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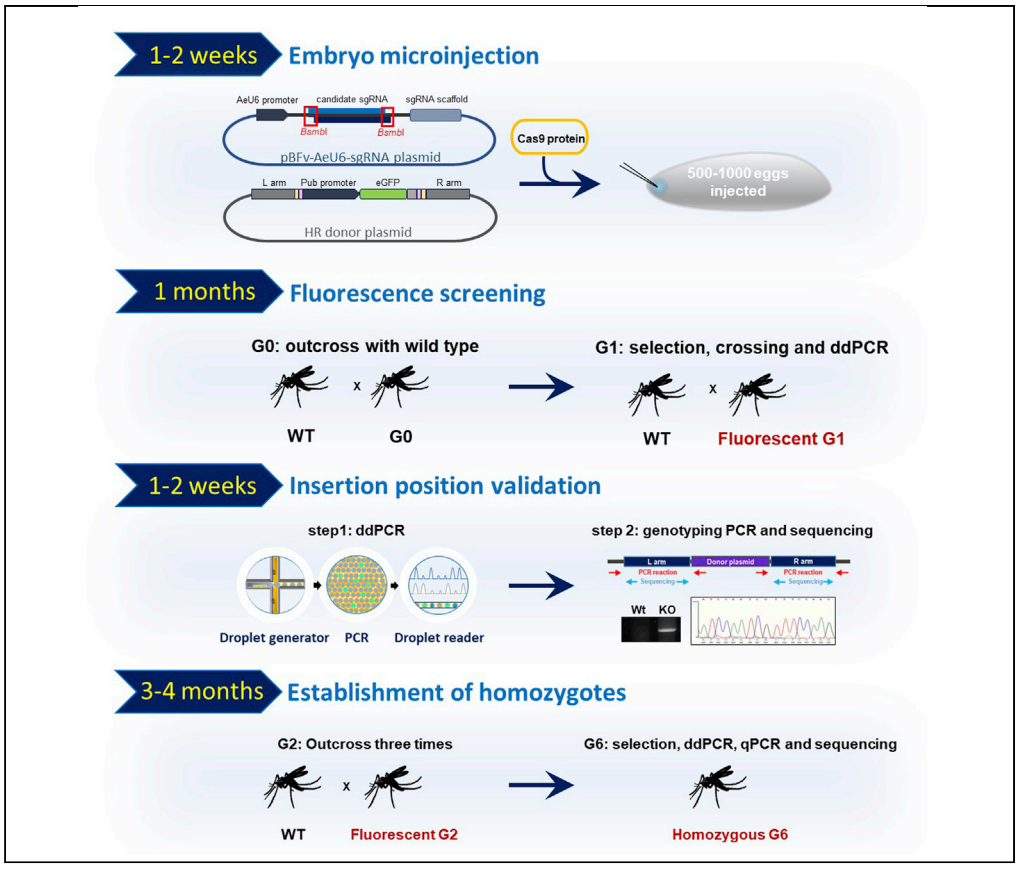
Protocol

Generating mutant *Aedes aegypti* mosquitoes using the CRISPR/Cas9 system



Implementation of CRISPR/Cas9 methodologies for mosquito gene editing has not yet become widespread. This protocol details the procedure for *Aedes aegypti* mosquito gene editing using homology-directed repair and fluorescent marker insertion, which facilitates the generation and screening of mutant mosquito lines for gene function testing. We describe optimized methods for single guide RNA plasmid preparation, homologous recombination donor plasmid construction, embryo microinjection, and precise gene knock-in confirmation. We also provide general guidance for establishing mutant mosquito lines.

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Highlights

Design and production of target-specific single guide RNA (sgRNA) and donor plasmids

Microinjection of sgRNA/donor plasmid/Cas9 mix into mosquito embryos

Use of fluorescent markers to hasten the establishment of mutant lines

Droplet digital PCR-based genotyping of indels *in vivo*

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Generating mutant *Aedes aegypti* mosquitoes using the CRISPR/Cas9 system

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SUMMARY

Implementation of CRISPR/Cas9 methodologies for mosquito gene editing has not yet become widespread. This protocol details the procedure for *Aedes aegypti* mosquito gene editing using homology-directed repair and fluorescent marker insertion, which facilitates the generation and screening of mutant mosquito lines for gene function testing. We describe optimized methods for single guide RNA plasmid preparation, homologous recombination donor plasmid construction, embryo microinjection, and precise gene knock-in confirmation. We also provide general guidance for establishing mutant mosquito lines.
For details on the practical use and execution of this protocol, please refer to Li et al. (2020).

BEFORE YOU BEGIN

Timing: 4–14 days

In the two weeks prior to embryo microinjection, single guide (sgRNA) and donor plasmids must be generated and female mosquitoes prepared for egg laying.

pBFv-AeU6-sgRNA plasmid generation

Timing: 1–2 weeks

We created a pBFv-AeU6 backbone plasmid for single guide RNA (sgRNA) cloning and expression to generate an expression system *in vivo* driven by the AeU6 promoter in *Aedes aegypti*.

1. The pBFv-AeU6 backbone plasmid was modified from the pBFv-U6.2 plasmid obtained from the Cas9 reagents provided by the Fly Stocks of the National Institute of Genetics (NIG-Fly) in Japan (Kondo and Ueda, 2013). The *Drosophila* U6.2 promoter was replaced with an *Ae. aegypti* U6 promoter to generate a pBFv-AeU6 backbone plasmid using an In-Fusion HD Cloning Kit (Clontech).
   a. We took 520 base pairs (bp) of the AeU6 promoter from the AAEL017763 locus of *Ae. aegypti* genomic DNA, amplified these bps via polymerase chain reaction (PCR) and fused them with a

gRNA backbone sequence to obtain an AeU6-gRNA DNA fragment by primer extension. The following primers were used:

Name	Sequence
AeU6-gRNA-F1	GCTTGATATCGAATTCCTATATAATTTAATTCCACTAGAGT
AeU6-gRNA-R1	TAGCTCTAAAACGGAGACGAACTCCGTCTCCA TTTCACTACTCTTGCCTCTGCTCTTATA
AeU6-gRNA-R2	TTTCAAGTTGATAACGGACTAGCCTTATTTTA ACTTGCTATTTCTAGCTCTAAAACGGAG

⚠ CRITICAL: Primers designed for In-Fusion HD cloning must have two characteristics: (1) the 5′ end of the primer must contain 15 homologous bases to coincide with the end of the DNA fragment to which it will be joined (i.e., the vector or another insert); (2) the 3′ end of the primer must contain sequences specific to the target gene.

i. Use the following mixtures to run the PCR:

Reagents	Amount
Genomic DNA (50 ng)	2 μL
AeU6-gRNA-F1 (50 μM)	1 μL
AeU6-gRNA-R1 (50 μM)	1 μL
dNTPs (10 mM)	2 μL
10× FFwd Buffer	10 μL
FFwd DNA Polymerase (2U/μL)	1 μL
Nuclease-free water	83 μL

ii. Use the following program conditions for PCR experiments.

Step	Temperature	Time
Initial denaturation	98°C	5 min
30 cycles	98°C	30 sec
	55°C	30 sec
	72°C	30 sec
Final extension	72°C	10 min
Hold	12°C	∞

iii. The first PCR products act as a template for secondary PCR using AeU6-gRNA-F1 and AeU6-gRNA-R2 primers.

b. Linearize the pBFv-U6.2 plasmid via double digestion with *Eco*RI/*Not*I (NEB).

i. Prepare the following reagents for restriction enzyme digestion.

Reagent	Amount
pBFv-U6.2 plasmid	5 μg
*Eco*RI (20U/μL)	1 μL
*Not*I (10U/μL)	2 μL
10× NEBuffer 3.1	4 μL
Nuclease-free water	Up to 40 μL

ii. Incubate at 37°C for 3–4 h for enzyme digestion. Alternatively, the digested plasmid can be incubated for 12–16 h at 37°C.

c. Purify the PCR products and digested plasmids using the procedures described in the Zymoclean Gel DNA Recovery Kit (Zymo Research, Cat#d4008; https://files.zymoresearch.com/protocols/_d4001t_d4001_d4002_d4007_d4008_zymoclean_gel_dna_recovery_kit.pdf).

d. Clone fragments using the In-Fusion HD Cloning Kit.
   i. Prepare the following mixtures for In-Fusion PCR:

Reagent	Amount
5× In-Fusion HD Enzyme Premix	3 μL
AeU6-gRNA DNA fragment	500 ng
*Eco*RI/*Not*I digested pBFv-U6.2 plasmid	1 μg
Nuclease-free water	Up to 15 μL

   ii. Incubate at 37°C for 15 min, followed by 15 min at 50°C and store at 4°C.

e. Transform 5 μL of the In-Fusion PCR products into 50 μL of Stellar Competent Cells (Clontech) for colony selection.
   i. Prepare the following reagents for colony PCR:

Reagents	Amount
Bacteria (from single colony)	A few
AeU6-gRNA-F1 (5 μM)	1 μL
AeU6-gRNA-R2 (5 μM)	1 μL
dNTPs (2.5 mM)	1 μL
10× PCR buffer	1 μL
Taq DNA polymerase (5U/μL)	0.1 μL
Nuclease-free water	5.9 μL

   ii. Use the following PCR program conditions:

Step	Temperature	Time
Initial denaturation	95°C	3 min
30 cycles	95°C	20 sec
	55°C	20 sec
	72°C	20 sec
Final extension	72°C	5 min
Hold	12°C	∞

f. Finally, use Sanger sequencing to confirm that the candidate pBFv-AeU6 backbone plasmids are correct.
g. Prepare the pBFv-AeU6 backbone plasmid using the QIAGEN Plamid Midi Kit (QIAGEN, Cat#12145) for the future generation of the pBFv-AeU6 sgRNA plasmid (Figure 1).

Pause point: Plasmid can be stored at −80°C more than a year prior to use for the cloning of annealed sgRNA oligo sets.

2. Select an optimal target sequence for CRISPR/Cas9 recognition. For this protocol we used the *Ae. Aegypti* target gene *GCTL-3* (AaegL3_AAEL000535, AaegL5_AAEL029058) as an example for gene knock-in. We have described this process in our recent publication (Li et al., 2020).

Note: The original ID of *GCTL-3* was AAEL000535 in AaegL3, but has been changed to AAEL029058 with an annotation and assembly in AaegL5.

a. Identify specific sgRNA sequences from target genes for CRISPR/Cas9 recognition. This can be done by inputting around 200 bases of the specific coding sequence (CDS) within one exon region into the CRISPR Optimal Target Finder web tool available at the flyCRISPR website. This tool can both identify CRISPR target sites and evaluate their specificity. We

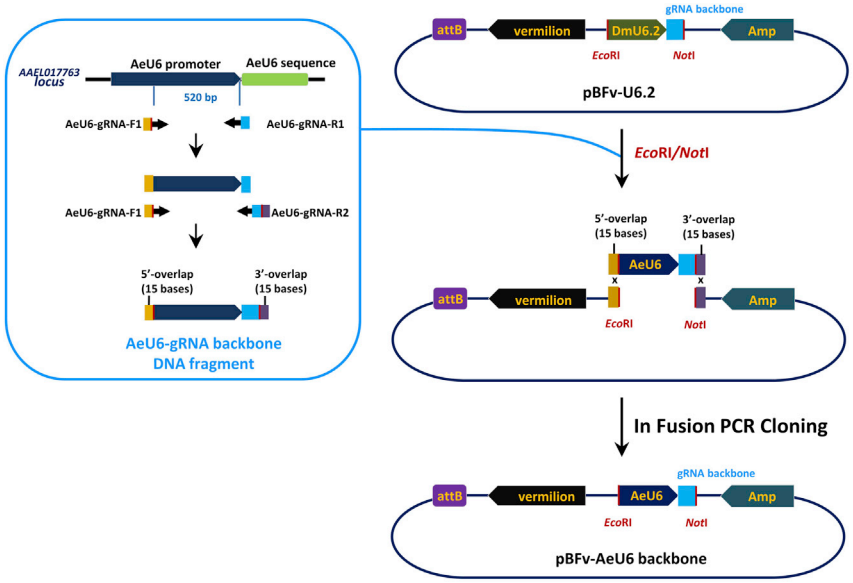

Figure 1. Cloning strategy for the pBFv-AeU6 backbone plasmid

suggesting using the finder available at https://flycrispr.org/target-finder/ (Gratz et al., 2014) and adjusting the parameter panels as follows:

i. Select genome: *Aedes aegypti* (AaegL3)
ii. Select guide length (nt): 20
iii. Find: All CRISPR targets

b. Balance selecting targets that are as close as possible to the transcription start site with those that minimize the risk of off-target effects.
   i. Try to find any mismatched base pairs within the seed regions of off-target binding sites.
   ii. Exclude target sites with four continuous T bases as this is the termination signal for RNA polymerase III (Gao et al., 2018).

Note: The risk of off-target effects for a specific target site is highly dependent on the genome database edition. The genome database of *Ae. aegypti* is not a complete assembly and is not completely annotated. For the same target site, we found a significant difference in the risk of off-target effects when using the AaegL3 or AaegL5 editions. In addition to this, the specific strain used can also present risks. For example, a large number of sequence variations have been found when comparing the Higgs and Liverpool *A. aegypti* strains.

Optional: Other websites such as CHOPCHOP (http://chopchop.cbu.uib.no/) or Benchling (https://benchling.com/) can also be used for sgRNA design and off-target site prediction (Montague et al., 2014).

3. Generate sgRNA fragments by annealing oligonucleotides and then cloning into the *Bsm*BI site of the pBFv-AeU6 backbone plasmid for pBFv-AeU6-sgRNA plasmid generation.
   a. Select a target site without a PAM sequence (NGG) when designing sgRNAs.
   b. Add an additional G base to the 5′ end of the sgRNA sequence (provided it is not already a G base).

⚠ CRITICAL: The G in the sense sequence corresponds to the first nucleotide of the sgRNA which is necessary for efficient U6-driven expression.

   c. Using the sgRNA sequence as a template, design the oligonucleotides for sgRNA fragment generation as follows:

i. Sense oligonucleotide: 5′–**AAAT** (sgRNA or G-sgRNA) –3′
ii. Anti-sense oligonucleotide: 3′– (sgRNA or C-sgRNA) **CAAA**–5′
Example: GCTL-3 target site (sgRNA sequence is underlined)
Genomic sequence: 5′–GCCCAGTTGGTGTAGTTGAC**GGG**–3′
Sense oligonucleotide: 5′–**AAAT**GCCCAGTTGGTGTAGTTGAC–3′
Anti-sense oligonucleotide: 5′–**AAAC**GTCAACTACACCAACTGGGC–3′

Note: This target site was created based on the AaegL3 edition of the genome database.

d. Dilute the oligonucleotide to 100 μM in 1× Tris-EDTA (TE) buffer and set up an annealing reaction.
i. Prepare the following reagents for oligonucleotide annealing reactions:

Reagents	Amount
Sense oligo (from 100 μM stock)	18 μL
Anti-sense oligo (from 100 μM stock)	18 μL
10× FFwd buffer	4 μL

ii. Heat the mixture to 95°C and allow the sample to slowly cool to 23°C –25°C.

e. Linearize the pBFv-AeU6 backbone plasmid by *Bsm*BI digestion (NEB).
i. Prepare the following reagents for restriction enzyme digestion, and incubate at 55°C for 3–4 h.

Reagent	Amount
pBFv-AeU6 backbone plasmid	5 μg
*Bsm*BI (10U/μL)	2 μL
10× NEBuffer 3.1	4 μL
Nuclease-free water	Up to 40 μL

ii. Purify the linearized pBFv-AeU6 backbone using the protocols described in the DNA Clean & Concentrator-5 Kit (Zymo Research, Cat#d4014; https://files.zymoresearch.com/protocols/_d4003t_d4003_d4004_d4013_d4014_dna_clean_concentrator_-5.pdf).

4. Ligate the annealed oligonucleotides to the *Bsm*BI cut pBFv-AeU6 backbone plasmid to generate the required pBFv-AeU6-sgRNA plasmid.
a. Prepare 10 μL total volume of the ligation mixture using the following reagents:

Reagents	Amount
Annealed oligo set	2 μL
*Bsm*BI digested pBFv-AeU6 backbone plasmid (50 ng/μL)	2 μL
10× T4 DNA ligase buffer	1 μL
T4 DNA ligase (200U/μL)	1 μL
Nuclease-free water	4 μL

b. Incubate at 4°C for 6 h for the ligation reaction. Afterward, transform 3 μL of the ligation product into 50 μL of DH10B Competent Cells for colony selection.
i. Prepare the following reagents for colony PCR:

Reagents	Amount
Bacteria (from a single colony)	A few
sgRNA sense oligo (5 μM)	1 μL
pFBv-com reverse primer (5 μM)	1 μL
dNTPs (2.5 mM)	1 μL
10× PCR buffer	1 μL
Taq DNA polymerase (5U/μL)	0.1 μL
Nuclease-free water	5.9 μL

ii. Use the following pFBv-com reverse primer sequence:
5′ –GGAAACAGCTATGACCATGA–3′
iii. Use the following PCR program conditions

Step	Temperature	Time
Initial denaturation	95°C	3 min
30 cycles	95°C	20 sec
	55°C	20 sec
	72°C	20 sec
Final extension	72°C	5 min
Hold	12°C	∞

c. Finally, use Sanger sequencing to confirm that the candidate pBFv-AeU6-sgRNA plasmid is correct.
d. Prepare the pBFv-AeU6-sgRNA plasmid using the EndoFree Pasmid Maxi Kit (QIAGEN, Cat#12362) for future embryo microinjection (Figure 2).

⚠ CRITICAL: Following plasmid cloning for embryo microinjection, we recommend that plasmids should be obtained using an endotoxin-free kit in order to achieve high-quality, low toxicity DNA. Furthermore, we strongly recommend to use either midi- or maxi-prep kits, as mini-plasmid preparation is inadequate for embryo microinjection. Minipreps kit based

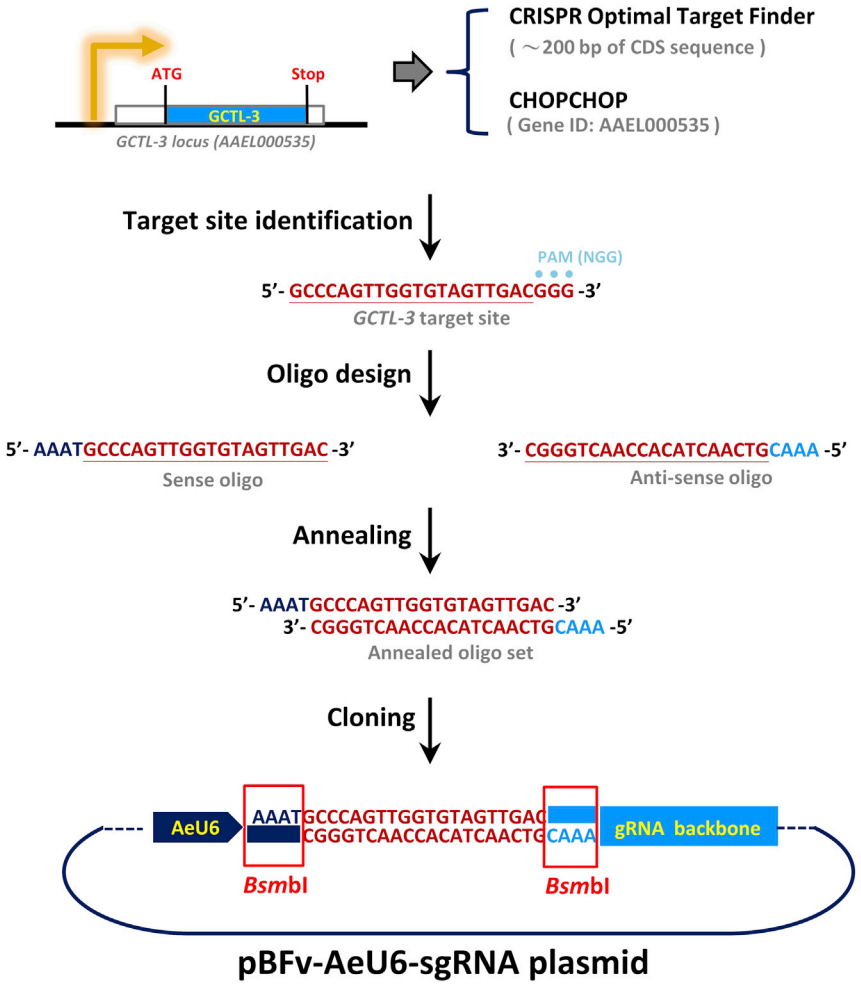

Figure 2. sgRNA design and cloning strategy for the pBFv-AeU6-sgRNA plasmid

on columns without endotoxin removal capabilities, isopropanol precipitation, and ethanol washing of DNA can leave substantial levels of toxic contaminants.

Pause point: Plasmid can be stored at −80°C more than a year before being used for embryo microinjection.

pCR2-TOPO-attp-loxp-Pub-eGFP HR donor plasmid generation

Timing: 1–2 weeks

In order to improve efficiency while screening multiple mutant lines, we generated a fluorescent marker donor plasmid, pCR2-TOPO-attp-loxp-Pub-eGFP, for genome insertion. The plasmid needs HR to replace the gene knock-out with the knock-in. This fluorescent marker HR donor plasmid introduces two pairs of site-specific sequence elements recognized by recombinase. The first is the loxp sequence to remove the fluorescent marker cargo via Cre recombinase, and the second is the attP sequence, to allow for cargo exchange by PhiC31 integrase.

5. Modify the pCR2-TOPO-attP-loxp-Pub-eGFP plasmid from the pCR2-TOPO plasmid of the Invitrogen TOPO-TA Cloning Kit.
   a. Step 1: Cloning eGFP-CDS and SV40-polyA fragments.
      The eGFP-CDS and SV40-polyA fragments were generated from the pMOS1_AePUb-Den3-4miR_3xp3-eGFP plasmid. They were then cloned into the *SpeI*/*XhoI* sites of pCR2-TOPO to generate a pCR2-TOPO-eGFP-SV40 transition plasmid using the In-Fusion HD Cloning Kit (Yen et al., 2018).
      i. Create the *SpeI*/*NheI*/*AvrII*/*NotI*_eGFP-CDS_*NruI* fragment using the following PCR primers. The first PCR product, F1R1, acts as the template for the secondary PCR using the F2 and R1 primers.

Name	Sequence
pCR2 fusion-1 eGFP-SV40_eGFP-F1	CCTAGGATTTGCGGCCGCATGGTG AGCAAGGGCGAGGAGCTGTT
pCR2 fusion-1 eGFP-SV40_eGFP-F2	GCTCGGATCCACTAGTGCTAGCTCT ACCTAGGATTTGCGGCCGCATGGTGA
pCR2 fusion-1 eGFP-SV40_eGFP-R1	TATGGCTGATTATGATCTCGCGATAT TACTTGTACAGCTCGTCCATGCCG

      ii. Create the SV40-polyA_*NdeI*/*XhoI* fragment using the following PCR primers:

Name	Sequence
pCR2 fusion-1 eGFP-SV40_SV40-F1	TCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGC
pCR2 fusion-1 eGFP-SV40_SV40-R1	TAGATGCATGCTCGAGATCATATGG TACGCGTATTCGATAAGCTTTAAG

      iii. Prepare 100 μL total volume of the following reagents for the PCR:

Reagents	Amount
pMOS1_AePUb-Den3-4miR_3xp3-eGFP plasmid (50 ng/μL)	1 μL
Forward primer (50 μM)	1 μL
Reverse primer (50 μM)	1 μL
dNTPs (10 mM)	2 μL
10× FFwd buffer	10 μL
FFwd DNA polymerase (2U/μL)	1 μL
Nuclease-free water	84 μL

iv. Set the following PCR program conditions.

Step	Temperature	Time
Initial denaturation	98°C	5 min
30 cycles	98°C	30 sec
	55°C	30 sec
	72°C	1 min
Final extension	72°C	10 min
Hold	12°C	∞

v. Purify PCR products using the Zymoclean Gel DNA Recovery Kit.

vi. Linearize the pCR2-TOPO plasmid via double digestion by *SpeI*/*XhoI* at 37°C for 3–4 h.

Reagent	Amount
pCR2-TOPO plasmid	5 μg
SpeI (10U/μL)	2 μL
XhoI (20U/μL)	1 μL
10× NEBuffer 2.1	4 μL
Nuclease-free water	Up to 40 μL

vii. Purify linearized plasmids using the DNA Clean & Concentrator-5 Kit.

viii. Prepare 20 μL total volume of the following reagents for In-Fusion PCR to generate the pCR2-TOPO-eGFP transition plasmid.

Reagent	Amount
5× In-Fusion HD Enzyme Premix	3 μL
SpeI/*NheI*/*AvrII*/*NotI*_eGFP-CDS_*NruI* fragment	400 ng
SV40-polyA_*NdeI*/*XhoI* fragment	400 ng
SpeI/*XhoI* digested pCR2-TOPO plasmid	1 μg
Nuclease-free water	Up to 20 μL

ix. Incubate at 37°C for 15 min, followed by 15 min at 50°C, and store at 4°C.

x. Transform 10 μL of the In-Fusion PCR reaction products into 100 μL of Stellar Competent Cells for colony selection.

xi. Prepare the following reagents for colony PCR:

Reagents	Amount
Bacteria (from a single colony)	A few
pCR2 fusion-1 eGFP-SV40_eGFP-F2 (5 μM)	1 μL
pCR2 fusion-1 eGFP-SV40_SV40-R1 (5 μM)	1 μL
dNTPs (2.5 mM)	1 μL
10× PCR buffer	1 μL
Taq DNA polymerase (5U/μL)	0.1 μL
Nuclease-free water	5.9 μL

xii. Use the following PCR program conditions.

Step	Temperature	Time
Initial denaturation	95°C	3 min
30 cycles	95°C	20 sec
	55°C	20 sec
	72°C	1 min
Final extension	72°C	5 min
Hold	12°C	∞

xiii. Finally, use Sanger sequencing to confirm that the candidate pCR2-TOPO-eGFP-SV40 transition plasmid is correct.

b. Step 2: Pub-promoter cloning

1382 bp of the Pub promoter was generated from the pMOS1_AePUb-Den3-4miR_3xp3-eGFP plasmid, amplified via PCR and cloned into the *AvrII*/*NotI* sites of the pCR2-TOPO-eGFP-SV40 transition vector using the In-Fusion HD Cloning Kit.

i. Generate the Pub promoter fragment using the following PCR primers:

Name	Sequence
pCR2 fusion-2 AePUb-pro-F1	GCTAGCTCTACCTAGGTATCTTTAC ATGTAGCTTGTGCATTGAATCC
pCR2 fusion-2 AePUb-pro-R1	GCTCACCATGCGGCCGCGTTGAAA TCTCTGTTGAGCAGAAAAAGAAACGAG

ii. Prepare 100 μL total volume of the following reagents for PCR:

Reagents	Amount
pMOS1_AePUb-Den3-4miR_3xp3-eGFP plasmid (50 ng/μL)	1 μL
pCR2 fusion-2 AePUb-pro-F1 (50 μM)	1 μL
pCR2 fusion-2 AePUb-pro-R1 (50 μM)	1 μL
dNTPs (10 mM)	2 μL
10× FFwd buffer	10 μL
FFwd DNA polymerase (2U/μL)	1 μL
Nuclease-free water	84 μL

iii. Use the following PCR program conditions.

Step	Temperature	Time
Initial denaturation	98°C	5 min
30 cycles	98°C	30 sec
	55°C	30 sec
	72°C	2 min
Final extension	72°C	10 min
Hold	12°C	∞

iv. Purify PCR products using the Zymoclean Gel DNA Recovery Kit.

v. Linearize the pCR2-TOPO-eGFP-SV40 transition plasmid via double digestion with *AvrII*/*NotI* at 37°C for 12–16 h.

Reagent	Amount
pCR2-TOPO-eGFP-SV40 transition plasmid	5 μg
AvrII (4U/μL)	2.5 μL
NotI (10U/μL)	1 μL
10× NEBuffer 3.1	4 μL
Nuclease-free water	Up to 40 μL

vi. Purify the linearized plasmids using the DNA Clean & Concentrator-5 Kit.

vii. Prepare 15 μL total volume of the following reagents for In-Fusion PCR to generate the pCR2-TOPO-Pub-eGFP-SV40 transition plasmid:

Reagent	Amount
5× In-Fusion HD Enzyme Premix	3 μL
NheI/*AvrII*_Pub-promoter_*NotI* DNA fragment	500 ng
AvrII/*NotI* digested pCR2-TOPO-eGFP-SV40 transition plasmid	1 μg
Nuclease-free water	Up to 15 μL

viii. Incubate at 37°C for 15 min, followed by 15 min at 50°C, and store at 4°C.

ix. Transform 5 μL of In Fusion PCR products into 50 μL of Stellar Competent Cells for colony selection.

x. Prepare the following reagents for colony PCR:

Reagents	Amount
Bacteria (from a single colony)	A few
pCR2 fusion-2 AePUb-pro-F1 (5 μM)	1 μL
pCR2 fusion-2 AePUb-pro-R1 (5 μM)	1 μL
dNTPs (2.5 mM)	1 μL
10× PCR buffer	1 μL
Taq DNA polymerase (5U/μL)	0.1 μL
Nuclease-free water	5.9 μL

xi. Use the following PCR program conditions.

Step	Temperature	Time
Initial denaturation	95°C	3 min
30 cycles	95°C	20 sec
	55°C	20 sec
	72°C	2 min
Final extension	72°C	5 min
Hold	12°C	∞

xii. Finally, use Sanger sequencing to confirm that the candidate pCR2-TOPO-Pub-eGFP-SV40 transition plasmid is correct.

c. Step 3: Cloning sequences, recognized by recombinase (attP and loxp), and three reading frames with stop sequences.

The DNA fragments containing two site-specific recombinase sequences, attP and loxp, and three reading frames with stop sequences were generated by PCR primer extension, and then each one was cloned into the 5′ and 3′ ends of the Pub-eGFP-SV40 cargo.

i. Generate the 5′ end attP-stop-loxp fragment containing an extra restriction enzyme, *Xma*I, using the following PCR primers. The first PCR product, F1R1, acts as the template for secondary PCR using the F2 and R2 primers.

Name	Sequence
pCR2 fusion-3 5′-attP-loxp-F1	CGGGAGTAGTGCCCCAACTGG GGTAACCTTTGAGTTCTCTCAGT TGGGGGCGTAGGGTCG
pCR2 fusion-3 5′-attP-loxp-F2	ATCCACTAGTGCTAGCCTGACCC GGGCCCAGGTCAGAAGCGGTTT TCGGGAGTAGTGCCC
pCR2 fusion-3 5′-attP-loxp-R1	GTTATTTATTAATCACTAATCAT TACAACCCCTTGTGTCATGTCGG CGACCCTACGCCCC
pCR2 fusion-3 5′-attP-loxp-R2	TGTAAAGATACCTAGGATAACT TCGTATAGCATACATTATACGAAG TTATTTATTAATC

ii. Prepare 100 μL total volume of the following reagents for PCR:

Reagents	Amount
Forward primer (50 μM)	1 μL
Reverse primer (50 μM)	1 μL
dNTPs (10 mM)	2 μL
10× FFwd buffer	10 μL
FFwd DNA polymerase (2U/μL)	1 μL
Nuclease-free water	85 μL

iii. Set the following PCR program conditions.

Step	Temperature	Time
Initial denaturation	98°C	5 min
30 cycles	98°C	30 sec
	55°C	30 sec
	72°C	30 sec
Final extension	72°C	5 min
Hold	12°C	∞

iv. Purify PCR products using the Zymoclean Gel DNA Recovery Kit.

v. Linearize the pCR2-TOPO-Pub-eGFP-SV40 transition plasmid via double digestion with *Nhe*I/*Avr*II at 37°C for 12–16 h.

Reagent	Amount
pCR2-TOPO-Pub-eGFP-SV40 transition plasmid	5 μg
*Nhe*I (10U/μL)	1 μL
*Avr*II (4U/μL)	2.5 μL
10× NEBuffer 1.1	4 μL
Nuclease-free water	Up to 40 μL

vi. Purify the linearized plasmids using the DNA Clean & Concentrator-5 Kit.

vii. Prepare 15 μL total volume of the following reagents for In-Fusion PCR to introduce the *Nhe*I/*Xma*I-attP-stop-loxp-*Avr*II DNA fragment into the 5′ end of the Pub-eGFP-SV40 cargo.

Reagent	Amount
5× In-Fusion HD Enzyme Premix	3 μL
*Nhe*I/*Xma*I-attP-stop-loxp-*Avr*II DNA fragment	500 ng
*Nhe*I/*Avr*II digested pCR2-TOPO-Pub-eGFP-SV40 transition plasmid	1 μg
Nuclease-free water	Up to 15 μL

viii. Incubate at 37°C for 15 min, followed by 15 min at 50°C, and store at 4°C.

ix. Transform 5 μL of the In-Fusion PCR products into 50 μL of Stellar Competent Cells for colony selection.

x. Conduct colony selection using the following primers for colony PCR:

Name	Sequence
pCR2-TOPO vector colony check F	CGTATGTTGTGTGGAATTGTG
AePUb-pr colony check R	CTTTCTTGTCTGGCTCAGCT

xi. Prepare the following reagents for colony PCR:

Reagents	Amount
Bacteria (from a single colony)	A few
pCR2-TOPO vector colony check F (5 μM)	1 μL
AePUb-pr colony check R (5 μM)	1 μL
dNTPs (2.5 mM)	1 μL
10× PCR buffer	1 μL
Taq DNA polymerase (5U/μL)	0.1 μL
Nuclease-free water	5.9 μL

xii. Use the following PCR program conditions.

Step	Temperature	Time
Initial denaturation	95°C	3 min
30 cycles	95°C	20 sec
	55°C	20 sec
	72°C	20 sec
Final extension	72°C	5 min
Hold	12°C	∞

xiii. Finally, use Sanger sequencing to confirm that the candidate pCR2-TOPO-Pub-eGFP transition plasmid containing the *Xma*I-attP-stop-loxp sequence at the 5′ end of the Pub-eGFP-SV40 cargo is correct.

xiv. Generate the 3′ end loxp-stop-attP fragment, also containing an extra restriction enzyme, *Nde*I, using the following PCR primers. The first PCR product, F1R1, acts as the template for secondary PCR using the F2 and R2 primers.

Name	Sequence
pCR2 fusion-3 3′-loxp-attP-F1	CGGGAGTAGTGCCCCAACT GGGGTAACCTTTGAGTTCTCT CAGTTGGGGGCGTAGGGTCG
pCR2 fusion-3 3′-loxp-attP-F2	ATCCACTAGTGCTAGCCTGACC CGGGCCCAGGTCAGAAGCGGTT TTCGGGAGTAGTGCCC
pCR2 fusion-3 3′-loxp-attP-R1	GTTATTTATTAATCACTAATCAT TACAACCCCTTGTGTCATGTCG GCGACCCTACGCCCC
pCR2 fusion-3 3′-loxp-attP-R2	TGTAAAGATACCTAGGATAACT TCGTATAGCATACATTATACGA AGTTATTTATTAATC

Note: We disrupted the *Nde*I site of the pCR2-TOPO-(5′-attP-stop-loxp)-Pub-eGFP-SV40 transition plasmid, and created a *Nde*I site in the 3′ end loxp-stop-attP fragment.

xv. Prepare 100 μL total volume of the following reagents for PCR:

Reagents	Amount
Forward primer (50 μM)	1 μL
Reverse primer (50 μM)	1 μL
dNTPs (10 mM)	2 μL
10× FFwd buffer	10 μL
FFwd DNA polymerase (2U/μL)	1 μL
Nuclease-free water	85 μL

xvi. Use the following PCR program conditions.

Step	Temperature	Time
Initial denaturation	98°C	5 min
30 cycles	98°C	30 sec
	55°C	30 sec
	72°C	30 sec
Final extension	72°C	5 min
Hold	12°C	∞

xvii. Purify PCR products using the Zymoclean Gel DNA Recovery Kit.

xviii. Linearize the pCR2-TOPO-Pub-eGFP-SV40 transition plasmid containing the *Xma*I-attP-stop-loxp sequence at the 5′ end of the Pub-eGFP-SV40 cargo via double digestion by *Nde*I/*Xho*I at 37°C for 3–4 h.

Reagent	Amount
pCR2-TOPO-(5′-attP-stop-loxp)-Pub-eGFP-SV40 transition plasmid	5 μg
*Nde*I (20U/μL)	1 μL
*Xho*I (20U/μL)	1 μL
10× NEBuffer 2.1	4 μL
Nuclease-free water	Up to 40 μL

xix. Purify the linearized plasmids using the DNA Clean & Concentrator-5 Kit.

xx. Prepare 15 μL total volume of the following reagents for In-Fusion PCR to introduce the loxp-stop-attP-*Nde*I/*Xho*I DNA fragment into the 3′ end of the Pub-eGFP-SV40 cargo.

Reagent	Amount
5× In-Fusion HD Enzyme Premix	3 μL
loxp-stop-attP-*Nde*I/*Xho*I DNA fragment	500 ng
*Nde*I/*Xho*I digested pCR2-TOPO-(5′-attP-stop-loxp)-Pub-eGFP transition plasmid	1 μg
Nuclease-free water	Up to 15 μL

xxi. Incubate at 37°C for 15 min, followed by 15 min at 50°C, and store at 4°C.

xxii. Transform 5 μL of the In-Fusion PCR products into 50 μL of Stellar Competent Cells for colony selection.

xxiii. Conduct colony selection using the following primers for colony PCR:

Name	Sequence
SV40-polyA colony check F	GTGGTTTGTCCAAACTCATCAA
pCR2-TOPO vector colony check R	ACGTTGTAAAACGACGGCCAG

xxiv. Prepare the following reagents for colony PCR:

Reagents	Amount
Bacteria (from a single colony)	A few
SV40-polyA colony check F (5 μM)	1 μL
pCR2-TOPO vector colony check R (5 μM)	1 μL
dNTPs (2.5 mM)	1 μL
10× PCR buffer	1 μL
Taq DNA polymerase (5U/μL)	0.1 μL
Nuclease-free water	5.9 μL

xxv. Use the following PCR program conditions.

Step	Temperature	Time
Initial denaturation	95°C	3 min
30 cycles	95°C	20 sec
	55°C	20 sec
	72°C	20 sec
Final extension	72°C	5 min
Hold	12°C	∞

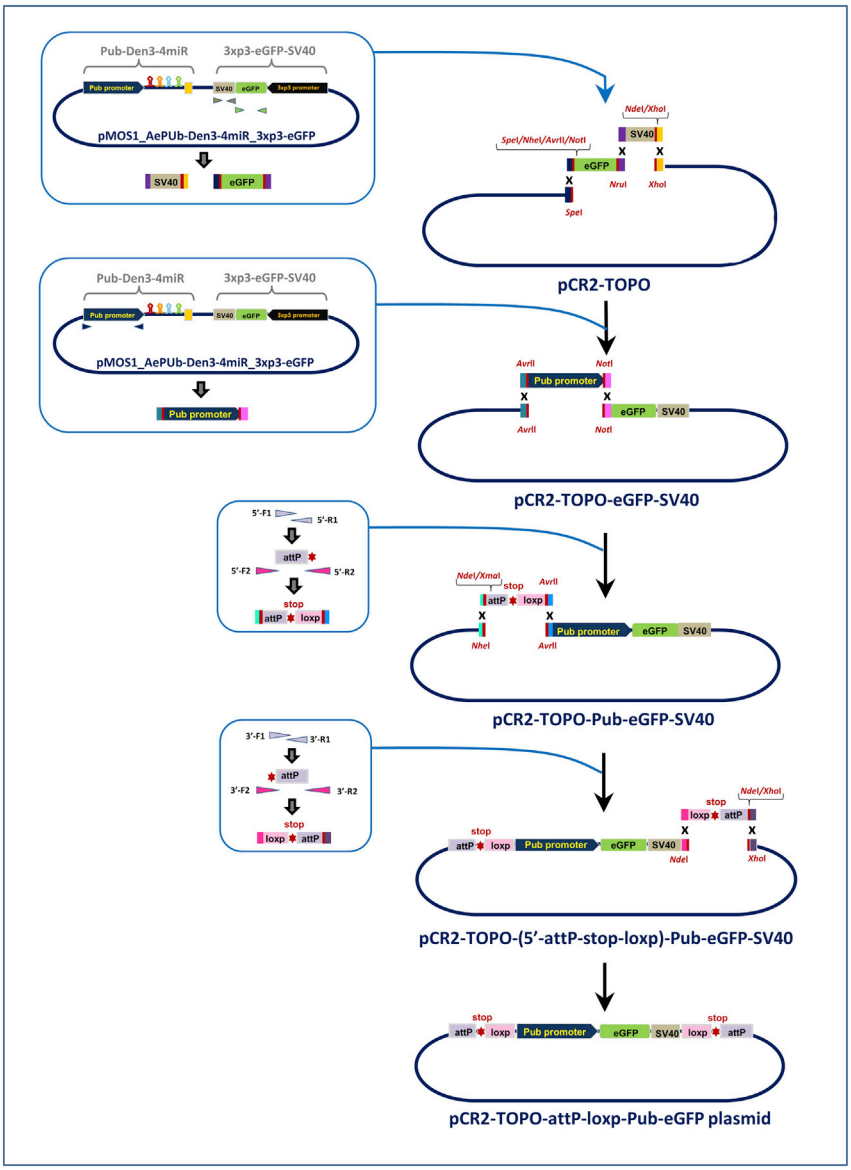

Figure 3. Cloning procedure for the pCR2-TOPO-attP-loxp-Pub-eGFP plasmid using the In-Fusion HD Cloning Kit

xxvi. Finally, use Sanger sequencing to confirm that the candidate pCR2-TOPO-attP-loxp-Pub-eGFP plasmid containing two pairs of the attP-stop-loxp sequence at the 5′ and 3′ ends of the Pub-eGFP-SV40 cargo is correct.

xxvii. Prepare the pCR2-TOPO-attP-loxp-Pub-eGFP plasmid using the QIAGEN Plamid Midi Kit (QIAGEN, Cat#12145) for the future cloning of the pCR2-TOPO-[Gene of Interest]-attP-loxp-Pub-eGFP HR donor plasmid (Figure 3).

Pause point: This plasmid can be stored at −80°C more than a year before being used for the future cloning of HR flanking sequences.

6. Generate the pCR2-TOPO-[Gene of Interest]-attP-loxp-Pub-eGFP HR donor plasmid by cloning the left (L) and right (R) HR flanking sequences of the Gene of Interest into the pCR2-TOPO-attP-loxp-Pub-eGFP plasmid.

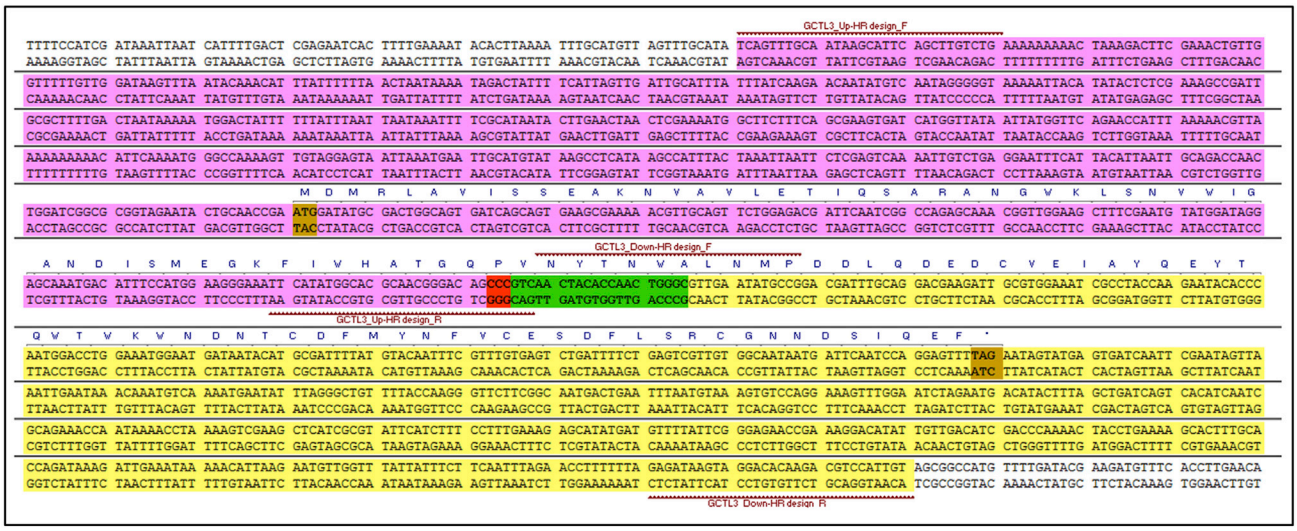

Figure 4. Design of the HR flanking sequences for the GCTL-3 gene

The HR region L-arm is highlighted in pink and the R-arm in yellow. Design of the HR flanking sequences for the GCTL-3 gene. The HR region L-arm is highlighted in pink and the R-arm in yellow. Green represents sgRNA target site. Red represents PAM sequence (NGG).

a. Step 1. Design the HR flanking sequences (Figure 4).
   Cas9 cutting site
   NNNNNNNNNNNNNNNNN^NNN**NGG**
   <- - - - LLLLLLLLLLLLLLLLLLLLLLLLLLLLL RRRRRRRRRRRRRRRRRR - - - ->
   500 - 1000 bp 500 - 1000 bp
   (L = left homology arm, R = right homology arm)
   Example: GCTL-3 target site **CCCGTC^AACTACACCAACTGGGC**
   GGGCAG^TTGATGTGGTTGACCCG
   L arm <- - - -LLLLLLLLLLLLLLLL RRRRRRRRRRRRRRRRRR- - - -> R arm
b. Step 2. Cloning of the HR flanking sequences cloning.
   i. The L-arm (678 bp) and R-arm (602 bp) HR flanking sequences of the *GCTL-3* gene were generated using the following primers for the PCR experiment.

Name	Sequence
PUb-eGFP HR fusion GCTL-3-Up HR_F1	ATCCACTAGTGCTAGCTCAGTT TGCAATAAGCATTCAGCTTGTCTG
PUb-eGFP HR fusion GCTL-3-Up HR_R1	CTGACCTGGGCCCGGGGACGTG CTGTCCCGTTGCGTGCCATATGAA
PUb-eGFP HR fusion GCTL-3-Down HR_F1	TCTGACCTGGGCATATGAACTA CACCAACTGGGCGTTGAATATGCCG
PUb-eGFP HR fusion GCTL-3-Down HR_R1	TAGATGCATGCTCGAGACAAT GGACGTCTTGTGTCCTACTTATCTC

   ii. Prepare 100 μL total volume of the following reagents for PCR:

Reagents	Amount
Genomic DNA (50 ng)	2 μL
Forward primer (50 μM)	1 μL
Reverse primer (50 μM)	1 μL
dNTPs (10 mM)	2 μL
10× FFwd buffer	10 μL
FFwd DNA polymerase (2U/μL)	1 μL
Nuclease-free water	83 μL

iii. Use the following PCR program conditions.

	Temperature	Time
Initial denaturation	98°C	5 min
30 cycles	98°C	30 sec
	55°C	30 sec
	72°C	1 min
Final extension	72°C	10 min
Hold	12°C	∞

iv. Purify PCR products using the Zymoclean Gel DNA Recovery Kit.

⚠ CRITICAL: If the HR flanking sequences contain the interferential restriction enzyme sites for HR cloning, you must pay particular attention to the cloning procedures.

v. Clone the down strand HR (R-arm) DNA fragment into the *NdeI*/*XhoI* sites of the pCR2-TOPO-attP-loxp-Pub-eGFP vector.

vi. Linearize the pCR2-TOPO-attP-loxp-Pub-eGFP plasmid via double digestion with *NdeI*/*XhoI* at 37°C for 3–4 h.

Reagent	Amount
pCR2-TOPO-attP-loxp-Pub-eGFP plasmid	5 μg
NdeI (20U/μL)	1 μL
XhoI (20U/μL)	1 μL
10× NEBuffer 2.1	4 μL
Nuclease-free water	Up to 40 μL

i. Purify the linearized plasmids using the DNA Clean & Concentrator-5 Kit.

ii. Prepare 15 μL total volume of the following reagents for In-Fusion PCR to introduce the down strand HR (R-arm) DNA fragment into the 3′ end of the attP-loxp-Pub-eGFP cargo of the pCR2-TOPO-attP-loxp-Pub-eGFP plasmid.

Reagent	Amount
5× In-Fusion HD Enzyme Premix	3 μL
NdeI-down strand HR (R-arm)-*XhoI* DNA fragment	500 ng
NdeI/*XhoI* digested pCR2-TOPO-attP-loxp-Pub-eGFP plasmid	1 μg
Nuclease-free water	Up to 15 μL

iii. Incubate at 37°C for 15 min, followed by 15 min at 50°C, and store at 4°C.

iv. Transform 5 μL of the In-Fusion PCR products into 50 μL of Stellar Competent Cells for colony selection.

v. Prepare the following reagents for colony PCR:

Reagents	Amount
Bacteria (from a single colony)	A few
SV40-polyA colony check F (5 μM)	1 μL
pCR2-TOPO vector colony check R (5 μM)	1 μL
dNTPs (2.5 mM)	1 μL
10× PCR buffer	1 μL
Taq DNA polymerase (5U/μL)	0.1 μL
Nuclease-free water	5.9 μL

i. Use the following PCR program conditions:

Step	Temperature	Time
Initial denaturation	95°C	3 min
30 cycles	95°C	20 sec
	55°C	20 sec
	72°C	20 sec
Final extension	72°C	5 min
Hold	12°C	∞

ii. Finally, use Sanger sequencing to confirm that the candidate pCR2-TOPO-(R arm)-attP-loxp-Pub-eGFP plasmid containing the down strand HR (R-arm) sequence at the 3′ end of the attP-loxp-Pub-eGFP-SV40 cargo is correct.

iii. After the down strand HR fragment has been created, clone the up strand HR (L-arm) DNA fragment into the *Nhe*I/*Xma*I sites of the pCR2-TOPO-(R arm)-attP-loxp-Pub-eGFP transition plasmid.

iv. Linearize the pCR2-TOPO-(R arm)-attP-loxp-Pub-eGFP transition plasmid via double digestion with *Nhe*I/*Xma*I at 37°C for 3–4 h.

Reagent	Amount
pCR2-TOPO-(R arm)-attP-loxp-Pub-eGFP transition	5 μg
*Nhe*I (10U/μL)	2 μL
*Xma*I (10U/μL)	2 μL
10× NEBuffer CutSmart	4 μL
Nuclease-free water	Up to 40 μL

v. Purify the linearized plasmids using the DNA Clean & Concentrator-5 Kit.

vi. Prepare 15 μL total volume of the following reagents for In-Fusion PCR to introduce the up strand HR (L-arm) DNA fragment into the 5′ end of the attP-loxp-Pub-eGFP cargo of the pCR2-TOPO-(R arm)-attP-loxp-Pub-eGFP plasmid.

Reagent	Amount
5× In-Fusion HD Enzyme Premix	3 μL
*Nhe*I-up strand HR (L-arm)-*Xma*I DNA fragment	500 ng
*Nhe*I/*Xma*I digested pCR2-TOPO-(R arm)-attP-loxp-Pub-eGFP plasmid	1 μg
Nuclease-free water	Up to 15 μL

vii. Incubate at 37°C for 15 min, followed by 15 min at 50°C, and store at 4°C.

viii. Transform 5 μL of the In-Fusion PCR products into 50 μL of Stellar Competent Cells for colony selection.

ix. Prepare the following reagents for colony PCR:

Reagents	Amount
Bacteria (from a single colony)	A few
pCR2-TOPO vector colony check F (5 μM)	1 μL
AePUb-pr colony check R (5 μM)	1 μL
dNTPs (2.5 mM)	1 μL
10× PCR buffer	1 μL
Taq DNA polymerase (5U/μL)	0.1 μL
Nuclease-free water	5.9 μL

i. Use the following PCR program conditions.

Step	Temperature	Time
Initial denaturation	95°C	3 min
30 cycles	95°C	20 sec
	55°C	20 sec
	72°C	20 sec
Final extension	72°C	5 min
Hold	12°C	∞

ii. Finally, use Sanger sequencing to confirm that the candidate pCR2-TOPO-GCTL-3-attP-loxp-Pub-eGFP HR donor plasmid is correct.

iii. Prepare the pCR2-TOPO-GCTL-3-attP-loxp-Pub-eGFP HR donor plasmid using the EndoFree Pasmid Maxi Kit (QIAGEN, Cat#12362) for future embryo microinjection (Figure 5).

Pause point: This plasmid can be stored at −80°C more than a year before being used for embryo microinjection.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial		
ECOS 10B Competent Cells (DH10B strain)	YB Biotech	Cat#FYE508-80VL
Stellar Competent Cells	Clontech	Cat#639649
Chemicals, peptides, and recombinant proteins		
Taq DNA polymerase	Bernardo Scientific	Cat#TA110150
T4 DNA ligase	NEB	Cat#M0202L
Restriction enzyme	NEB	n/a
FFwd DNA polymerase	Bernardo Scientific	Cat#FF110350
Critical commercial assays		
In-Fusion HD Cloning Kit	Clontech	Cat#639649
TrueCut Cas9 Protein v2	Thermo Fisher	Cat#A36497
EndoFree Plasmid Max Kit (10)	QIAGEN	Cat#12362
QIAGEN Plasmid Midi Kit (100)	QIAGEN	Cat#12145
Zymoclean Gel DNA Recovery Kit	Zymo Research	Cat#D4008
DNA Clean & Concentrator-5 Kit	Zymo Research	Cat#D4014
GeneAmp PCR System 9700	Applied Biosystems	n/a
Applied Biosystems 3730XL DNA Analyzer	Thermo Fisher Scientific	n/a
Ver C	Bulletin	Cat#10033173
EasyPure Genomic DNA Mini Kit	Bioman Scientific	Cat#EP-500
Ribonuclease A from bovine pancreas	Sigma	Cat#R5503
Proteinase K from Tritirachium album	Sigma-Aldrich	Cat#P2308
Phenol:chloroform:IAA (25:24:1) pH 8.0	Sigma	Cat#P3803
Droplet Digital™ PCR (ddPCR™) Technology	Bio-Rad	n/a
Experimental models: Mosquitoes		
Aedes aegypti (Higgs strain)	Omar Akbari Lab	n/a
Oligonucleotides		
Integrated DNA Technologies	IDT	n/a
Recombinant DNA		
pBFv-U6.2 plasmid	NIG	n/a
Invitrogen TOPO TA Cloning Kit	Thermo Fisher	Cat#K451022

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Electric controlled pneumatic microinjector	Narishige	Cat#IM-31
Flaming/Brown Micropipette Puller	Sutter Instrument Company	Cat#P-97
Aluminosilicate glass	Sutter Instrument Company	Cat#AF100-64-10
Millex-GV	Millipore	Cat#SLGVR04NL
Halocarbon oil 700	Sigma	Cat#H8898
Filter paper-110 mm	Advantec	Cat#CT-01110

MATERIALS AND EQUIPMENT

10× Injection buffer (storage at 25°C)		
Reagent	Final concentration	Amount
Sodium phosphate (0.1 M)	0.1 mM	50 μL
KCL (2 M)	2 mM	125 μL
ddH_2O	n/a	4825 μL
pH	6.8	n/a
Total	**n/a**	**5 mL**

Injection mix (storage at −20°C)		
Reagent	Final concentration	Amount
pBFv-AeU6 sgRNA plasmid (1 μg/μL)	200 ng/μL	2 μL
HR donor plasmid (2.5 μg/μL)	500 ng/μL	2 μL
Cas9 protein (2 μg/μL)	400 ng/μL	2 μL
Injection buffer (10×)	1×	1 μL
ddH_2O	n/a	3 μL
Total	**n/a**	**10 μL**

Alternatives: If more than one sgRNA plasmid is used simultaneously, increase the final concentration of the sgRNA plasmid mix to $N \times 100$ ng/μL, where N represents the number of sgRNA plasmids injected.

Preparation of female mosquitoes for egg laying

Timing: 1 week

1. Prepare 50–100 wild-type female adult *Ae. aegypti* for egg laying— preferably young mated female mosquitoes less than 14 days old.
2. Feed mated female mosquitoes a blood meal three days prior to embryo injection (day -3).

STEP-BY-STEP METHOD DETAILS

Once injection plasmids have been prepared, it becomes possible to generate mutant strains via microinjection of mosquito embryos.

Note: This protocol is an optimized and amalgamated version of previously reported protocols (Jasinskiene et al., 2007; Kistler et al., 2015; Lobo et al., 2006).

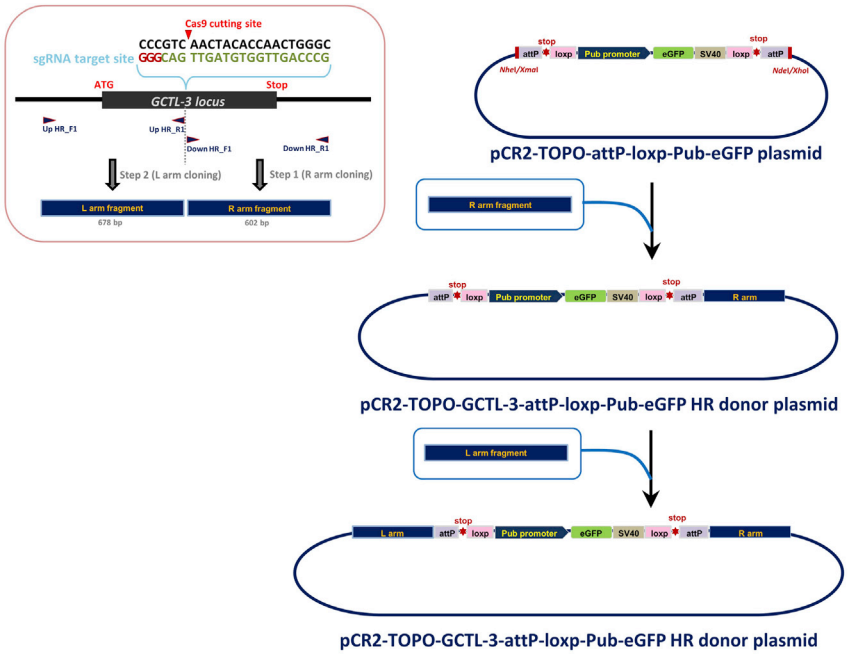

Figure 5. Cloning procedure for the pCR2-TOPO-GCTL-3-attP-loxp-Pub-eGFP HR donor plasmid

Microinjection of mosquito embryos

Timing: 2–4 h

1. Prepare microinjection needles containing the injection mix.
   a. Using a micro-pipette puller, create multiple glass needles with unbroken tips.
      i. The optimal puller program differs according to the choice of glass needle; for the suggested micro-pipette type we recommend using the following settings: HEAT = 517; PULL = 20; VEL = 110; TIME = 250.
   b. Before loading the injection mix into the needle, centrifuge the solution (excluding the Cas9 protein) at 16,000 × *g* for 10 min at 4°C to remove impurities.
   c. Load 1 μL of the injection mix into the needle while avoiding bubble formation.
   d. Using two overlapping microscope slides, carefully break the tip of the glass needle (Figure 6).
      i. Place the needle horizontally into a micromanipulator.
      ii. Move the microscope plate slowly towards the needle tip until it is sufficiently broken so that the injection mix can drip. Monitor the slide movement using a microscope.

⚠ CRITICAL: Pay particular attention to the glass needle tip after breaking. If it is too blunt, microinjection will be difficult.

2. Prepare embryos for injection (Figure 7).
   a. Allow the prepared female mosquitoes to lay eggs 40–50 min prior to microinjection. We suggest aspirating 12–15 females into vials containing wet filter paper before leaving the vial in darkness for at least 40 min. Wild-type female should lay over 100 eggs each during this time period.
   b. Using forceps, gently align 20–60 embryos on wet filter paper (Figure 7, left panel). Embryos should optimally appear light gray (Figure 7, right panel, red circle).

⚠ CRITICAL: In step 2b, take under 20 min to align the embryos to prevent melanization.

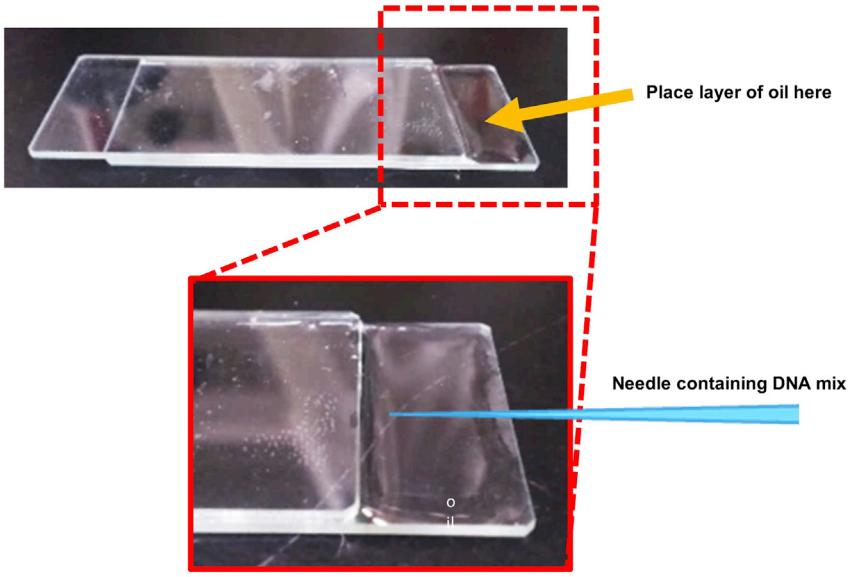

Figure 6. Needle preparation for microinjections
Two overlapping microscope slides are used to carefully break the tip of the glass needle.

Note: For this step we suggest using a maximum of 15 wild-type females per 28.5 × 95 mm *Drosophila* vial to collect eggs.

Note: We do not recommend using embryos collected from females blood fed more than five days prior to the start of the experiment. In our experience, the yolk of these eggs is too thick and easily blocks the needle.

c. Dehydrate mosquito eggs delicately using filter paper before utilizing Scotch double sided tape to minimize egg movement. Tape should be placed over the anterior section of the eggs, as the posterior section must remain clear for injection.
d. Add approximately 200–500 μL oil to cover all embryos.

⚠ CRITICAL: In step 2c, dehydrated mosquito eggs should not be exposed to air for more than five minutes before oil is added to prevent embryo melanization.

3. Inject embryos with injection mix (Figure 8).
   a. Using a micromanipulator, allow the needle to penetrate the posterior section of each embryo. Typical injection volume should reach around 500 pL per embryo. For optimal results, do not allow the needle tip to go beyond the yellow dotted line.
   b. Retract the needle from the embryo slowly by moving the microscope plate laterally, minimizing yolk loss.
   c. Repeat this injection procedure for the entire row of embryos.

⚠ CRITICAL: Identifiable transparent droplets near the posterior of the egg after injection are a signature of a successful injection; the mixed droplets indicate outflow of yolk.

4. Move injected embryos to fresh wet filter paper using forceps and wash the eggs using MilliQ-water to remove excess oil.
5. Keep the eggs humid and attached to wet filter paper for four days, then hatch and rear as normal.

⚠ CRITICAL: Injected embryos require at least one day for repair. We suggest keeping the eggs on wet filter paper for at least four days before hatching.

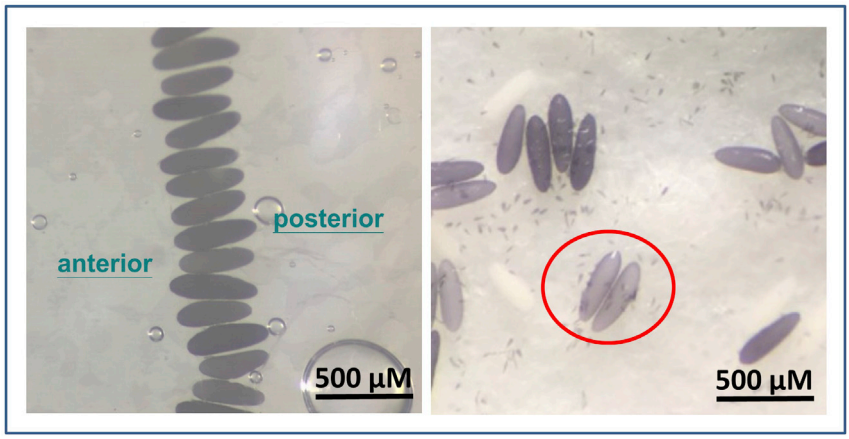

Figure 7. Collection and Alignment of *Aedes* mosquito embryos
Embryos are aligned in identical orientations to facilitate microinjection. The anterior end of the embryo is wide, whilst the posterior end is narrow (Left panel). Light gray mosquito embryos should be selected for alignment (right panel, red circle, scale bar 500 µm).

Pause point: Keep the injected embryos on filter paper for up to a maximum of two weeks.

Generation of mutant mosquitoes - day 5

Timing: 3–4 months

Establishing heterozygous and homozygous mutants (Figure 9).

6. After injected eggs have hatched and as the larvae are reared to adulthood, collect and sort male and female pupae prior to emergence to prevent mating from occurring.
7. Prepare crosses between adult micro-injected and wild-type mosquitoes in cages. Cross micro-injected males with wild-type females and vice versa. Add an excess of wild-type mosquitoes in each cross to increase the likelihood that microinjected mosquitoes will mate; the suggested minimal ratios are one microinjected male to three wild-type females, and one microinjected female to one wild-type male.

   Note: Add an excess of wild-type mosquitoes independent of sex to ensure injected individuals mate and produce progeny. To ensure all microinjected females are mated, a 1:1 ratio or excess of males is suggested.

8. After allowing mosquitoes to mate for three days, provide all females with a blood meal to allow for egg laying.

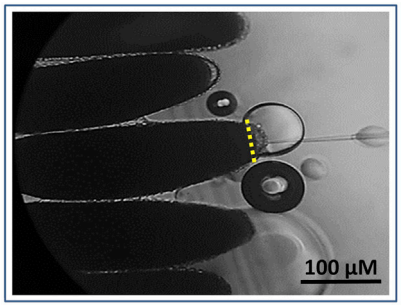

Figure 8. *Aedes* mosquito embryo microinjection
Mixture should be injected into the posterior end of the mosquito eggs, with the needle suggested not to pass the yellow dotted line, scale bar 100 µm.

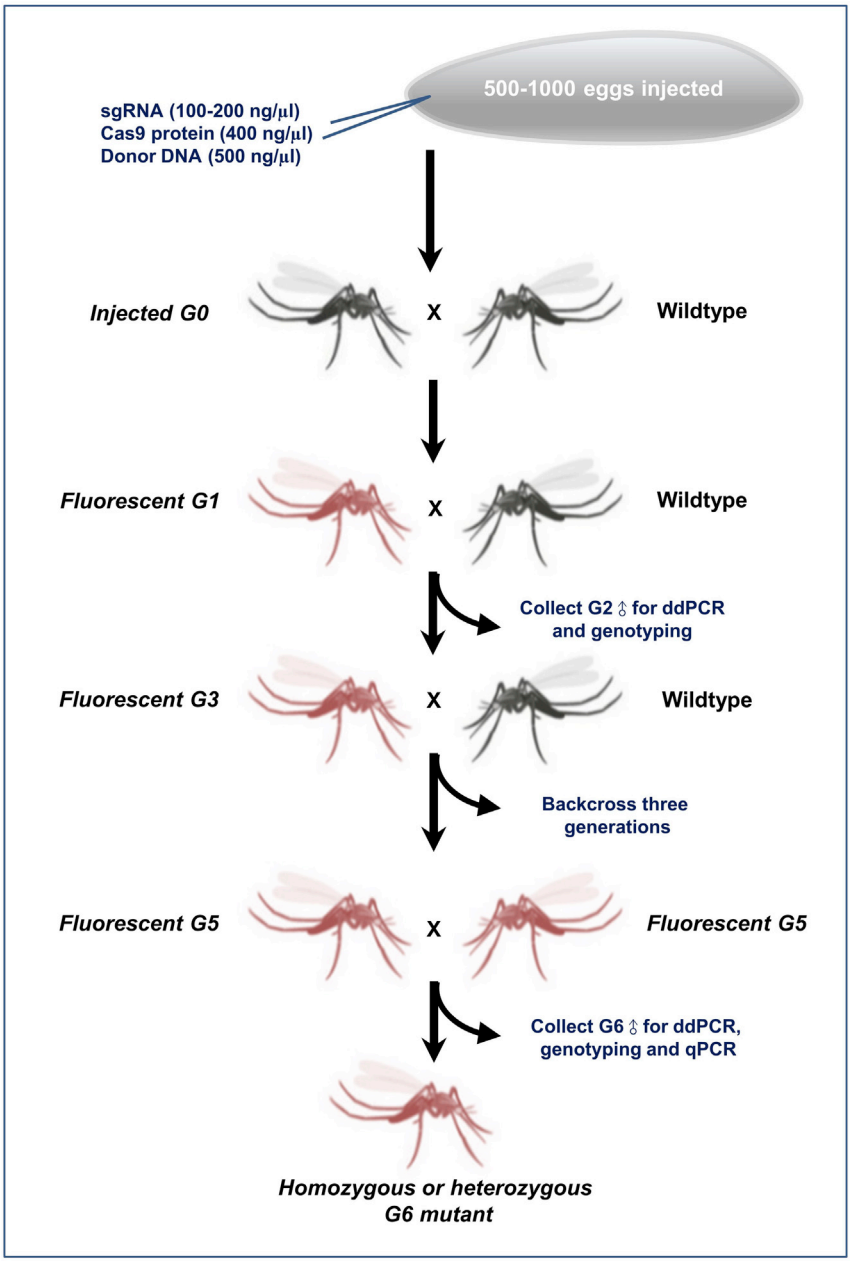

Figure 9. Schema of mutant mosquito generation

sgRNA: single guide RNA. G: generation. ddPCR: droplet-digital PCR. qPCR: reverse transcription polymerase chain reaction.

9. After three more days, transfer females into individual vials containing wet filter paper. Allow females to lay eggs onto the filter paper.
10. Hatch the generation 1 (G1) eggs using separate containers for each individual female. Screen mosquito larvae for fluorescent markers either throughout the whole body (for markers driven by the Pub promoter) or specific to the eyes, peripheral nerves, and siphon tubes (for markers driven by the 3xP3 promoter).
11. Outcross adults emerging from screened larvae with control mosquitoes for five generations, screening for fluorescence during the larval stage of each generation.

Note: Fluorescent mosquitoes can be collected and tested using digital droplet PCR (ddPCR), genotyping PCR, sequencing, or other methods to screen for precise gene knock-in or potential off-target effects (please see details provided in the next step).

Pause point: Store eggs on dry filter paper for up to a maximum of three months.

Precise gene knock-in mutant line confirmation by ddPCR

Timing: 1 week

We originally tried to amplify the junction sequences of the integration between the marker cargo and flanking regions of the target gene (*GCTL-3*). Unfortunately, this attempt ultimately failed. Based on this experience, we tried to answer a basic question: How can we ensure the precise integration of a marker cargo? We thus utilized ddPCR technology as part of the gene knock-in mutant line screening procedure to check the copy number variation (CNV) of the target site and eGFP cargo.

12. Isolate genomic DNA from a single fluorescent mosquito.

Note: The following procedure for genomic DNA isolation is modified and optimized from Bioman Scientific Company's suggested protocols.

a. Add a single fluorescent mosquito (male or virgin female) to 200 μL of Cell Lysis Solution (Bioman Scientific) and homogenize with a small Homogenizer.
b. Incubate the lysate at 65°C for 30 min.
c. Add 2 μL Proteinase K solution (Stock: 10 mg/mL) to the lysate. Mix well by inverting and incubate at 55°C incubator with an inverting or shaking mixer for at least three h and up to 12–16 h.
d. Allow the 55°C lysate sample to cool to 23°C–25°C, and then add 1 μL RNase A solution (Stock: 4 mg/mL) to the Proteinase K-treated lysate and mix well by inversion. Incubate the mixture at 37°C for 30 min.
e. Add 67 μL of Protein Precipitation Solution (Bioman Scientific) to the RNase A-treated lysate sample and vortex vigorously at high speed for 20 s.
f. Extract with 40 μL Phenol/Chloroform/IAA (PCI) and then centrifuge at 15000 × *g* for 10 min at 23°C–25°C.

⚠ CRITICAL: Insect genomic DNA is very delicate at this point. Mix well with PCI via gentle inversion, and avoid vortexing as this can cause fragmentation.

g. Carefully remove the supernatant containing the DNA (leaving the protein pellet and PCI behind) and transfer to a clean 1.5 mL Eppendorf tube.
h. Add 250 μL of isopropanol to the supernatant and gently mix the solution by inversion.
i. Centrifuge for 15 min at 15000 × *g*. The DNA will be visible as a small white pellet. Carefully decant the supernatant.
j. Add 250 μL of 70% EtOH to wash the DNA pellet and incubate for 5 min. Centrifuge for 5 min at 15000 × *g*.
k. Decant the supernatant and dry the pellet for 5–10 min in a DNA Speed Vacuum machine.
l. Resuspend in 20 μL nuclease-free H2O and keep at 4°C for long term storage.
m. Calculate the genomic DNA concentration using a NanoDrop 2000 Spectrophotometer.

Note: DNA concentrations of single male and single virgin female mosquitoes are about 60 ng/μL and 130 ng/μL, respectively.

13. Design the primer/probe sets for the ddPCR assay (Figure 10).

⚠ CRITICAL: Confirm the sequence of the 500 bp at either side of the strand flanking the Cas9 target site sequence before designing the primer/probe sets.

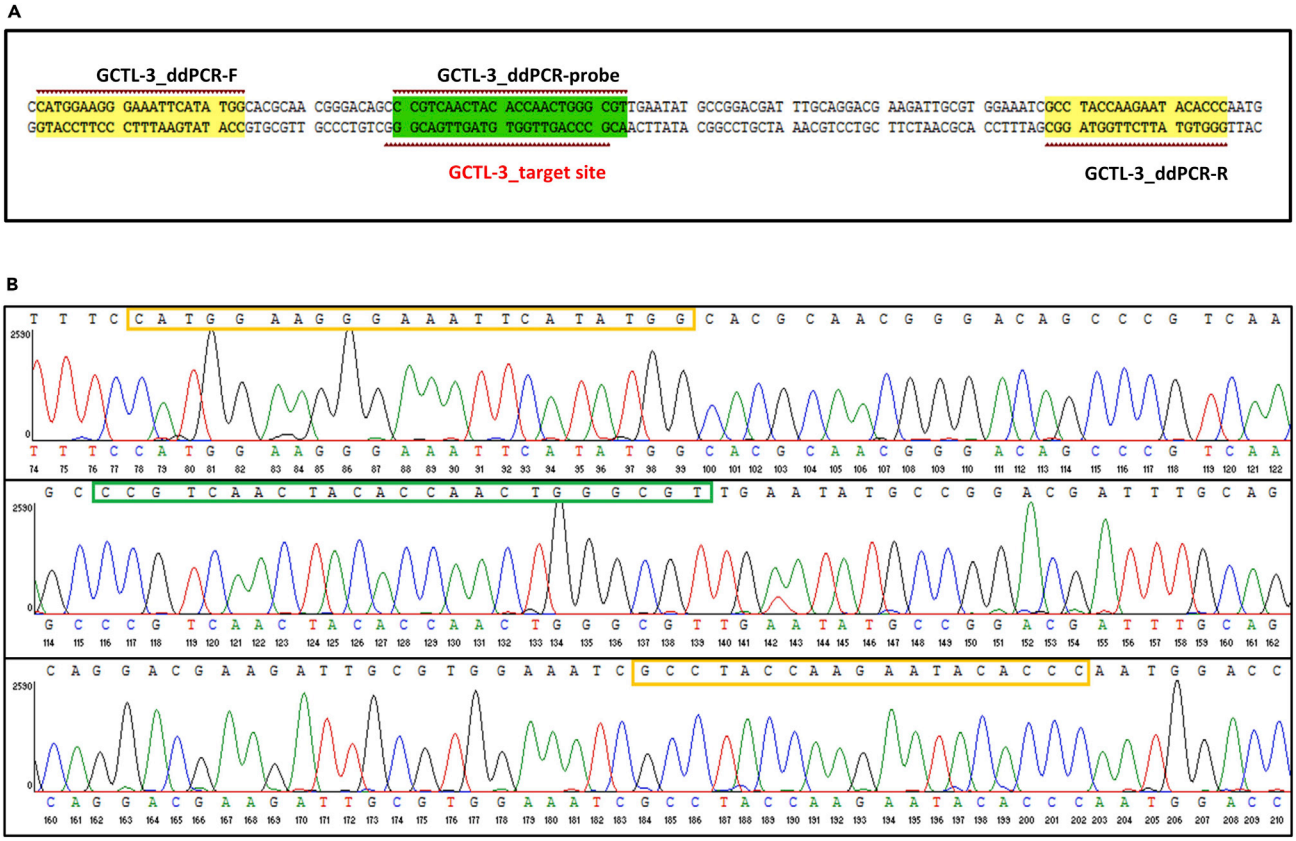

Figure 10. Design of the ddPCR primer/probe set

(A) Relative position and sequences of the *GCTL-3* primer/probe set and target site.

(B) Flanking sequences around the target site after double confirmation using Sanger sequencing.

a. The primer/probe sets of the target genes (GCTL3 and eGFP) were obtained using the Beacon Designer software (PREMIER Biosoft).

⚠ CRITICAL: Design probe sequences located as close as possible to the Cas9 target site region.

b. The primer/probe set of the reference gene (nk) was obtained from (Hall et al. 2015).

c. The probe was synthesized following the ZEN quencher technology protocol from the Integrated DNA Technologies (IDT) Company.

Note: The ZEN quencher developed by IDT can promote a lower background and increase signal detection (available at https://sg.idtdna.com/pages/education/decoded/article/modification-highlight-zen-internal-quencher).

d. The primer/probe set sequences are:

Name	Sequence
GCTL-3_ddPCR-F	CATGGAAGGGAAATTCATATGG
GCTL-3_ddPCR-R	GGGTGTATTCTTGGTAGGC
GCTL-3_ddPCR-probe	56-FAM-CCGTCAACT-ZEN-ACACCAACTGGGCGT-3IABkFQ
eGFP_ddPCR-F	CAACGAGAAGCGCGATCA
eGFP_ddPCR-R	CGCGATATTACTTGTACAGCTC

(*Continued on next page*)

Continued	
Name	Sequence
eGFP_ddPCR-probe	56-FAM-CCTGCTGGA-ZEN-GTTCGTGACCGCC-3IABkFQ
Rf-nk_ddPCR- F	CGTGGTGCAGATAGTGAACG
Rf-nk_ddPCR- R	CATGTTAAGTTTGCCATAAAATTCG
Rf-nk_ddPCR-probe	Hex-TGGTGACTT-ZEN-GGGAAGGATGAAGTA-3IABkFQ

14. Prepare samples for ddPCR.

Note: All relative ddPCR reagents and procedures follow the protocols from Bio-Red ddPCR Technology (available at https://www.bio-rad.com/en-tw/applications-technologies/droplet-digital-pcr-ddpcr-technology?ID=MDV31M4VY).

Note: Detailed information regarding the ddPCR Applications Guide can be obtained at https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf.

a. Ensure the use of optimal genomic DNA concentrations of 20–40 ng/20 μL per reaction (*Ae. aegypti* genome size is approximately 1.38 Gbp).
b. Identify optimal melting temperature (Tm) for primer/probe annealing condition testing with test runs to find the most suitable conditions.
c. Prepare the following reagents for ddPCR:
Example: CNV detection of GCTL-3 target site.

Reagent	Amount
2× ddPCR Supermix for Probes (No dUTP)	10 μL
GCTL-3_ddPCR-Probe (10 μM)	0.25 μL
GCTL-3_ddPCR-F (18 μM)	1 μL
GCTL-3_ddPCR-R (18 μM)	1 μL
Rf-nk_ ddPCR-Probe (10 μM)	0.25 μL
Rf-nk_ ddPCR-F (18 μM)	1 μL
Rf-nk_ ddPCR-R (18 μM)	1 μL
Restriction enzyme-*Hae*III (10U/μL)	0.2 μL
Nuclease-free water	4.3 μL
DNA sample (20ng/μL)	1 μL
Total	**20 μL**

d. Incubate the reagent mixtures at 23°C–25°C for 10 min for restriction enzyme digestion of the sample DNA.

⚠ CRITICAL: It is particularly important to fragment the genomic DNA by restriction enzyme digestion prior to droplet generation. This will enable optimal accuracy by separating tandem gene copies, reducing sample viscosity, and improving template accessibility.

⚠ CRITICAL: Each ddPCR amplicon should not involve the expected restriction enzyme sites (e.g., *Hae*III for the GCTL-3 ddPCR amplicon).

15. Perform droplet generation using a QX200 Droplet Generator (Bio-Red).
a. All steps follows the guide in the QX200 Droplet Generator Instruction Manual (available at https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10031907.pdf).
b. The following is a summary of the steps:
i. Transfer 20 μL of each prepared sample into sample wells (middle row) of the DG8 cartridge.
ii. Using a multichannel pipette, fill each oil well (bottom row) with 70 μL Droplet Generation Oil.
iii. Hook the gasket over the cartridge holder using the holes on both sides.

iv. Open the QX200 Droplet Generator and place the cartridge holder into the instrument.
v. When droplet generation is complete, the top wells of the cartridge will contain droplets, and the middle and lower wells will be nearly empty, with only a small amount of residual oil.
vi. Carefully transfer 40 μL of the contents of the top wells (the droplets) of the DG8 cartridge into a single column of a 96-well PCR plate.
vii. Cover the 96-well plate with one sheet of pierceable foil sealed using PX1 PCR plate sealer (Bio-Red).
viii. Once the 96-well plate containing the droplets is sealed, place it into the thermal cycler for PCR amplification.

16. Run PCR reaction using a T100 Thermal Cycler (Bio-Red).
    a. Put the sealed 96-well plate containing the droplets into the thermal cycler for PCR amplification.
    b. Run the following PCR program.

Cycling step	Temperature	Time	Ramp rate	Cycles
Enzyme activation	95°C	10 min	2°C/s	1
Denaturation	94°C	30 s		40
Annealing/extension	57°C	1 min		
Enzyme deactivation	98°C	10 min		1
Hold	4°C	Infinite	1°C/s	1

⚠ CRITICAL: When using each ddPCR primer/probe set for the first time, it is essential to run a gradient annealing temperature to find the optimal conditions.

17. Read the droplets using a QX200 Droplet Reader (Bio-Red).
    a. Run a CNV Assay (Figure 11).
        i. Select "Copy Number Variation (CNV)" as the experiment type in the QuantaSoft software when loading wells.
        ii. Double click on the experiment name in the main software window to set the ploidy for reference.
        iii. CNV analysis by ddPCR involves quantifying target and reference loci through the use of duplex target and reference assays. In the QuantaSoft software, copy number is determined by calculating the ratio of target molecule concentration to reference molecule concentration, multiplied by the number of copies of reference loci in the genome (usually 2).

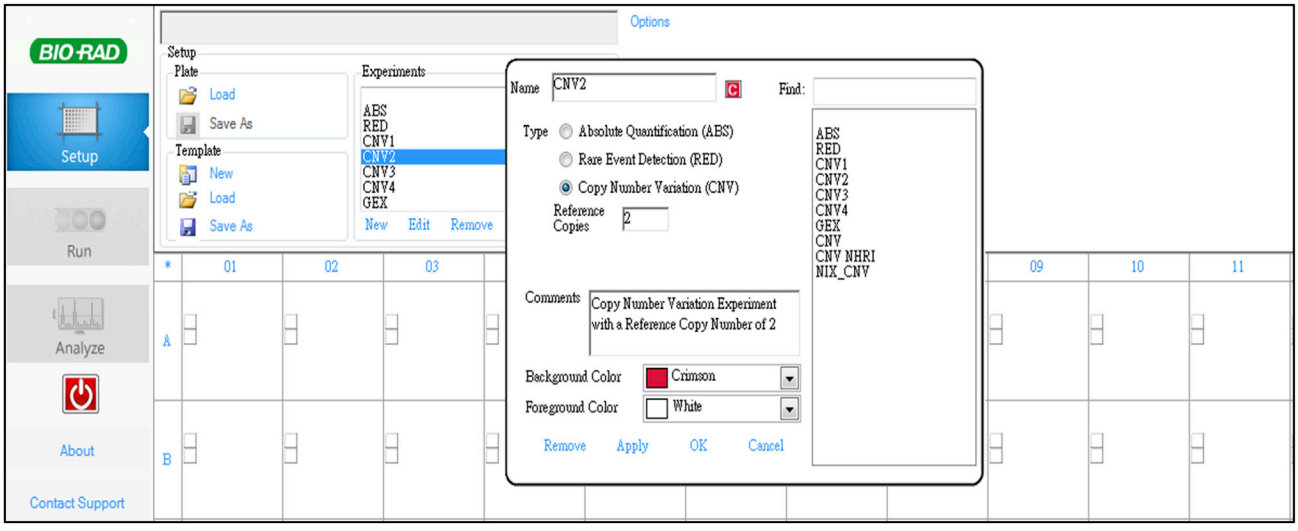

Figure 11. Select "Copy Number Variation (CNV)" as the experiment type and set the ploidy for reference in QuantaSoft software

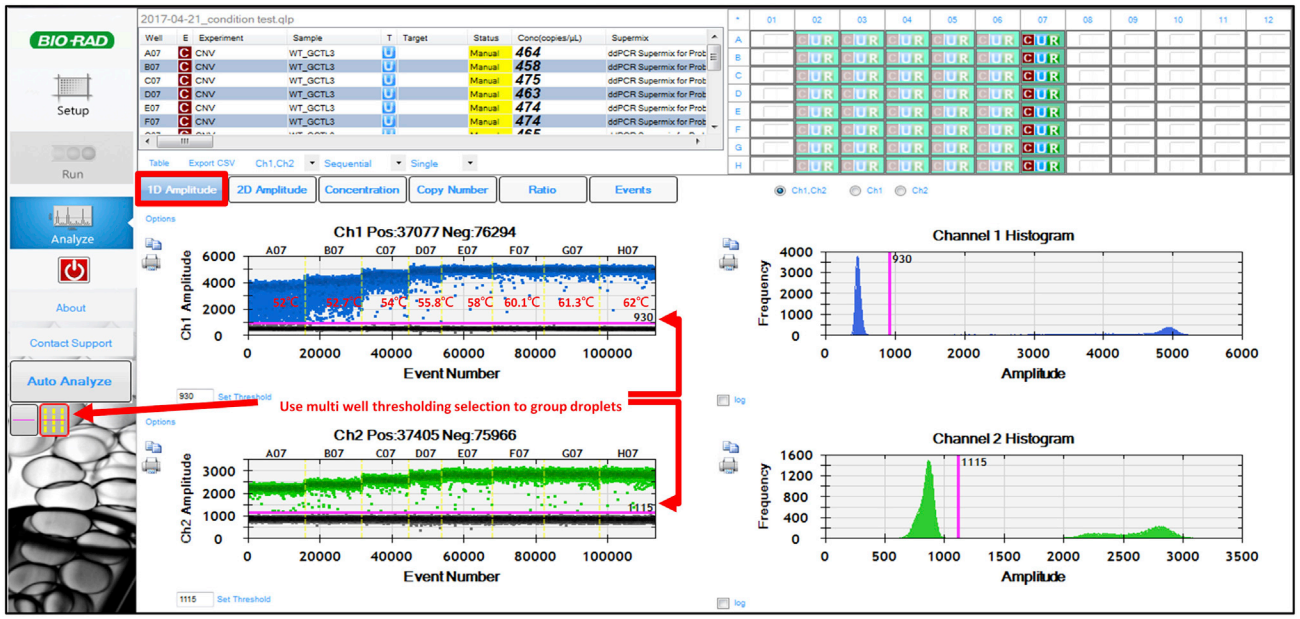

Figure 12. Select multi-well thresholding to group droplets of GCTL-3 and Rf-nk

Identify optimal Tm for testing *GCTL-3* (blue) and Rf-nk (green) primer/probe annealing conditions by running a gradient annealing temperature from 52°C–62°C. The optimal Tm conditions are when the amplitudes are constant and concentrated (shown from E07_58°C to H07_62°C).

iv. Apply the CNV formula:

b. Select "1D Amplitude" for threshold design from the Analyze panel (Figure 12).
   i. Display amplitudes and histograms by individual assay (1D Amplitude).
   ii. Select single or multi-well thresholding to group droplets.
   iii. Example: Optimal Tm condition identification for GCTL-3 primer/probe test.

c. Select Copy Number to display CNV for allele detection (Figure 13).

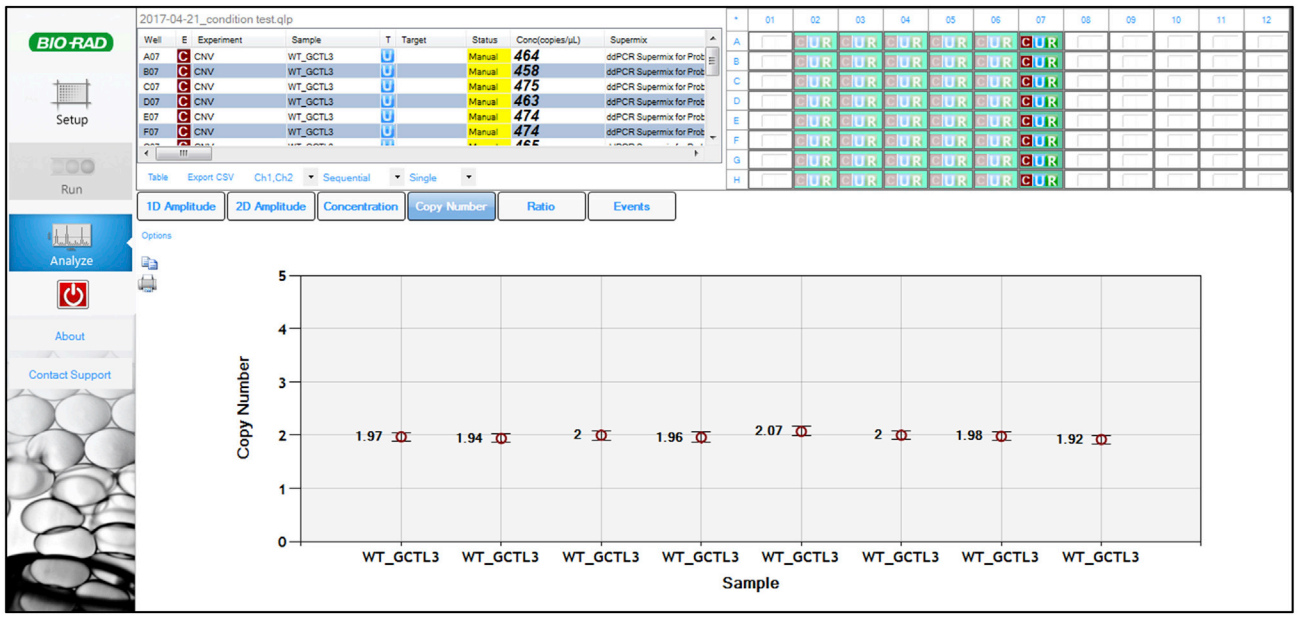

Figure 13. Display CNV for allele detection by selecting Copy Number

Use genotyping PCR to confirm the break point site

Timing: 3–4 h

After performing fluorescent marker analysis via ddPCR technology, candidate mutant line break point sites should be confirmed by genotyping. This requires amplifying the junction sequences of the integrations between the marker cargo and flanking regions of target gene (*GCTL-3*).

18. Design appropriate primers for genotyping.
    a. For GCTL-3, we used the following sequences of primer sets for genotyping PCR:

Region	Name	Sequence
L arm breaking point checking	*GCTL-3*_L arm-F1	CGACCATCTTCGATTTGCAGTG
	*GCTL-3*_L arm-R1	GTGCAGTCAGCAAAGTGACG
R arm breaking point checking	*GCTL-3*_R arm-F3	CCGACAACCACTACCTGAGC
	*GCTL-3*_R arm-R3	CCAATATGCAGGGAAAAAGCAGG

⚠ CRITICAL: Primer design for the genotyping PCR amplicon needs to cover the junction sequences of the integrated cargo and the flanking regions of the target gene (*GCTL-3*).

    b. Prepare the following reagents for the PCR experiment:

Reagents	Amount
Genomic DNA (50 ng)	2 μL
Forward primer (50 μM)	1 μL
Reverse primer (50 μM)	1 μL
dNTPs (10 mM)	2 μL
10× FFwd buffer	10 μL
FFwd DNA polymerase (2U/μL)	1 μL
Nuclease-free water	83 μL

    c. Set the following PCR program conditions:

Step	Temperature	Time
Initial denaturation	98°C	5 min
30 cycles	98°C	30 s
	55°C	30 s
	72°C	1 min
Final extension	72°C	10 min
Hold	12°C	∞

    d. Purify PCR products following the protocols established by the Zymoclean Gel DNA Recovery Kit.
    e. Confirm the purified PCR products with Sanger sequencing by using designed primers:

Region	Name	Sequence
L arm to Pub promoter	*GCTL-3*_L arm-F2	ATCGGCGCGGTAGAATACTG
L arm to genome	*GCTL-3*_L arm-R2	TTAGTCAAAAGCGCAATCGGC
R arm to genome	*GCTL-3*_R arm-F4	ATTCGGGAGAACCGAAAGGA
R arm to eGFP	*GCTL-3*_R arm-R4	TCTTTATCTGGTGCAAAGTGCT

Figure 14. Primer sets for PCR and sequencing confirmation (red and blue arrows, respectively) for break point detection at the insertion site

⚠ CRITICAL: Design a sequencing primer that crosses the breakpoint and donor plasmid of the insertion site and reads from the L and R arms to ensure the marker inserts at the correct site (Figure 14, blue arrows).

Off-target confirmation

Timing: 3–4 h

No matter the sgRNA design, it is not possible to fully rule out the possibility of off-target effects in *A. aegypti* because both genome assembly and annotation are incomplete. All of the genotype confirmed *GCTL-3* mutant lines were therefore back crossed with the wild-type strain for three generations to eliminate the chance of off-target effects. Sanger sequencing can be used to check the sequence states of the potential off-target sites.

a. Use the VectorBase BLAST tool to find potential off-target sites for the sgRNA sequence.
b. Download the off-target genes.
c. Design a series of primer sets to amplify approximately 500 bp around the flanking sequences that cover either strand of the off-target site.
d. Use nest primers to confirm the sequence states with Sanger sequencing.
e. Example: GCTL-3 target site (Figure 15).

Optional: Other online tools, such as Cas-OFFinder (Bae et al., 2014) (http://www.rgenome.net/cas-offinder/) and CHOP-CHOP (https://chopchop.cbu.uib.no/), not only allow for the use of the Aedes aegypti genome as a reference, but are also able to predict off-target sites based on similarity, likelihood of cleavage based on the position of the mismatches, number of mismatches, etc.

EXPECTED OUTCOMES

1. Pre-screened G0 mosaic fluorescent larvae occur at rates of about 30%–80%; the success rates for fluorescent G1 adults is about 1%; the success rates for germline mutant mosquitoes is about 0.2%. The efficiencies of two attempts at GCTL-3 microinjection germline mutant generation are shown in Table 1.

LIMITATIONS

1. Some fluorescent markers gradually become less obvious during following generations. For example, in the case of *GCTL-3* microinjection, we obtained ten G1 fluorescent mutants but only three fluorescent mutants (Figure 16; larvae labeled♀7 , ♀8 and ♂5) still displayed eGFP fluorescence in G2 larvae. We also found that only non-mosaic fluorescent mutants could maintain the eGFP marker (Figure 16).
2. Some non-mosaic fluorescent mutants have one GCTL-3 copy number (heterozygotes) but multiple eGFP copy numbers (Figure 17C).

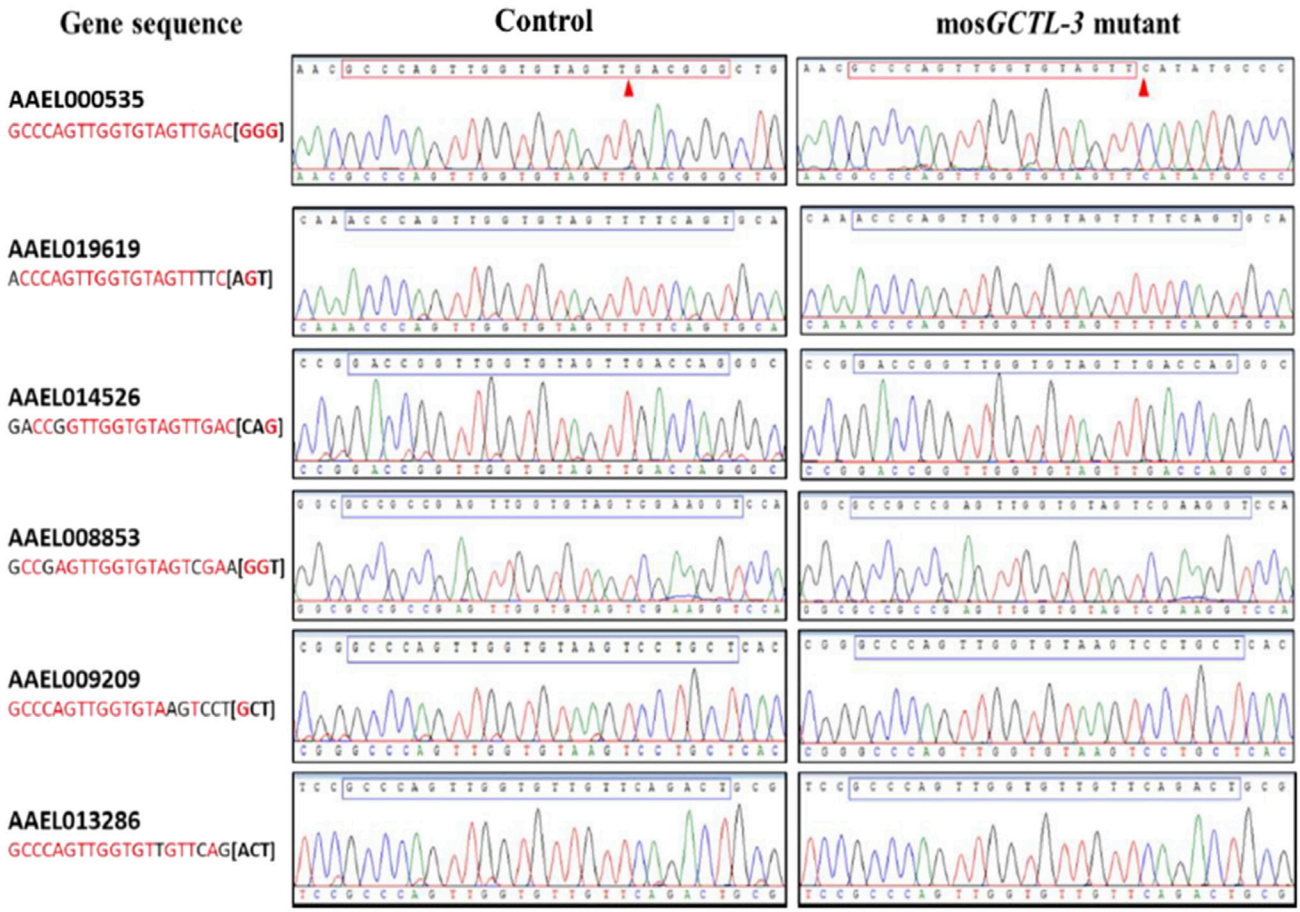

Figure 15. Sequencing analyses of the five potential target sites identified for GCTL-3 knock-in
Figure reprinted with permission from Li et al., 2020.

TROUBLESHOOTING

1. In "pCR2-TOPO-attp-loxp-Pub-eGFP HR donor plasmid generation" step:

Table 1. Microinjection results

Generation	G0				G1	G2
Stage	Embryos	Larvae		Adult	Larvae	Larvae-Adult
State	Microinjection	Survival	Fluorescent	Eclosion	Visible eGFP	Germline mutant
Batch A	795	210/795 (26%)	168/210 (80%)	176/795 (22%)	8/795 (1%)	2/795 (0.25%)
Batch B	467	77/467 (16%)	47/77 (61%)	69/467 (14.5%)	2/467 (0.43%)	1/467 (0.21%)

2. When using the Pub promoter, only fully fluorescent larvae can transmit the marker to the next generation. Some mosaic groups will disappear during the G2 generation (Figure 16).
3. Although we introduced the HR donor plasmid with a fluorescent marker for gene knock-in, we found that the fluorescent marker was not a true indicator of precise integration (Figure 17).
4. Run the PCR gel to allow for visualization of the major bands of predicted sizes both in the 5′ and 3′ end break points (Figure 18).
5. Sequence the break points (both ends) of the genomic DNA in mutant mosquitoes (Figure 19).

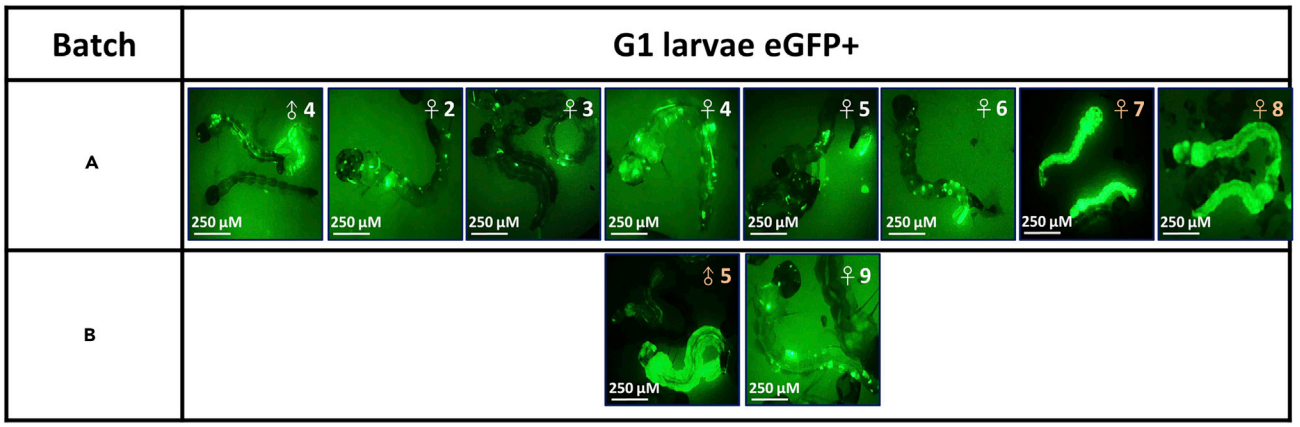

Figure 16. Examples of G1 fluorescent larvae

In the case of GCTL-3, three fluorescent G1 groups (♀7, ♀8 and ♂5) maintained eGFP in the G2 generation. In other mosaic groups, eGFP fluorescence disappeared in the following generation. ♂4, ♀2–6 and ♀9 are mosaic fluorescent; ♀7–8 and ♂5 are non-mosaic fluorescent mutants, scale bar 250 μm.

a. Problem: Low transformation efficiency.
   i. Transformation with too much In-Fusion reaction mixture (or ligation products) will lead to disruption of the ionic conditions of competent cells. Do not add more than 5 μL of the In-Fusion reaction mixture to 50 μL of competent cells.
   ii. Competent cells are sensitive to the In-Fusion enzyme. Dilute the In-Fusion reaction mixture with TE buffer 3–5 times prior to transformation (add 20–40 μL of TE buffer to 10 μL of the In-Fusion reaction mixture).
   iii. Use fresh or highly transformation efficiency competent cells ($\geq 1 \times 10^8$ cfu/μg).
   iv. Regions of homology may not be long enough for efficient cloning of multiple fragments at once. Extend the overlapping homologous region from 15 bp to 20 bp.
b. Problem: Large numbers of colonies were obtained with no insert.
   v. Vector may be incompletely linearized. Increase the incubation time or enzyme units during restriction enzyme digestion.
   vi. Ensure that the antibiotic plates are fresh and correct.
   vii. Use Competent Cells with few or no recombination effects (DH10B, Stellar or SURE) to enhance the cloning efficiency of HR fragments.

2. In "Microinjection of mosquito embryos" step:

Large outflow of mosquito embryo yolk during microinjection will likely lead to reduced egg hatching rates.

3. In "Generation of mutant mosquitoes" step:

Fluorescent markers can be viewed in G0 larvae during the pre-screening process. For example, if fluorescence is identifiable (>30% mosquito larvae with some fluorescence) in the abdomens of the mosquito larvae (Figure 20, red circle), it means the pBFv-AeU6-sgRNA plasmid is functioning and there is a high probability of obtaining mutant strains. If no fluorescence is identifiable at the G0 stage, another sgRNA target site should be used.

4. In "Precise gene knock-in mutant line confirmation by ddPCR" step:
   a. Raining droplets are usually caused by primer degradation, probe hydrolysis or a disorderly PCR reaction (Figure 21B).
      i. Run a gradient Tm to identify the optimal annealing temperature.
      ii. Use a fresh primer/probe set.

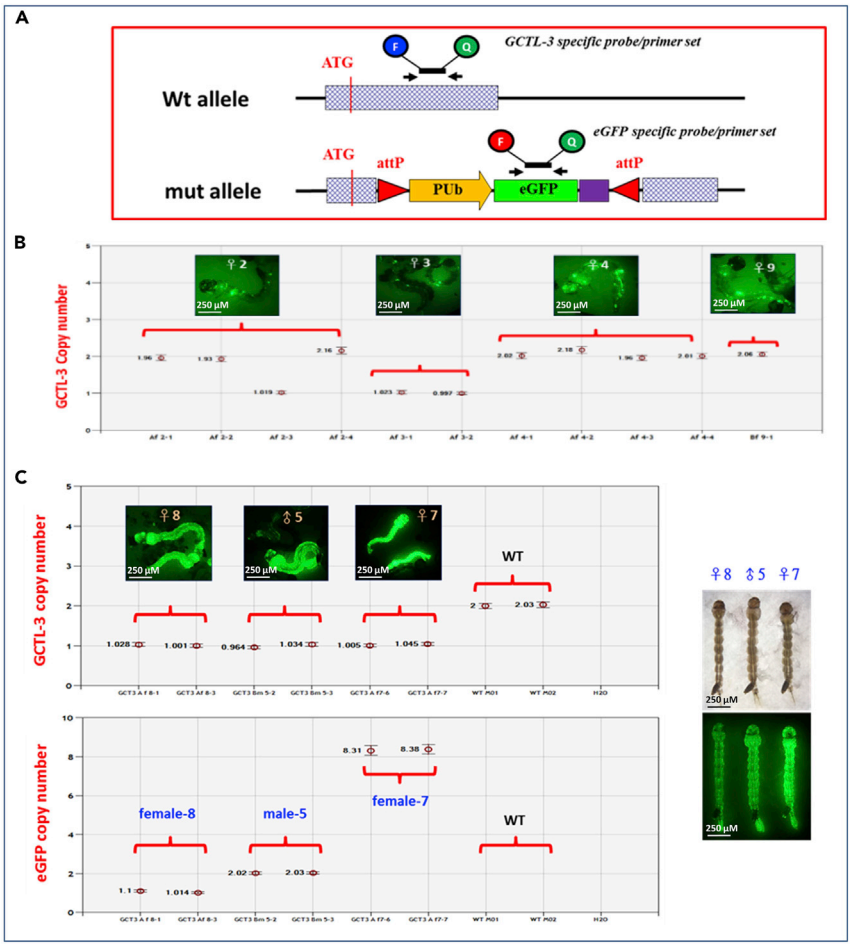

Figure 17. Detecting CNV of *GCTL-3* and eGFP using ddPCR technology

(A) The principle of *GCTL-3* and eGFP CNV detection using a specific primer/probe set.

(B) The mosaic fluorescent strains shown *GCTL-3* allele diversity in individual G1 offspring from the same G0 parental generation, scale bar 250 μm.

(C) The non-mosaic fluorescent strains demonstrate a precise and constant integration for GCTL-3 gene knock-in. Based on the eGFP CNV detection, multiple integrations also exist in the homology-directed repair (HDR) system, scale bar 250 μm.

iii. Redesign another primer/probe set.

b. Not obtaining negative droplets can be caused by an excess of templates (Figure 21C).

iv. Dilute the concentration of genomic DNA for ddPCR.

5. In "Use genotyping PCR to confirm the break point site" step:

If you cannot see the major band of the PCR result, you can identify the optimal Tm for the primer annealing conditions by running a gradient annealing temperature from 52°C–62°C. If this is unsuccessful, try another primer set.

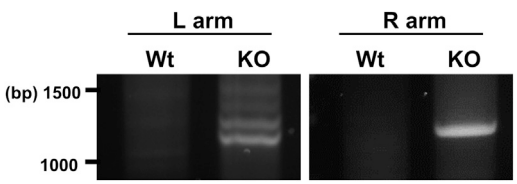

Figure 18. The 5′ end (L arm) and 3′ end (R arm) genomic DNA break points in mutant mosquitoes detected by PCR

Wt: wild-type. KO: knock-out. Figure reprinted with permission from Li et al., 2020.

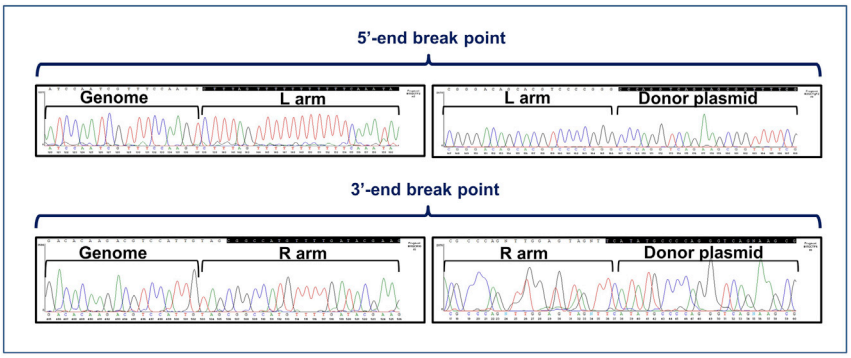

Figure 19. The 5′ end (L arm) and 3′ end (R arm) genomic DNA break points in mutant mosquitoes detected by PCR; Wt: wild-type
KO: knock-out. Figure reprinted with permission from Li et al., 2020.

RESOURCE AVAILABILITY

Lead contact

Requests for further information, resources, and reagents should be directed to and will be fulfilled by the lead contact, Chun-Hong Chen (chunhong@gmail.com)

Materials availability

All materials generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

The published article includes all data generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.-H.L., J.-C.L., and C.-H.C.; methodology, H.-H.L. and J.-C.L.; investigation, H.-H.L., J.-C.L., and K.-L.L.; resources, C.-H.C.; data curation, H.-H.L.; writing – original draft, H.-H.L., J.-C.L., and M.P.S.; writing – review & editing, H.-H.L., J.-C.L., M.P.S., and C.-H.C.

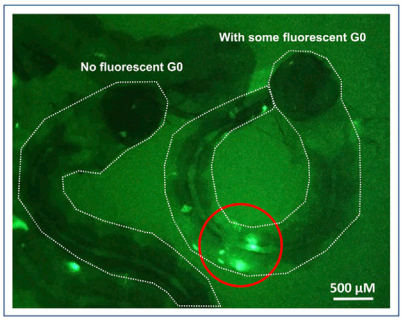

Figure 20. Sequencing 5′ end (L arm) and 3′ end (R arm) insertion sites
Figure reprinted with permission from Li et al., 2020.

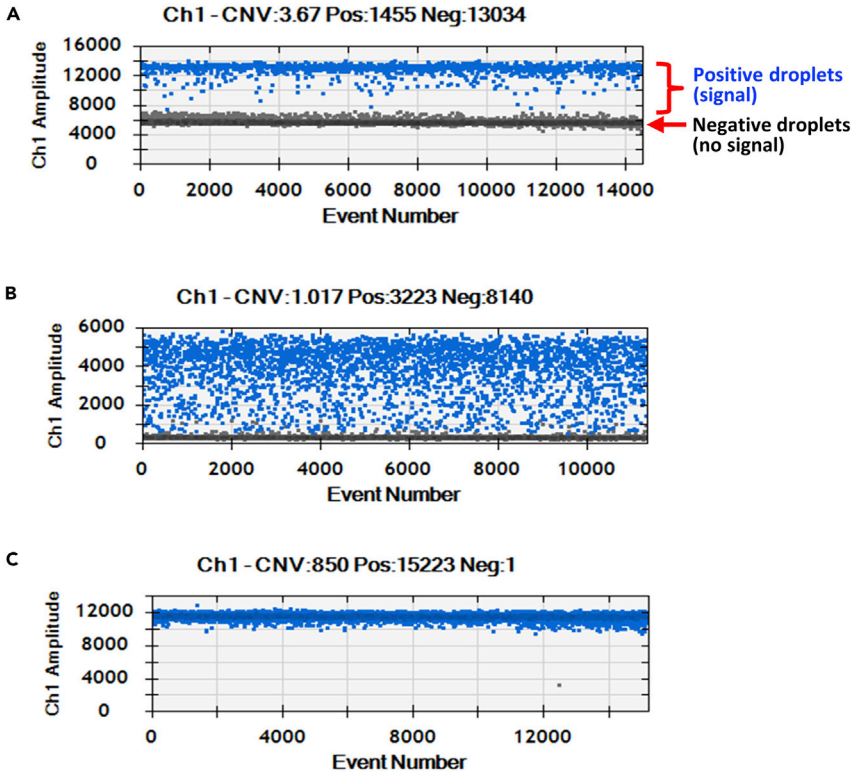

Figure 21. Fluorescent mosquito larvae

eGFP fluorescence driven by the Pub promoter in G0 mosquito larvae, scale bar 500 μm.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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