the PCR products from step 14 in a 1% agarose gel.
16. Gel purify all the PCR products from step 14 with a gel extraction kit such as the NucleoSpin Gel and PCR Clean-up kit following the manufacturer's instructions (http://bioke.com/blobs/manuals/MN/NS/UM_PCRcleanup_Gelex_NSExII.pdf).
17. Assemble all the purified PCR products using the NEBuilder HiFi DNA Assembly kit following the manufacturer's instructions. We used a 1:1 vector:insert ratio with 0.2 pmol total amount of each fragment to be assembled (https://international.neb.com/protocols/2014/11/26/nebuilder-hifi-dna-assembly-reaction-protocol).
18. Transform NEB 5-α *E. coli* cells with 2 μL of the chilled assembled product, following the manufacturer's transformation protocol (https://international.neb.com/protocols/0001/01/01/high-efficiency-transformation-protocol-c2987).
19. Validate by PCR the plasmid assembly and the mutated PAMs. First, check the pHD-ScarlessDsRed backbone - 5′ homology arm - 3xP3-DsRed marker cassette junction (PCR 1, Figure 2A).
    a. Pick 10–20 single colonies with a pipet tip or plastic inoculation needle.
    b. Dissolve the individual picked colonies in the following PCR master mix.

    Note: for this diagnostic PCR, use an inexpensive PCR system such as DreamTaq Green PCR Master Mix (Thermo Fisher) and the primers pHD-BB2 and pHD-HSP70-R (primer sequences can be found in the "key resources table").

    ⚠ CRITICAL: circle and label the picked colonies on the original plate and let them grow for 16 h again at 37°C to keep them to use as a template in step 20. Alternatively, after dissolving the individual picked colonies in the PCR master mix, they can be replated in a new LB plate and grown for 16 h at 37°C. After that, store the plate in the fridge at 4°C until further use.

PCR reaction master mix	
Reagent	Amount
DreamTaq Green PCR Master Mix (2×)	12.5 μL
Forward primer, 10 μM (pHD-BB2)	1 μL
Reverse primer, 10 μM (pHD-HSP70-R)	1 μL
Picked colony	-
ddH_2O	up to 25 μL

    c. Follow the PCR cycling conditions of the Thermo Scientific DreamTaq Green PCR Master Mix (2×) (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0012704_DreamTaq_Green_PCR_MasterMix_K1081_UG.pdf).

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1

(*Continued on next page*)

Continued			
Steps	Temperature	Time	Cycles
Denaturation	95°C	30 s	25–35 cycles
Annealing	50°C–72°C (see Note)	30 s	
Extension	72°C	1 min/kb	
Final extension	72°C	5 min	1
Hold	4°C–15°C	forever	

Note: recommended annealing temperature is 57°C.

d. Run the PCR products on a 1% agarose gel. Verify the insertion of the homology arms and the mutated PAM regions by Sanger sequencing the PCR product with pHD-BB2 or with pHD-HSP70-R primers (primer sequences can be found in the "key resources table").

Note: if the assembly has not been validated in any of the colonies tested, repeat step 19.

20. Validate by PCR the 3xP3-DsRed marker cassette - 3′ homology arm - pHD-ScarlessDsRed backbone junction (PCR 2, Figure 2A).
    a. Pick all the verified colonies in step 19 with a pipet tip or plastic inoculation needle from the replated plate.

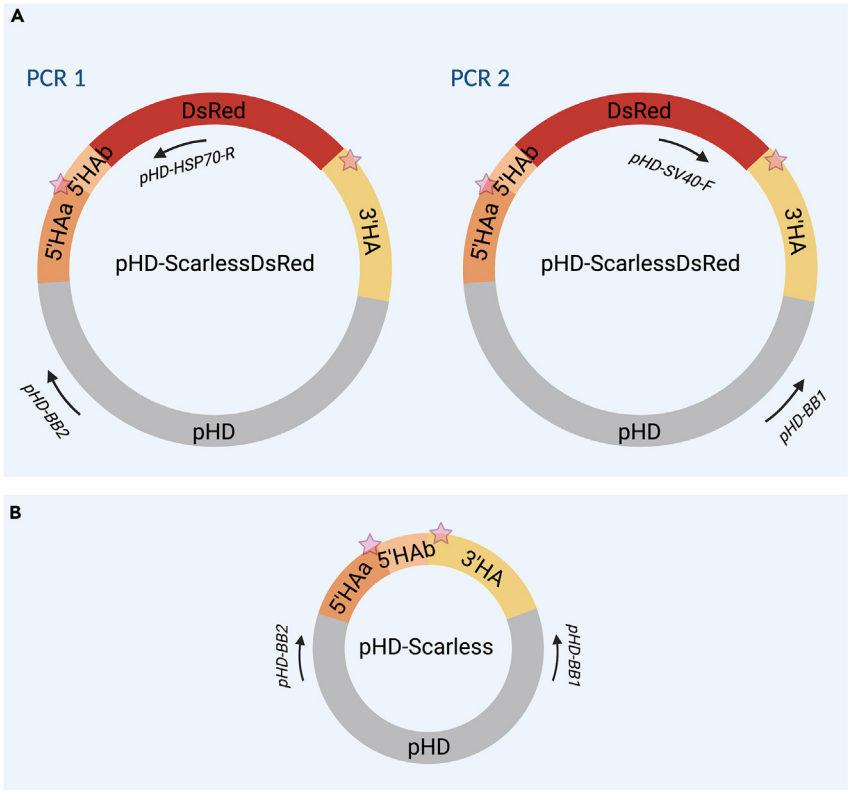

Figure 2. Schematic representation of the PCR validation of the repair plasmids

(A) Schematic representation of the PCR validation to check the pHD-ScarlessDsRed repair plasmid. PCR 1 allows the validation of the pHD-ScarlessDsRed backbone - 5′ homology arm - 3xP3-DsRed marker cassette junction and the mutated PAM sequence. PCR 2 allows the validation of the 3xP3-DsRed marker cassette - 3′ homology arm - pHD-ScarlessDsRed backbone junction and the other mutated PAM sequence.

(B) Schematic representation of the PCR validation to check the pHD-Scarless repair plasmid. Stars represent PAM sequences mutated.

b. Repeat steps 19b-d with the primers pHD-SV40-F and pHD-BB1 (primer sequences can be found in the "key resources table").

21. Do a 16 h culture of the selected verified colony and purify the assembled repair plasmid with a Maxiprep kit (GenElute Plasmid Maxiprep kit) following the manufacturer's instructions (https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/586/617/00007361w-mk.pdf).

Note: make a bacterial glycerol stock of the selected colony and keep it at −70°C for long-term storage of plasmids.

22. Quantify the purified plasmid (Figure 2B) and check its quality.

Pause point: repair plasmid containing the homology arms and the visual marker can be stored at −20°C until injection. Repeated freeze-thaw cycles should be avoided.

Injection, screening for transposable element deletion, and generation of the mutant strain

Timing: ~3–5 months (depending on the number of backcrosses; see below)

The pCFD5 plasmid containing the gRNAs can be co-injected with the pHD-ScarlessDsRed repair plasmid together with a plasmid that specifically expresses Cas9 in the germline (pnos-Cas9-nos plasmid) into embryos of the desired *D. melanogaster* natural population (Figure 3). The injection mix can be sent to companies for injection if in-house injection facilities are not available.

23. Expand the *D. melanogaster* natural population (following the example, the *FBti0019985* outbred population) needed for microinjection (if it was not done before you started the protocol as recommended).
24. Microinject a minimum of 500 embryos with the injection mix containing the pCFD5 plasmid (100 ng/μL), the pHD-ScarlessDsRed repair plasmid (500 ng/μL) and the pnos-Cas9-nos plasmid (250 ng/μL) (Figure 3). For *FBti0019985* deletion, a total of 536 embryos were microinjected, and of those, 318 hatched.

Note: recommended concentrations are 100–500 ng/μL for pCFD5, 500 ng/μL for pHD-ScarlessDsRed repair plasmid, and 250–500 ng/μL for pnos-Cas9-nos plasmid. Prepare a final volume of 20 μL of injection mix. This injection mix can be stored at −20°C until further use (Figure 3). A detailed protocol for embryo microinjection can be found in http://gonzalezlab.eu/two-step-crispr-cas9-protocol

Note: several batches of embryos can be microinjected to increase the probability to find transformants.

25. Injected embryos are grown to adulthood in vials with fresh fly food medium and individually crossed to flies of the opposite sex from the maternal natural population (Figure 3). For *FBti0019985* deletion, a total of 149 flies emerged and 135 fertile crosses were established.
26. Screen for DsRed fluorescent signal in the next generation (F1) adult eyes/ocelli (Figure 3). For *FBti0019985* deletion, we obtained 47 F1 flies showing fluorescence in the eyes/ocelli belonging to 8 individual crosses (between 2–19 flies per individual cross). Success rate was then 1.5% (8 F0 individuals out of 536 embryos microinjected).
27. Select and cross individual transformants (F1) between them (Figure 3).

Note: F1 transformants can be individually crossed with the maternal natural population if there are not enough F1 transformants to perform male-female crosses between them. Then, go back to step 26–27 to screen F2 individuals and cross the selected flies between them.

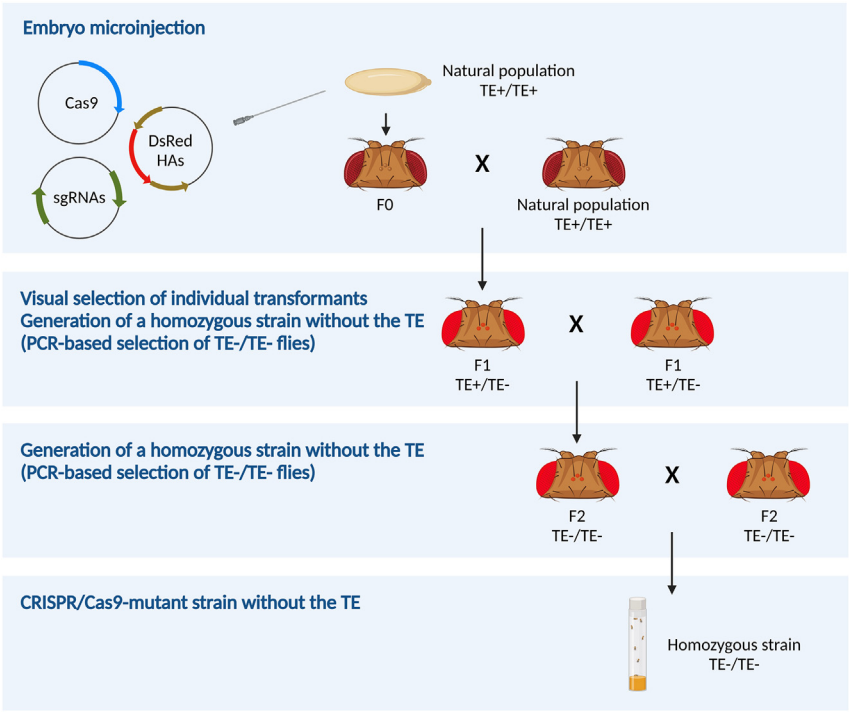

Figure 3. Crossing scheme after injection to obtain a homozygous CRISPR/Cas9 mutant strain for the substitution of the TE by the DsRed visual marker

Optional: select individual transformants and backcross them individually with the maternal natural population for 5 generations to remove possible off-target mutations (Figure 3). This step is optional if the backcrosses are performed in step 45.

28. To validate by PCR the TE deletion, extract genomic DNA from F1 transformant flies after laying eggs.
    a. Harvest the flies and place them individually in empty Eppendorf tubes.
    b. Homogenize the flies with a pestle and follow the "Single fly DNA prep for PCR' protocol found at http://francois.schweisguth.free.fr/protocols/Single_fly_DNA_prep.pdf.
29. Design three primers for the PCR verification, one pair binding just outside the regions that correspond to the 5′ and 3′ homology arms (for *FBti0019985* deletion validation; TE_verif_fwd and TE_verif_rev), and the other primer binding to the DsRed coding sequence (pHD-SV40-F) (Figure 4A). Primer sequences can be found in the "key resources table".
30. Validate by PCR that the TE substitution by the visual marker has been produced, and in the genomic region desired, performing two PCR reactions. One with the forward TE_verif_fwd and reverse TE_verif_rev primer pair spanning the whole substituted region (PCR 1, Figure 4A); and another PCR reaction with the forward TE_verif_fwd and the pHD-SV40-F primer (inside DsRed sequence) to verify that the previous PCR 1 result is due to the TE substitution by the DsRed fluorescent marker and not due to other undesired rearrangement events (PCR 2, Figure 4A).

Note: PCR 1 of selected transformants is expected to give two bands, as F1 transformants should be heterozygous for the mutation. One band corresponds to the substituted allele TE-, and the other band to the TE+ allele from the maternal natural population. However, PCR 2 is expected to give a single band, meaning that the TE insertion has been substituted by the DsRed fluorescent marker. For PCR reaction mixture and PCR conditions, refer to step 11. Verify the TE substitution by Sanger sequencing the PCR products.

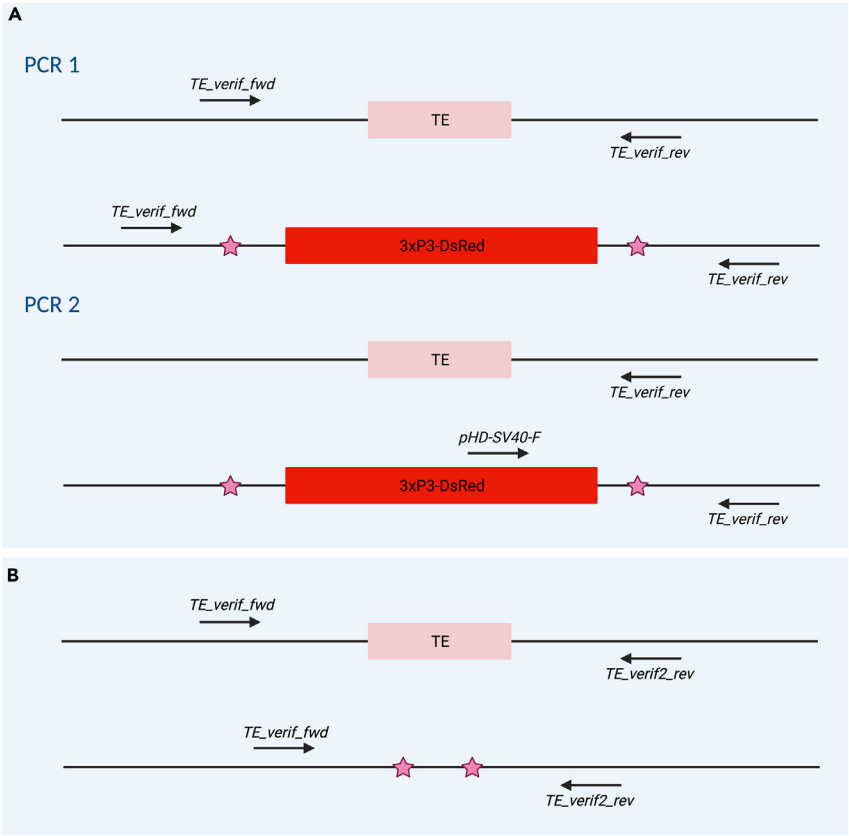

Figure 4. Schematic representation of the PCR validation of the transformants

(A) Schematic representation of the PCR validation to check the TE substitution for the DsRed visual marker in the genomic DNA of the transformant flies. PCR1 allows the validation by obtaining different band sizes, while PCR2 only gives a band when the DsRed visual marker is present.

(B) Schematic representation of the PCR validation to check for the absence of the TE in the genomic DNA of the transformant flies.

31. Generate a homozygous strain absent for the TE insertion (Figure 3).
    a. F2 flies can be TE+/TE+, TE+/TE-, or TE-/TE-. TE+/TE+ F2 flies do not display red fluorescence and thus, they can be discarded.
    b. Self-cross flies from F2 that display red fluorescence (TE+/TE- or TE-/TE-) individually. We recommend performing at least 10 crosses to maximize the chances to obtain TE-/TE- x TE-/TE- crosses.

Note: sometimes TE+/TE+ homozygous flies can be carefully selected based on the brightness on the eyes.

    c. Repeat step 27 to extract the genomic DNA from the F2 flies (both males and females).

Note: TE+/TE- flies will produce two bands in PCR 1, and a single band in PCR 2 (Figure 4A). However, TE-/TE- flies will produce a single band in PCR 1, and also a single band in PCR 2 (Figure 4A).

    d. Repeat step 30 to validate by PCR the TE deletion in both alleles.
    e. Keep the cross in which both F2 parents are homozygous for the TE deletion.

Pause point: the homozygous CRISPR-mutant without the TE insertion can be kept in vials with fresh food as many generations as needed until the next injection (step 38). However, if you are going to proceed with the protocol, you can start to expand it (step 37).

Note: steps 32–36 can be done simultaneously with step 31.

sgRNA selection and primer design for visual marker deletion

Timing: 45 min

Select two sgRNAs for DsRed deletion and order primers for cloning them into the pCFD5 plasmid. This time, sgRNAs should be designed to target the 3xP3-DsRed marker cassette flanking regions.

32. Get the nucleotide sequence of the 3xP3-DsRed marker cassette (1691 bp) from the previously generated pHD-ScarlessDsRed repair plasmid (obtained in step 30) including 500 bp of each flanking region (corresponding to the homology arms).
33. Follow the steps 2 and 3 to design and order primers for cloning the sgRNAs into the pCFD5 plasmid. For DsRed deletion, we chose the following sgRNA pair: sgRNA3 5′-ttgaacactaatgac aattt-3′ and sgRNA4 5′-gagctgcgcagttgtgagct-3′. The forward primer for cloning sgRNA3 is 5′-gcggcccgggttcgattcccggccgatgcttgaacactaatgacaatttgttttagagctagaaatagcaag-3′ and the reverse primer for cloning the sgRNA4 is 5′-attttaacttgctatttctagctctaaaacagctcacaactgcgc agctctgcaccagccgggaatcgaaccc-3′. These primers contain the sgRNA3 and sgRNA4 sequences (underlined), respectively.

Repair plasmid and primer design for visual marker deletion

Timing: 1 h

Again, the new repair plasmid for homology-directed repair will be derived from the pHD-ScarlessDsRed plasmid. This time, the 3xP3-DsRed marker cassette is deleted and only two homology arms for homology-directed repair are inserted. Both homology arms must be ~1000 bp each. The boundaries of the right (5′) and left (3′) homology arms must be the right and left boundaries of the 3xP3-DsRed marker cassette in the previously generated CRISPR/Cas9-mutated strain without the TE insertion, respectively. Crucially, the PAM sequence (NGG) corresponding to the designed sgRNAs must be modified in the new homology arms to avoid Cas9 cutting the repair plasmid. The introduction of this plasmid thus allows the removal of the DsRed fluorescent marker producing, at the end of the protocol, a precise deletion of the desired TE insertion in *D. melanogaster* natural populations.

34. Design and order primers for the removal of the 3xP3-DsRed marker cassette and the insertion of the 5′ and 3′ homology arms into the pHD-ScarlessDsRed plasmid (Figure 1B).
    a. Identify ~1000 bp of the nucleotide sequence of both flanking regions of the 3xP3-DsRed marker cassette in the previously generated CRISPR-mutant strain without the TE insertion. These sequences have been obtained in step 30.
    b. Use the NEBuilder Assembly Tool (https://nebuilder.neb.com/#!/) to design the primers for the assembly of the homology arms into the pHD-ScarlessDsRed plasmid (Figure 1B).
        i. Introduce the nucleotide sequence of all the different fragments to be assembled (5′ homology arm, 3′ homology arm and pHD-ScarlessDsRed plasmid backbone without the 3xP3-DsRed marker cassette).

Note: be sure that the fragments are in the correct order (Figure 1B).

        ii. Follow the NEBuilder Assembly Tool instructions to obtain the primer sequences that will allow the introduction of both homology arms into the repair plasmid. For DsRed deletion, primer sequences can be found in the "key resources table" (Figure 1B).

⚠ CRITICAL: use the designed primers to modify the two PAM sequences in the homology arms (corresponding to the two target regions) in order to avoid Cas9 cutting the repair plasmid. We recommend changing the second nucleotide of the PAM region (NGG) since Cas9 can sometimes recognize other PAMs like NGA. If the PAM to be mutated is in a genic region, try not to create a non-synonymous mutation that could in turn affect the coding capacity of the gene. To modify the PAM sequences, two strategies can be followed depending on the position of the PAM in the homology arms. The first is to extend one primer sequence until the closest PAM region and modify it to not contain the NGG sequence (the total primer sequence should not exceed 75 bp). In the example, we modified the PAM region of the 3′ homology arm (G > A) following this strategy. However, if the PAM region is not close enough to the primer sequence, we recommend splitting the homology arm in two fragments. In that case, go back to step 34b and introduce the sequences of all fragments to be assembled. After that, modify the sequence of the desired primer to not contain the NGG sequence of the PAM. In the example, we modified the PAM region of the 5′ homology arm (G > A) following this strategy, thus having two fragments for this homology arm: 5′HAa and 5′HAb (Figure 1B).

sgRNA cloning for visual marker deletion

Timing: 3–4 days

In this step, sgRNAs for DsRed deletion are cloned into the pCFD5 plasmid following the protocol described in http://www.crisprflydesign.org/wp-content/uploads/2016/07/pCFD5cloningprotocol.pdf.

35. Use the same procedures as in the "sgRNA cloning for transposable element deletion" section (steps 5-13) to clone the sgRNAs for DsRed deletion into the pCFD5 plasmid using the forward sgRNA3 and reverse sgRNA4 primers.

Repair plasmid cloning for visual marker deletion

Timing: 1 week

In this step, pHD-ScarlessDsRed repair plasmid is modified to remove the 3xP3-DsRed marker cassette and to clone the two new homology arms using the NEBuilder HiFi DNA Assembly kit.

36. Use the same procedures as in the "repair plasmid cloning for transposable element deletion" section (steps 14-22) to assemble repair plasmid. Validate by PCR that both 3′ and 5′ homology arms have been assembled with the pHD-BB1 and pHD-BB2 primer pair (primer sequences can be found in the "key resources table") (Figure 2B).

Injection, screening for visual marker deletion, and generation of the mutant strain

Timing: ~5 months

The new pCFD5 plasmid containing the sgRNAs for DsRed removal can be co-injected with the new pHD-Scarless repair plasmid and the pnos-Cas9-nos plasmid into embryos of the previously generated CRISPR-mutant strain without the TE insertion (Figure 5). The injection mix can be sent to companies for injection if in-house injection facilities are not available.

37. Expand the previously generated CRISPR-mutant strain without the TE insertion to be used for microinjection (if it was not done before as recommended in step 31).

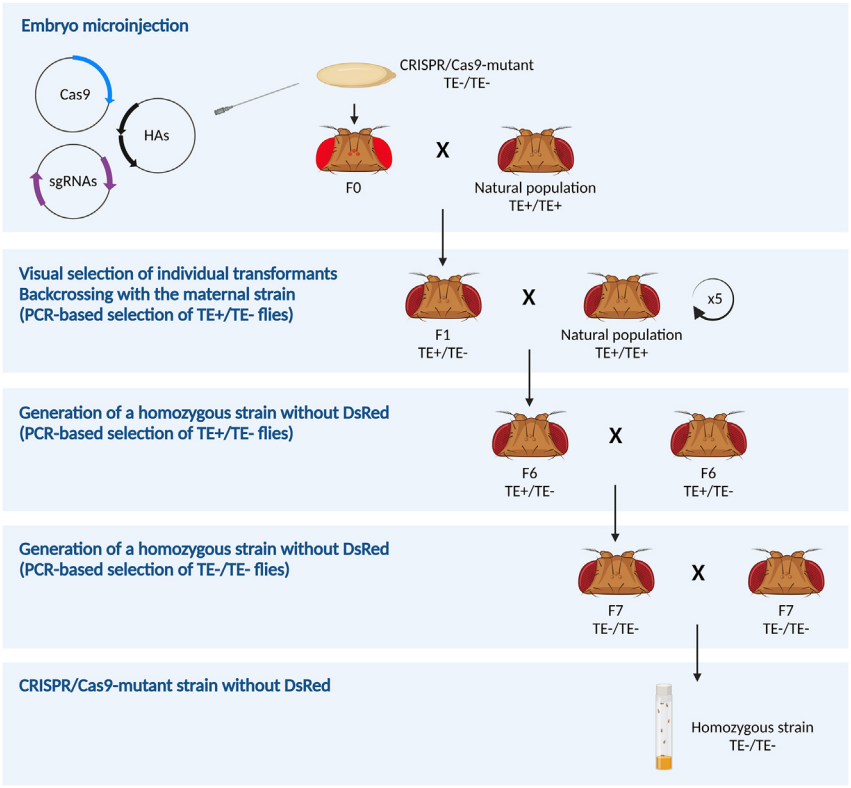

Figure 5. Crossing scheme after injection to obtain a homozygous CRISPR/Cas9 mutant strain for the deletion of the DsRed visual marker

38. Microinject a minimum of 500 embryos with the injection mix containing the new pCFD5 plasmid (100 ng/μL), the new pHD-Scarless repair plasmid (500 ng/μL) and the pnos-Cas9-nos plasmid (250 ng/μL) (Figure 5). For DsRed deletion, a total of 550 embryos were microinjected, and of those, 168 hatched. A detailed protocol for embryo microinjection can be found in http://gonzalezlab.eu/two-step-crispr-cas9-protocol

    Note: recommended concentrations are 100–500 ng/μL for pCFD5, 500 ng/μL for pHD-Scarless repair plasmid, and 250–500 ng/μL for pnos-Cas9-nos plasmid. Prepare a final volume of 20 μL of injection mix. This injection mix can be stored at −20°C until further use.

    Note: several batches of embryos can be microinjected to increase the probability to find transformants.

39. Injected embryos are grown to adulthood in vials with fresh fly food medium and individually crossed to flies of the opposite sex from the natural population (Figure 5). For DsRed deletion, a total of 94 flies emerged and 67 fertile crosses were established.
40. Screen for no DsRed fluorescent signal in the next generation (F1) adult eyes/ocelli (Figure 5). For DsRed deletion, we obtained 1 F1 fly showing no fluorescence in the eyes/ocelli belonging to 1 individual crosses (success rate 0.2%, 1 F0 individuals out of 550 embryos microinjected).
41. Select individual transformants (F1) and backcross them individually with the natural population (Figure 5).
42. Extract genomic DNA from F1 transformant flies after laying eggs.
    a. Harvest the flies and place them individually in empty Eppendorf tubes.

b. Homogenize the flies with a pestle and follow the "Single fly DNA prep for PCR' protocol found at http://francois.schweisguth.free.fr/protocols/Single_fly_DNA_prep.pdf.

43. Design a pair of primers for the PCR verification, such that one of each primer binds just outside the regions that correspond to the 5′ and 3′ homology arms. For DsRed deletion validation, we used the TE_verif_fwd and TE_verif2_rev primers (Figure 4B). Primer sequences can be found in the "key resources table".

44. Validate by PCR the visual marker deletion in F1 with the primers from the previous step.

Note: this PCR reaction of non-fluorescent selected transformants is expected to give two bands, as F1 transformants should be heterozygous for the mutation. One band corresponds to the TE- and DsRed deleted allele, while the other corresponds to the TE + allele from the maternal natural population (Figure 4B). For PCR reaction mixture and PCR conditions, refer to step 11. Verify the marker deletion and the subsequent homology-directed repair by Sanger sequencing the PCR product.

45. Backcross the selected flies for a minimum of 5 generations with the natural population to remove possible off-targets.

Note: in each generation, set up crosses with only the flies that present no fluorescence and keep vials only after the TE heterozygosity has been validated by PCR (Figures 4B and 5).

Note: it is necessary to validate by PCR that the flies are heterozygous for the presence of the TE insertion in every generation. The number of backcrossed generations can be reduced, reducing the time of generation of a homozygous strain, although the probability to keep off-target mutations will increase.

46. Generate a homozygous strain absent for the TE insertion (Figure 5).
    a. Self-cross flies from F6 (TE+/TE-) individually. Their offspring (F7) can be TE+/TE+, TE+/TE-, or TE-/TE-.
    b. Self-cross flies from F7 individually. We recommend performing at least 10 crosses to maximize the chances to obtain TE-/TE- x TE-/TE- crosses.
    c. Repeat step 42 to extract the genomic DNA from the F7 flies (both males and females). TE+/TE- flies will produce two bands, while TE-/TE- flies will produce a single band (Figure 4B).
    d. Repeat step 44 to validate by PCR the absence of the TE in both alleles.
    e. Keep the cross in which both F7 parents are homozygous for the absence of the TE.

EXPECTED OUTCOMES

This protocol generates a precise deletion of any TE (or genomic region) in natural populations of *D. melanogaster* using two steps of CRISPR-Cas9 homology-directed repair. In Merenciano and Gonzalez, 2023 [1], a precise deletion of the *FBti0019985* TE insertion was achieved following this protocol.

LIMITATIONS

This protocol uses two steps of CRISPR-Cas9 homology-directed repair to generate a precise deletion of a TE in *D. melanogaster* natural populations. Hence, one of the limitations of the process is the time invested in producing the CRISPR mutants. Here, to try to reduce possible off-targets produced by the CRISPR-Cas9 technique, flies were backcrossed for 5 generations. The number of backcrossed generations can be reduced, although the probability to keep off-target mutations will increase. Another limitation of the process is the generation of two SNPs in the PAM regions. These SNPs are introduced during the homology-directed repair and they are necessary to prevent an effective Cas9 cleavage of the repair plasmid. Because these SNPs were not present in the initial background, we are thus introducing genetic variation in the

genome. The fact that this protocol is focused on performing CRISPR-Cas9 deletions in natural populations (with red eyes) makes the screening of DsRed transformants (expressing red fluorescence in the eye and the ocelli) impossible to the naked eye. Thus, this protocol requires a fluorescence microscope for the screening. Furthermore, the deletion of the DsRed visual marker in the second CRISPR/Cas9 step of this protocol implies a PCR screening from F2 generation on as only non-fluorescent flies will be obtained. Finally, while this protocol has not been tested in other *Drosophila* species, some of the plasmids and promoters used have been tested in other *Drosophila* species like *D. simulans* or *D. suzukii*.[6–8]

TROUBLESHOOTING

Problem 1

No target sites found in the CRISPR target finder tool (Step-by-step Method step 2).

Potential solution

First, look for target sites selecting the option "*Low Stringency*". Consider that this would increase the probability to have off-target events. Additionally, the length of the guides can be reduced down to 17 bp.[9]

Problem 2

Not assembled gRNAs into the pCFD5 plasmid or duplicated gRNAs cloned (Step-by-step Method step 11 and 35).

Potential solution

Make sure that the assembly reaction uses at least50 ng of digested backbone and an ~two-fold molar excess of each insert. Double check primer sequences. Repeat again the assembly reaction.

Problem 3

Not assembled fragments into the pHD-ScarlessDsRed plasmid, or not all of them are assembled (Step-by-step Method step 19-20 and 36).

Potential solution

Try to increase (up to 20–30 bp) the overlap regions between each fragment. Make sure that all the fragments in the NEBuilder HiFi DNA Assembly reaction are in equimolar volumes (suggested: 0.2 pmol each).

Alternatively, if some fragments have been assembled but not all of them, new fragments can be obtained by PCR amplifying the regions already assembled. With this, it is possible to repeat the NEBuilder HiFi DNA Assembly reaction with a reduced number of fragments, thus increasing the efficiency of the process.

Problem 4

No CRISPR transformants after the visual screening of the F1 (Step-by-step Method step 26 and 40)

Potential solution

Double check that there are no SNPs in the chosen target sites in the gDNA of the natural population used for injection.

Check the predicted efficiency of sgRNAs using the online tool at https://www.flyrnai.org/evaluateCrispr/ or performing an *in vitro* cutting assay.[10]

Make sure all the plasmids microinjected have a good quality. If not, transform again the assembled plasmids and purify them. Moreover, make sure that the injected concentrations are correct.

Try to increase the number of microinjected embryos, thus increasing the probability to obtain a successful transformant.

Alternatively, choose new CRISPR target sites.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Josefa González (josefa.gonzalez@csic.es). All external protocols used have been downloaded and can be found in http://gonzalezlab.eu/two-step-crispr-cas9-protocol.

Materials availability

This study did not generate any unique reagents.

Data and code availability

This study did not generate any unique datasets or codes.

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AUTHOR CONTRIBUTIONS

M.M. participated in the protocol design, performed the experiments, and wrote the first draft of the manuscript. L.A. participated in the protocol design, set the first steps of the protocol, and performed embryo microinjections. J.G. supervised the project and reviewed the manuscript. All authors have edited and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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