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Generating and Validating Transgenic Mosquitoes with Transposon-Mediated Transgenesis

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Abstract

Transposon-mediated transgenesis has revolutionized both basic and applied studies of mosquito vectors of disease. Currently, techniques such as enhancer traps and transposon tagging, which rely on remobilizable insertional mutagenesis, are only possible with transposon-based vector systems. Here, we provide general descriptions of methods and applications of transposon-based mosquito transgenesis. The exact procedures must be adapted to each mosquito species and comparisons of some differences among different mosquito species are outlined. A number of excellent publications showing detailed and specific protocols and methods are featured and referenced.

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

Agarose

Deionized or double-distilled sterile H_2O

DNeasy Blood & Tissue Kit (QIAGEN 69504)

Donor and helper plasmid DNA for transposon-mediated transgenesis (see Introduction: **Mosquito Transposon-Mediated Transgenesis** [Bottino-Rojas and James 2023]).

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Helper plasmids are available by request from researchers who have published transgenesis studies and some may be available from Addgene (https://www.addgene.org).

concentration will depend on the experiment (see Step 4).

EndoFree Plasmid Maxi kit (QIAGEN 12362)

Ethanol, absolute, molecular-biology-grade (optional; see Step 2)

Gel electrophoresis buffer of choice

Halocarbon oil 27 (Sigma-Aldrich H8773)

Halocarbon oil 700 (Sigma-Aldrich H8898)

Injection buffer for mosquitoes <R>

Isotonic buffer <R>

Mosquitoes (species/strains specific for the study) (Benedict 2015)

Oligonucleotide primers to confirm transgenesis

Design oligonucleotide primers that amplify specific regions of the transposable element to confirm the presence of the construct-genome junctions in the genomes of apparent transgenic organisms.

PCR reagents

Equipment

Coverslips

Dissecting microscope (e.g., Leica MZ12)

This microscope is used for embryo alignment.

Double-sided Scotch tape

Fine paintbrush

Gel electrophoresis apparatus

Glass slides

Insectary maintained with the following conditions: 28°C temperature, 75% humidity, with a 12 h cycle of light and darkness

Inverted microscope (e.g., Leica DM 1000 LED or M165 FC)

This microscope is used for microinjection.

Larval trays (plastic, 33-cm×19-cm×11-cm)

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Microinjector and micromanipulator (e.g., Sutter Instrument—XenoWorks) Microinjection needles/micropipettes (pulled from Quartz glass capillaries [Sutter Instrument QF100-70-10] as described in **Mosquito Embryo Microinjection** [Harrell 2023a])

Microloader pipette tips (2-μL) (Eppendorf)

Micron filter (0.22-μm) or magnetic beads (optional; see Step 2)

Microscope with fluorescence filter set (e.g., Leica MZFLIII)

This microscope is used for larval screening.

Minimum-fiber filter paper (e.g., Fisher Brand 05-714-4)

Oviposition containers (conical; paper cup, *Drosophila* vials, or 50-mL centrifuge tubes)

Petri dishes (plastic, 90-mm×15-mm) or glass containers (125-mm × 65-mm) (Pyrex 3140)

Thermocycler

Transfer pipettes (e.g., Fisher Brand 13-711-7M)

METHOD

Users must follow all institutional procedures for working with mosquitoes and seek guidance from the relevant regulatory bodies for such matters.

Step-by-step instructions for making transgenic Aedes aegypti, Anopheles gambiae, Anopheles stephensi, and Culex quinquefasciatus, some of which include video images that allow replication and troubleshooting, are available online (Allen et al. 2001; Lobo et al. 2006; Jasinskiene et al. 2007; Terenius et al. 2007; Fuchs et al. 2013; Adolfi et al. 2021; Carballar-Lejarazú et al. 2021). Significant differences among species are detailed in Table 1. See the Discussion for further details.

Plasmid Preparation

1. Design and construct the desired donor plasmid with the following essential components: fluorescent marker (with an appropriate promoter); terminal-repeat sequences from transposable elements; desired transgene cargo (see Introduction: **Mosquito Transposon-Mediated Transgenesis** [Bottino-Rojas and James 2023]).

2. Purify donor and helper plasmids using an endotoxin-free plasmid purification kit (e.g., EndoFree Plasmid Maxi kit).

   Proceed with an additional purification (ethanol precipitation, magnetic beads, or 0.22 μm filtration) to remove any impurities that can poison the embryos and particulate matter that may clog the microinjection needles.

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3. Dilute the purified plasmids in injection buffer for mosquitoes into aliquots for microinjection (~1 µg/µL) that can be stored until needed at −70°C.

4. Mix donor and helper plasmids at a desirable ratio and microinject into preblastoderm mosquito embryos as described in the next section.

   The helper:donor ratio is adjusted for plasmid size and overall concentration. Typically, the total DNA concentration for injection should not exceed 1 mg/mL. Standard protocols suggest a higher proportion of donor to helper plasmid (two- to fourfold; e.g., 0.5 mg/mL donor:0.3 mg/mL helper) but successful applications have been reported for equal (1:1) ratios as well (Coates et al. 1998; Jasinskiene et al. 1998; Handler and O'Brochta 2011).

Microinjection Procedures

Detailed microinjections procedures can be found in Introduction: Mosquito Embryo Microinjection (Harrell 2023a) and Protocol: Mosquito Embryo Microinjection under Halocarbon Oil or in Aqueous solution [Harrell 2023b].

5. Collect preblastoderm embryos by allowing gravid females (3–5 d after blood feeding) to force-lay eggs on an oviposition container for 45–75 min at insectary conditions.

6. Allow eggs to mature until their color has changed from creamy yellow to gray. This color change is characteristic of the eggshell melanization process and it is important to not let the eggs turn dark brown or black, as these will be too difficult to inject and break the needles. Timing varies among species and general times are listed in Table 1.

7. Using a fine paintbrush, align individual eggs on a strip of double-sided tape mounted on a glass coverslip. Place the coverslip on a glass slide to elevate the coverslip and allow movement of the embryos on the microscope platform during injection.

   Keep a humid microenvironment to prevent desiccation (e.g., use a piece of moistened filter paper as a support for alignment).

8. Proceed with manual dechorionation of the eggs as needed.

   Some researchers use manual and chemical dechorionation of the eggs to make them easier to inject (Kumar and Puttaraju 2012). However, if the eggs are injected before melanization (Table 1), then dechorionation is not necessary. Forgoing dechorionation greatly increases embryo postinjection survival. Others have used delayed melanization, but this runs the risk of not getting the injected DNA into the germline cells before they develop membranes that would exclude the DNA (Catteruccia et al. 2000).

9. Use a microloader tip to fill the needles with 2 µL of DNA (donor and helper plasmids) mixture. Mount beveled prefilled microcapillary needles in the

microinjection apparatus at an appropriate angle and inject solution laterally targeting the posterior pole (region of formation of the future germline) of the embryo.

The volume injected depends proportionally on the degree of preinjection aging (Step 6). A successful injection will lead to a small movement of the cytoplasm within the egg.

See Troubleshooting

Postinjection Procedures

10. After microinjection, move the injected eggs undisturbed on the injection slides to insectary conditions (e.g., place the coverslip in a covered Petri dish with moist filter paper or in a glass slide box with water to incubate the embryos in a vertical orientation).

Hatching should start ~48 h after injection in some species. Since injection may cause a developmental delay, it is advisable to keep monitoring for late-hatching larvae for at least 3–5 d. We have kept some eggs for 10 d.

11. Isolate surviving injected (G_0) insects as pupae, sort according to sex, and cross resulting adult mosquitoes to an excess of fresh (5–7 d after emergence) wild-type adults (Adolfi et al. 2021; Carballar-Lejarazú et al. 2021).

Standard protocols call for establishing founder families in separate containers by mating each surviving G_0 male to five to 10 wild-type females of the strain of origin, and mating females in batches (of five to 10) with 20 or more wild-type males. Allow adults to mate for 4–5 d.

12. Blood-feed G_0 families, collect eggs, and rear emerging next generation (G_1) (Adolfi et al. 2021; Carballar-Lejarazú et al. 2021).

13. Screen G_1 larvae (third-/fourth-instar) for inheritance of the transgenes (see Fig. 1 in Introduction: **Mosquito Transposon-Mediated Transgenesis** [Bottino-Rojas and James 2023]). Verify expression of the visible marker using a stereomicroscope with an attached fluorescence module.

See Troubleshooting.

14. Transfer transformed G_1 individuals using a transfer pipette into a larval tray and rear to pupae (Benedict 2015). Discard nonfluorescent individuals.

15. Sort marker-expressing pupae by sex and prepare them for mating. Outcross *en masse* transformed G_1 individuals from each G_0 founder family with opposite-sex age-matched wild-type individuals.

Repeat the outcrossing procedures over the next generations until sufficient progeny numbers allow inter-crossing of putative transgenics and establishment of new transgenic pure-breeding colonies.

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16. Set aside a subset of next-generation marker-positive (fluorescent) individuals (G_2 onward) for molecular analysis.

17. Use PCR-based methods for transgenesis confirmation.

    i. Isolate genomic DNA from pupa or adult specimens of each colony of transgenic insects using a commercial genomic DNA extraction kit (e.g., DNeasy Blood & Tissue Kit) from animal tissues in accordance with the manufacturer's instructions.

    ii. Perform PCR using oligonucleotide primers that amplify specific regions of the transposable element to confirm the presence of the construct-genome junctions in the genomes of apparent transgenic organisms.

18. Visualize the presence of the expected diagnostic PCR amplicons through agarose gel electrophoresis using standard methods. Sequence PCR products to confirm anticipated sequences.

19. Upon establishment of transgenic pure-breeding (homozygous) colonies, further experiments can be performed to evaluate transgene copy number and chromosomal location of the insertion sites, as well as effects such as insertional mutagenesis and position-dependent differences in gene expression pattern and timing. Furthermore, expression profile studies can be used to link gene function with the sequence information from genomics data (see Discussion).

TROUBLESHOOTING

Problem (Step 9): The DNA solution is too viscous for the needles.

Solution: The purified plasmids must be injected at a defined concentration (<1 mg/mL total DNA).

Make sure the appropriate concentration is being used.

Problem (Step 13): Transformation efficiency is low and/or fitness of injected individuals is low.

Solution: In this protocol, the helper plasmid expresses transposase from a constitutive promoter. The ubiquitous presence of the enzyme may lead to transformation of somatic cells if microinjections are not directed precisely to the posterior region of the embryo where the germline forms. Although such transformation events are not heritable, somatic effects can decrease the transformation efficiency and the fitness of injected individuals. If this occurs, repeat the experiment with more precise injections.

DISCUSSION

Space limitations prevent the detailed and specific description of protocols; however, step-by-step instructions for making transgenic *Ae. aegypti*, *An. gambiae*, *An. stephensi*, and *Cx. quinquefasciatus*, some of which include video images that allow replication and

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troubleshooting, are available online (Allen et al. 2001; Lobo et al. 2006; Jasinskiene et al. 2007; Terenius et al. 2007; Fuchs et al. 2013; Adolfi et al. 2021; Carballar-Lejarazú et al. 2021). These protocols have been applied to other species that are closely related—for example, *Aedes albopictus*, *Aedes triseriatus*, and *Aedes fluviatilis* (Lobo et al. 2001; Rodrigues et al. 2006; Labbé et al. 2010). The procedures include embryo collection and preparation, injection needle specifics, injection microscope variations, injections, postinjection care, and screening (see Introduction: **Mosquito Embryo Microinjection** [Harrell 2023a] and Introduction: **Techniques for Identifying and Sorting Transgenic Mosquito Larvae** [Marois 2023]). Some of the differences among species are outlined in Table 1. For example, many Culicine mosquitoes must undergo a period of estivation before hatching and this must be accommodated in the postinjection handling procedures. Transgene insert validation or copy number and genomic insertion site and transgene validation by microscopy and functional tests also are provided in the reference materials.

A carefully examined plasmid design is paramount for the success of this experiment (see Introduction: **Mosquito Transposon-Mediated Transgenesis** [Bottino-Rojas and James 2023]). For example, a larger insert generally correlates negatively with transformation efficiency and transposons most successfully integrate DNA fragments of 10–15 kilobases in length (Volohonsky et al. 2015; Gregory et al. 2016).

Additionally, special care must be paid to the rearing and crossing of individuals derived from injected embryos to maximize the chances of recovering transformants. Lines derived from male founders are used as the numerator in determining transformation efficiencies, and extra care must be taken with female-derived lines as they may represent more than one independent transformation event. Recovery of "clusters" of transformed progeny can be expected if the transposon inserts into a germ cell early in its differentiation to multiple gametes. Once a line has been established with sufficient numbers to ensure its continued propagation, molecular approaches such as inverse polymerase chain reaction (iPCR) (Ochman et al. 1988) can be used to establish transgene copy number and chromosomal location of the insertion sites. Alternately, the confirmation of transgenesis and number of insertions can be verified with Southern blotting (Southern 2006). Depending on the other genes in the construct, fluorescence microscopy can be used to verify the correct expression profiles of DNA control sequences of interest linked to a fluorescent reporter construct (e.g., Nirmala et al. 2006; Chen et al. 2007; Mathur et al. 2010).

RECIPES

Injection Buffer for Mosquitoes

5 mM KCl

0.1 mM $NaPO_4$

Adjust pH to 7.2 and filter sterilize with a 0.22-µm filter. Store for 6 mo at −20°C.

Isotonic Buffer

150 mM NaCl

5 mM KCl

10 mM HEPES

2.5 mM $CaCl_2$

Adjust pH to 7.2 and filter sterilize with a 0.22-μm filter. Store for 1 mo at 4°C.

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TABLE 1.

Significant embryo microinjection procedure differences among mosquito species

	Anopheles stephensi	***Anopheles gambiae***	***Aedes aegypti***	***Culex quinquefasciatus***
Preparation for egg hyrng	Blood-feed mosquitoes twice and collect embryos 2–4 d after the second blood meal.	Blood-feed mosquitoes twice and collect embryos 2–4 d after the second blood meal.	Blood-feed mosquitoes once and collect embryos 3–4 d after feeding.	Blood-feed mosquitoes once and collect embryos 4–5 d after feeding.
Egg laying setup (embryo collection)	*Drosophila* vial tube with wet cotton and filter paper —forced egg laying for 1 h.	50-mL centrifuge tube with filter paper adapted on a Petri dish—forced egg laying for 45 min.	*Drosophila* vial tube with wet cotton and filter paper —forced egg laying for 1 h 15 min.	Egg rafts collected immediately after oviposition in a cup of water—allowed to mature for 30–45 min.
Manipulation solution	Isotonic buffer	Sterile deionized water	Sterile deionized water	Sterile deionized water
Halocarbon oil to prevent desiccation	Yes, 2:1 proportion (700:27)	No	Yes, 1:1 proportion (700:27)	Yes. 1:1 proportion (700:27)
Embryo microinjection angle	30°	15°	30°	45°
Handling of injected embryos	Remove excess oil and keep embryos in a Petri dish covered with isotonic buffer to allow hatching. Transfer hatched larvae to a rearing container.	Transfer eggs into a glass container lined with filter paper and filled with water. Transfer hatched larvae to a rearing container.	Incubate eggs in a humidified chamber, in a vertical, anterior-down, orientation. Hatch 4 d after injection and observe hatching for up to 10 d.	Incubate eggs in a humidifier chamber, in a vertical, anterior-down, orientation. Hatch 24–30 h after injection.
Reference	Terenius et al. 2007	Carballar-Lejarazú et al. 2021	Jasinskiene et al. 2007	Allen et al. 2001