Protocol

Generating *Aedes aegypti* Mutant Strains with Transgenic Cas9

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Here, we provide a protocol for generating *Aedes aegypti* mutant strains via end-joining (EJ) or homology-directed repair (HDR) mechanisms using genetically encoded Cas9.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Aedes aegypti

We use the Liverpool strain as the wild-type (BEI Resources 735) and a genetically encoded Cas9-expressing strain (e.g., expressed using the exuperantia promoter or other; also called Exu-Cas9 strain, marked with Opie2-dsRed [Li et al. 2017]).

Ambion RNaseZap (Sigma-Aldrich R2020)
DNA extraction kit (DNeasy Blood and Tissue Kit; QIAGEN 69506)
Donor DNA template synthesis reagents (see Step 5)
- Gibson assembly master mix (2×; New England BioLabs E2611S)
- piggyBac plasmid pBac-3xP3-dsRed (Addgene 100707)
- ZymoPURE II Plasmid Maxiprep Kit (Zymo Research D4202)

Embryonic microinjection
- Halocarbon oil solution for embryo microinjection <R>
- Nitrogen gas (research purity, 99.9999%; Matheson G2173112)

H_2O (deoxygenated deionized and ultrapure molecular grade [Invitrogen 10977-015])
Ice
Mosquito husbandry reagents
- Larval food (crushed TetraMin Tropical Flakes; Tetra Werke 77101)
- Mice (live, anesthetized intraperitoneally using ketamine/xylazine/sterile saline solution [82.5 mg/kg ketamine; 10 mg/kg xylazine])
- Sucrose (Sigma-Aldrich S0389; crystal form, purity ≥ 99.5%) or glucose (see Step 9)

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From the Mosquitoes collection, edited by Laura B. Duvall and Benjamin J. Matthews.

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PCR product purification kit (Zymoclean Gel DNA Recovery Kit; Zymo Research D4007)
Reagents for in-house sgRNA synthesis (optional; see Step 4)
- MegaScript T7 (Ambion AM1334)
- Primers for generating a DNA template of sgRNA using example target sequence (see Step 4)
- Q5 high-fidelity DNA polymerase (New England BioLabs M0491S)

RNA 6000 Nano Kit (Agilent 5067-1511)
Single-guide RNA (sgRNA)

sgRNA can be obtained via a commercial synthesis service (we use Synthego Corporation) or via in-house synthesis (see Step 4).

Equipment

Bioanalyzer (Agilent Bioanalyzer 2100)
Dissecting microscope with light source (optional; see Step 20)
Embryonic microinjection equipment
- Coverslip (disposable; Fisherbrand S17525C)
- Glass needles (quartz capillary glass tube; Sutter Instrument QF100-70-10)
- Microinjector (FemtoJet 4×; Eppendorf 5252000021)
- Microloader tips (Eppendorf 930001007)
- Micromanipulator (Sutter Instrument MM-33R)
- Micropipette puller (Sutter Instrument P-1000 or P-2000)
- Needle beveler (Sutter BV-10)
- Slides (Fisherbrand 12-550-A3)
- Tape (double-sided; Scotch 378988)
- Vacuum drying oven (ECO 250C 1.9 Cu Ft; Across International AT19e)

Filter paper (Whatman 1825-021)
Fluorescent stereomicroscope (Leica M165FC)
Items for in-house sgRNA synthesis (optional; see Step 4)
- Magnetic beads (AMPure XP; Beckman Coulter)
- MegaClear columns (Life Technologies AM1908)
- Kimwipes
- Microcentrifuge tubes (Fisher Scientific 05-408-130)

Mosquito husbandry equipment
- Cages (24.5-cm × 24.5-cm × 24.5-cm; Bugdorm DP1000)
- Containers (plastic, 34.6-cm × 21-cm × 12.4-cm; Sterilite 1642)
- Cups (plastic; 9-oz [Karat C-KC9] and 16-oz [Amazon B015TK4N30])
- Dehydration chamber (Nalgene transparent polycarbonate; Thermo Scientific 5311-0250) (optional; see Step 20)
- Desiccant (e.g., Drierite granules; Carolina 858963) (optional; see Step 20)
- Membrane feeding system (Hemotek SP6W1-3) (optional; see Step 10)
- Vials (narrow, polystyrene) with plugs (Genesee Scientific 32-116)

Paintbrush (fine-tipped; ZEM 25950)
Paper towels
PCR thermal cycler (T100 Series Touch; Bio-Rad 1861096)
Software to design sgRNA (e.g., CHOPCHOP V3.0.0) (see Step 3)
Spectrophotometer (NanoDrop 2000; Thermo Scientific ND-2000)
Transgenesis screening in G_1 microscopy (see Step 27)
- Camera (Leica DMC2900)
- Fluorescent microscope filters (ECFP, Leica 10447409; EGFP, Leica 10447408; EYFP, Leica 10447410; RFP/dsRed/tdTomato/mCherry, Leica 10447410; ECFP/EGFP/RFP, Leica 10450611)
- LED light source (Lumencor SOLA-III)

METHOD

1. Select regions within the target genes for editing based on project requirements, gene structures, transcripts, protospacer adjacent motif (PAM) requirement (e.g., NGG for Cas9), and exon–intron junction boundaries.

   It is highly recommended to validate the target region sequence using polymerase chain reaction (PCR) amplification (use DNA extraction kit, purification kit, and external primer synthesis service offered by Integrated DNA Technologies, Inc.) and Sanger sequencing (use external sequencing service offered by Integrated DNA Technologies, Inc.) before designing sgRNAs for the target region.

2. Rear *Ae. aegypti* Exu-Cas9 strain and wild-type larvae at an optimal density at 27°C ± 1°C, with 50%–80% humidity, and 12:12-h (light:dark) photoperiod (~200 larvae in 3 L of deoxygenated deionized H_2O) in plastic containers. Feed larvae with a standardized amount of larval food daily until pupation.

   Standardizing the rearing procedure and feeding regimen according to larval density is highly recommended to achieve synchronous pupation and uniform adult size. A standard food density is ~50 mg/200 larvae daily for first-instar (L1) and L2 larvae and ~1300 mg/200 larvae daily for L2–L4 larvae. While going through this protocol, it is important to keep rearing wild-type Ae. aegypti *continuously under the above-mentioned condition. Pupae from these wild-type mosquitoes will be needed for Step 23.*

3. Use CHOPCHOP V3.0.0 or CRISPR software to design the sgRNAs of interest.

4. Prepare sgRNAs in the form of in vitro–transcribed sgRNA or plasmid DNA to be used in the injection mixture.

   i. Thoroughly clean the work area including the microinjection apparatus, gloves, and pipettes with RNaseZap before conducting experiments.

   ii. Prepare in vitro sgRNAs, or sgRNA-expressing plasmid DNA, using ultrapure molecular grade nuclease-free deionized H_2O.
   Option 1: Use a commercial sgRNA synthesis service.
   Option 2: Perform in-house sgRNA synthesis using T7-mediated in vitro transcription. Use the reagents Q5 high-fidelity DNA polymerase, MegaClear columns, magnetic beads, Ambion MegaScript T7, and primers for generating DNA template of sgRNA (Li et al. 2017).

   The primers we use are sgRNA-F (replace underlined sequence with your target sequence, 5′-GAAATTAATACGACTCACTATAGGATCCTCATGACATTACATGTTTTAGAGCTAGAAATAGC-3′) and sgRNA-R (universal; 5′-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3′)

   Option 3: Synthesize an sgRNA-expressing plasmid according to Li et al. (2021a).

   iii. Determine the concentration of the sgRNA or plasmid DNA using a spectrophotometer.

   The target size of 5–100 µg is acceptable.

   iv. Verify the size and concentration using an Agilent Bioanalyzer with the RNA 6000 Nano Kit.

   The bioanalyzer's result of its size may not be accurate due to the nature of the sgRNA structure, but it is important to have a single sharp band/peak around the approximate size with little evidence of degradation

   v. Store the final solution in aliquots at 1 µg/µL of a volume 1–10 µL in microcentrifuge tubes at −80°C.

5. If HDR-mediated insertion is the desired editing method, design and assemble a donor DNA plasmid using Gibson assembly (Gibson et al. 2009).

   i. Assemble the donor DNA plasmid using Gibson assembly master mix and the piggyBac plasmid.

   ii. To obtain high-quality donor DNA plasmid for microinjection (ultrapure and endotoxin-free), isolate and purify the assembled HDR plasmid using the ZymoPURE II Plasmid Maxiprep kit.

The donor DNA plasmid template generally contains a user-defined DNA segment to be inserted, a transgenic marker sequence (we use 3xP3-dsRed), and two homology arms of 500–1000 bases flanking the target cleavage site. An additional marker can be added outside the homology arms and used as an indicator of proper insertion (e.g., CFP), as one would not expect an HDR-mediated insertion into the correct location to result in expression from both markers.

iii. After obtaining the donor plasmid, confirm the plasmid sequence with Sanger sequencing.

iv. Store plasmids for 1 yr+ at −80°C.

6. Prepare final injection mixtures with nuclease-free deionized H_2O, and store the mixture in aliquots (5–10 µL) in microcentrifuge tubes at −80°C until use (Fig. 1A,B).

   The recommended final concentrations for different components are as follows: sgRNA-expressing plasmid (300 ng/µL), sgRNA (100 ng/µL), and donor DNA plasmid (100 ng/µL, HDR only). Only one source of sgRNA is needed in the injection mixtures.

7. Manually collect 200–300 *Ae. aegypti* pupae from the plastic rearing container as the parental generation to be reared to adults and used for embryo collection.

8. Place the pupae into 9-oz cups containing ∼100 mL of ultrapure H_2O.

9. Place the pupal cups inside a holding cage maintained at 27°C ± 1°C, with 50%–80% humidity, and 12:12-h (light:dark) photoperiod. Provide the adults with sucrose or glucose in a 9-oz cup with a filter paper capable of saturation by capillary action for ad libitum adult feeding. Allow adults to mate freely in the cage for 4–5 d.

   Ensure no sources of moisture are present in the cage other than the sugar source, as adult mosquitoes may prematurely lay on this surface, preventing timed oviposition required for injections.

10. Blood feed the females from each cage with anesthetized mice until satiated (for ∼15 min daily on two consecutive days).

    Another way of blood feeding is to use the Hemotek membrane feeding system loaded with fresh animal blood. However, blood feeding with live anesthetized mice usually leads to more eggs being laid and higher egg survival rates.

11. Prepare an oviposition cup (9-oz) by filling it with H_2O to 2/3 of its volume, and place paper towels (soaked with H_2O) in the cup. Then, place the cup inside the cage on the day of embryonic microinjections.

    Females are ready to oviposit eggs 72 h after the second blood meal.

    Blowing on, or agitating the cage, in addition to heating the oviposition cup to just above room temperature, may help stimulate egg laying.

12. Collect 50 individual eggs that are white to light gray in coloration (within ∼15 min to 1 h of oviposition) and align them side-by-side on moistened filter paper (sufficiently wet with double-distilled H_2O to keep the eggs moist without a meniscus forming) using a paintbrush. Orient the narrow posterior poles (which is the narrower end of the two poles) of the embryos to the same direction for microinjection.

    After embryos are aligned, use fresh filter paper to absorb H_2O from the wet filter paper that is being used as an alignment substrate. This step soaks up residual moisture to help the embryos stick to the double-sided tape in Step 13.

13. Adhere double-sided tape to a glass coverslip and a microscope slide. Transfer aligned embryos to the taped coverslip by gently pressing the coverslip against the surface of the aligned embryos. Flip the glass coverslip so that the eggs are on top and adhere the untaped side of the coverslip to the taped microscope slide to immobilize the coverslip.

14. Immediately pour a little of the ultrapure H_2O-saturated halocarbon oil over the embryos so that they are immersed.

15. Thaw the injection mix on ice.

16. Prepare glass needles for injection.

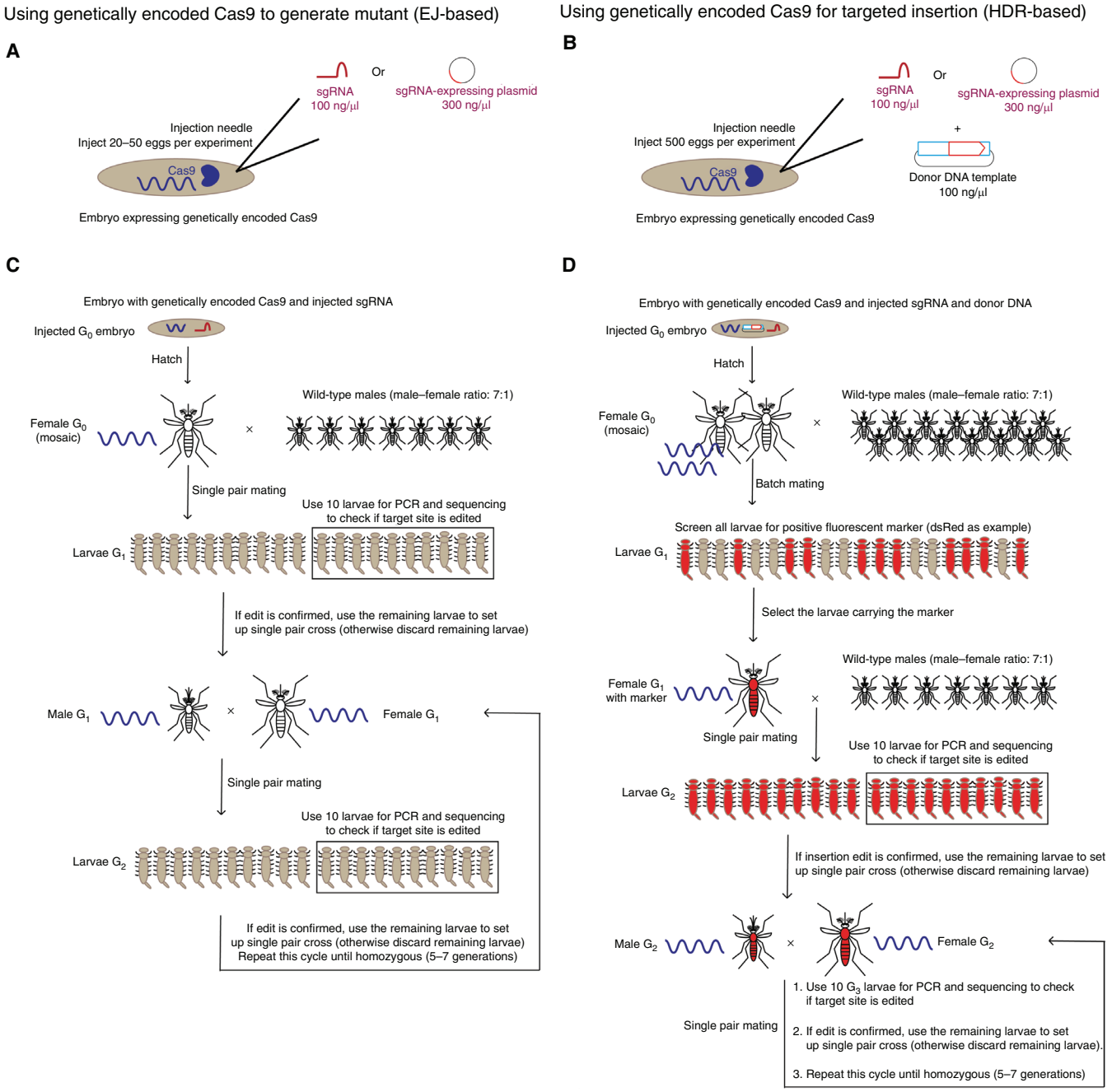

FIGURE 1. Injection and crossing steps when using genetically encoded Cas9 to generate site-directed mutations and insertions. (*A*) Injection mixture for end-joining (EJ)-based editing in mutant generation includes single-guide (sgRNA) or sgRNA-expressing plasmid only. (*B*) Injection mixture for homology-directed repair (HDR)-based editing in mutant generation includes sgRNA or sgRNA-expressing plasmid and donor DNA template. (*C*) Postinjection genetic crosses for obtaining homozygous lines of EJ-based editing. (*D*) Postinjection genetic crosses for obtaining homozygous lines of HDR-based editing include an additional screening and cross step compared to EJ-based editing.

We recommend using quartz capillary glass needles pulled by a Sutter P-2000 puller (heat: 750, filament: 4, velocity: 40, delay: 150, pull: 165). The tip of the needle needs to be beveled (using Sutter needle beveler) to create a sharp opening.

17. Set the Femtojet microinjector to a constant compensation pressure (P_c) of ~1 Psi.

    This pressure minimizes capillary backflow/blockage.

18. Load a glass needle with 2 µL of the injection mixture using microloader tips while avoiding bubble formation and then mount it on the microscope micromanipulator stage.

19. Examine embryos under a fluorescent stereomicroscope. If embryos are white/light gray (ideally <1 h old), carefully insert the needle into the posterior pole of the embryo and inject the mixture using an Eppendorf Femtojet 4× with a typical injection pressure (P_I) of 30–50 Psi (using nitrogen gas) and injection time (T_I) of 0.1–0.3 sec, which results in an injection quantity of ~10% of the volume of the embryo.

    For best results, keep the injection needle at an angle slightly greater than 45° relative to the surface of the posterior quarter of the embryo.

20. (Optional) If the injected embryo is leaking fluid, perform light desiccation of the eggs with one of the following options.
    - Place the slide containing embryos briefly under the illumination of a dissecting microscope and constantly monitor the embryos under high magnification until the slightest sign of a dimple or indentation is observed in the side of the embryos.
    - Place the slide containing aligned embryos in a dehydration chamber consisting of a sealed Nalgene jar with desiccant (e.g., Drierite) until liquid is no longer leaking from the embryos.

21. Carefully remove all halocarbon oil within 20 min postinjection using a Kimwipe. Remove the injected G_0 eggs gently from the coverslip with a clean paintbrush and transfer them to a moist filter paper to recover and develop for 4 d at 27°C.

22. After 4 d postinjection, hatch the injected G_0 eggs in deoxygenated deionized H_2O in a vacuum drying oven (27°C, 20 Psi, and 1 h). Follow normal larval rearing procedures as in Step 2, with special care to prevent interlarval competition due to underfeeding or high population density.

    For nonlethal gene targets, typically 40%–80% of injected embryos hatch.

23. Rear larvae to the pupal stage. Sort and separate surviving pupae (G_0) into 16-oz cups based on sex (use sex-specific cages for HDR-G_0). Add the opposite sex of wild-type pupae into each cup (or cage) at 7:1 male-to-female ratios (wild type:G_0) (Fig. 1C).

    If single EJ events need to be isolated, place only one injected G_0 pupa per cup. A 16-oz cup is the smallest cup size in which mosquitoes will reliably mate in single pairs or small-group crosses. Do not mate mosquitoes in smaller containers and vials (e.g., Drosophila *vials). If isolating single EJ events is not needed, batch mate G_0 with excess virgin wild-type mosquitoes in larger cages.*

24. Blood feed adult G_0 females with anesthetized mice until satiated at 4 d posteclosion.

25. (EJ only) Use an empty vial to capture an individual blood-fed adult G_0 female and transfer it directly to another single narrow polystyrene vial with a moist filter paper cone for egg-laying (G_1 eggs) 3 d post-blood meal.

    For HDR editing, put a 9-oz cup in each cage for oviposition.

26. Hatch G_1 eggs in deoxygenated deionized H_2O using a vacuum drying oven (27°C, 20 Psi, and 1 h) after 4 d. Then follow normal larval rearing procedures as in Step 2.

    If a vacuum drying oven is not available, at 2 d postoviposition, condition the moist egg papers for synchronous hatching by removing them individually from their vials and drying them for 5 min at room temperature. Dry the inside of the original oviposition vials with Kimwipes and place the moist oviposition papers that have been drying back into their respective polystyrene vials and plug using cotton stoppers. Allow an additional 2 d to let these egg papers in each vial to desiccate in the insectary. Hatch conditioned eggs in deoxygenated deionized H_2O that has been boiled, capped in a bottle, and cooled to room temperature, and rear mosquitoes as specified in Step 2.

27. (EJ only) Select 10 larvae (G_1) randomly from each isolated colony (out of 40–80 larvae per female) and then pool and extract DNA from these larvae using a DNA extraction kit. Conduct PCR and Sanger sequencing (using commercial PCR primer synthesis and sequencing service) to check for CRISPR–Cas9-induced mutations in the genomic target site (Fig. 1C). Keep colonies with confirmed mutant genotypes and discard other colonies.

    For HDR, screen all G_1 larvae of L3 and older under a fluorescent stereomicroscope (with filters, an LED light source, and camera) for positive fluorescent markers (Fig. 1D). Perform Step 23 on the selected larvae using 16-oz cups. Next, perform Steps 24–26 to obtain G_2 larvae carrying fluorescent markers. Select and pool 10 G_2 larvae carrying fluorescent markers for each sample, perform DNA extraction and then conduct PCR and Sanger sequencing to validate HDR donor DNA insertion. Keep the G_2 colonies with confirmed insertion.

28. (EJ only) Use sexed pupae from each G_1 colony with confirmed CRISPR-induced mutations or insertion to set up multiple single pair crosses of G_1 mosquitoes (mate one female only with one male) (Fig. 1C). Blood-feed mated G_1 females and let them lay eggs. Genotype the candidate G_1 mutant parents using DNA extraction, followed by PCR and Sanger sequencing or restriction fragment analysis after egg laying. Keep G_2 colonies with confirmed mutant genotypes/HDR-mediated insertions and discard other colonies. Continue intercrossing this way until a homozygous mutant/transgenic line is obtained, which can take up to 5–10 generations.

    For HDR, either set up multiple single crosses using G_2 mosquitoes (single intercross) with the confirmed insertion or outcross the G_2 mosquitoes with wild-type mosquitoes (Fig. 1D). For a single intercross, repeat this step until homozygous transgenic lines are obtained. For outcrossing, it is recommended to outcross for five to seven generations to remove any potential off-target editing. After outcrossing, a single intercross is still needed.

    Homozygosity, the presence of two identical copies of the edited gene location, should be determined via sequencing.

    For all PCR and Sanger sequencing steps, it is recommended to sequence for any putative off-target site editing.

DISCUSSION

In the process of generating mutant strains of mosquitoes with CRISPR–Cas9, researchers should keep several key items in mind. Validation of target DNA sequences is important as the reference genome sequence of *Ae. aegypti* is imperfect. Single-nucleotide polymorphisms, insertions, and deletions are common in the genomes of in-house laboratory strains relative to the genome assembly strains. The use of genetically encoded Cas9 strains streamlines the generation of mutant strains and site-directed insertions. Additionally, researchers using this tool in mosquitoes will help unravel the genes important for basic physiological functions and also may prove useful for generating innovative population control strategies that can leverage genetically encoded CRISPR reagents such as gene drives (Champer et al. 2016; Li et al. 2020; Raban et al. 2020; Verkuijl et al. 2022) and precision-guided sterile insect techniques (Kandul et al. 2019; Li et al. 2021b) to develop transformative technologies to prevent the spread of deadly mosquito-borne pathogens.

RECIPE

Halocarbon Oil Solution for Embryo Microinjection

Reagent	Amount
Halocarbon oil 700 (Sigma-Aldrich H8898)	9 mL
Halocarbon oil 27 (Sigma-Aldrich H8773)	1 mL
Deoxygenated deionized H_2O	20 mL

Store in a tightly sealed container in a dry, well-ventilated space at room temperature.

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REFERENCES

Champer J, Buchman A, Akbari OS. 2016. Cheating evolution: engineering gene drives to manipulate the fate of wild populations. *Nat Rev Genet* **17:** 146–159. doi:10.1038/nrg.2015.34

Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA III, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* **6:** 343–345. doi:10.1038/nmeth.1318

Kandul NP, Liu J, Sánchez C HM, Wu SL, Marshall JM, Akbari OS. 2019. Transforming insect population control with precision guided sterile males with demonstration in flies. *Nat Commun* **10:** 84. doi:10.1038/s41467-018-07964-7

Li M, Bui M, Yang T, Bowman CS, White BJ, Akbari OS. 2017. Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector, *Aedes aegypti*. *Proc Natl Acad Sci* **114:** E10540–E10549. doi:10.1073/pnas.1711538114

Li M, Yang T, Kandul NP, Bui M, Gamez S, Raban R, Bennett J, Sánchez C HM, Lanzato GC, Schmidt H, et al. 2020. Development of a confinable gene drive system in the human disease vector *Aedes aegypti*. *eLife* **9:** e51701. doi:10.7554/eLife.51701

Li H-H, Li J-C, Su MP, Liu K-L, Chen C-H. 2021a. Generating mutant *Aedes aegypti* mosquitoes using the CRISPR/Cas9 system. *STAR Protoc* **2:** 100432. doi:10.1016/j.xpro.2021.100432

Li M, Yang T, Bui M, Gamez S, Wise T, Kandul NP, Liu J, Alcantara L, Lee H, Edula JR, et al. 2021b. Suppressing mosquito populations with precision guided sterile males. *Nat Commun* **12:** 5374 doi:10.1038/s41467-021-25421-w

Raban RR, Marshall JM, Akbari OS. 2020. Progress towards engineering gene drives for population control. *J Exp Biol* **223:** jeb208181. doi:10.1242/jeb.208181

Verkuijl SAN, Gonzalez E, Li M, Ang J, Kandul NP, Anderson MAE, Akbari OS, Bonsall MB, Alphey L. 2022. A CRISPR endonuclease gene drive reveals two distinct mechanisms of inheritance bias. *Nat Commun* **13:** 7145. doi:10.1038/s41467-022-34739-y

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