Submission ID #: 62146
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Project Page Link: https://www.jove.com/account/file-uploader?src=18954578

Title: Site-directed ϕC31-mediated integration and cassette exchange in Anopheles vectors of malaria

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera? **No**

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Olympus MVX10 stereoscope with fluorescence filters

   Nikon Eclipse TE2000-U Inverted Microscope

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? **Different rooms located on the same floor**

Current Protocol Length

Number of Steps: 19
Number of Shots: 36

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Adriana Adolfi:** This protocol supports the characterization of the role of mosquito genes involved in a variety of physiological pathways including, for example, insecticide resistance and malaria parasite development.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Adriana Adolfi:** The method drives transgene insertion into single pre-characterized genomic locations, and therefore allows to directly compare phenotypes resulting from alternate transgenes, effectively avoiding the issue of positional effect.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 4.5.1, 4.5.2*

OPTIONAL:

1.3. **Amy Lynd:** This method can be applied to a variety of insect species of public health and agricultural importance and its applications extend widely, from bacteria to mammalian cells.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 3.4.1*

Protocol

2. Preparation of plasmids for the microinjection mix

2.1. Begin by designing *attB (pronounce 'at-B')* donor plasmids carrying the dominant fluorescent marker, *attB* recombination sites, and the desired transgene cargo **[1]**. Use a single *attB* site for integration of the whole plasmid carrying the transgenic cassette **[2]** or two inverted *attB* sites for cassette exchange **[3]**.

2.1.1. WIDE: Establishing shot of talent at the computer designing the plasmids.

2.1.2. LAB MEDIA: Figure 3 A.

2.1.3. LAB MEDIA: Figure 3 B.

2.2. Purify donor and helper plasmids using an endotoxin-free purification kit **[1]**.

2.2.1 Talent opening the purification kit and/or holding a bacterial flask.

2.3. Combine the *attB*-tagged donor plasmid carrying the transgene of interest and the helper plasmid carrying the integrase to obtain a mix with a final concentration of 350 nanograms per microliter of the donor plasmid and 150 nanograms per microliter of the helper plasmid **[1]**.

2.3.1. Talent combining the plasmids.

2.4. Precipitate the DNA by adding 0.1 volume of 3 molar sodium acetate and 2.5 volumes of ice-cold 100% ethanol **[1]**, then vortex **[2]**. A white precipitate should be immediately visible **[3]**. *Videographer: This step is important!*

2.4.1. Talent adding sodium acetate and ethanol to the mix.

2.4.2. Talent vortexing the tube. Videographer's NOTE: 2.4.1 and 2.4.2 combined in one shot.

2.4.3. ECU: White precipitate. Videographer's NOTE: Audio slate action at 00:15 or 00:45 for shot 2.4.3.

2.5. Wash the pellet and resuspend it in injection buffer to reach a total final concentration of 500 nanograms per microliter **[1]**, then prepare aliquots of 10 to 15 microliters each **[2]** and store them at -20 degrees Celsius **[3]**.

2.5.1. Talent resuspending the DNA pellet.

2.5.2. Talent preparing aliquots.

2.5.3. Talent putting the aliquots in the freezer.

NOTE: VO narration for step 2.5 was edited by authors

3. Microinjection of embryos from an *Anopheles* docking line

3.1. Blood feed 4 to 7-day-old mosquitoes from the desired docking line and their wild-type counterparts 72 hours prior to microinjection **[1]**.

3.1.1. Talent blood feeding the mosquitoes.

3.2. Perform *Anopheles gambiae* embryo microinjections in 25 millimolar sodium chloride by targeting the posterior pole of the embryo at a 45-degree angle **[1]**. Perform *Anopheles stephensi* embryo microinjections in halocarbon oil by targeting the posterior pole at a 30-degree angle **[2]**. *Videographer: This step is difficult and important!*

3.2.1. SCOPE: Talent injecting an *Anopheles gambiae* embryo.

3.2.2. SCOPE: Talent injecting an *Anopheles stephensi* embryo.

Videographer's NOTE: 3.2.2 was combined with 3.2.1

Videographer's NOTE for 3.2.1 and 3.2.2: Scope shot was not possible, inverted microscope did not have sufficient light so subject was in silhouette, author has suggestions for alternative footage for this shot, use this footage only if alternatives not viable.

Author's NOTE for 3.2.1 and 3.2.2: Issues with camera fitting and quality of the image in the inverted microscope Microinjections in Anopheles is a separate protocol in itself and JoVE already possesses of these:
An.stephensi:https://www.jove.com/t/216/injection-an-stephensi-embryos-to-generate-malaria-resistant
An.gambiae:https://www.jove.com/t/62591/microinjection-method-for-anopheles-gambiae-embryos

The last author of these protocols is the same as this protocol so, in the absence of footage from us, would it be possible to introduce some short footage from there or to reference this work in the video?

3.3. Immediately after injection, transfer the eggs to a Petri dish filled with sterile distilled water **[1]** and return them to insectary conditions **[2]**.

3.3.1. Talent placing eggs in a Petri dish.

3.3.2. Talent placing the eggs in insectary conditions.

3.4. Upon hatching, transfer G_0 larvae into a tray with salted distilled water daily and rear to pupae **[1]**.

3.4.1. Talent transferring larvae into a tray.

4. Crossing and screening of transformed individuals

4.1. Sort G_0 pupae by sex under a stereoscope **[1]**. Allow the males to emerge in separate cages in groups of 3 to 5 and add a 10-fold excess of age-matched wild type females **[2]**. *Videographer: This step is important!*

4.1.1. SCOPE: Talent sorting the pupae. Videographer's NOTE for 4.1.1: audio slate. action from 00:55

4.1.2. Talent adding females to the males.

4.2. Allow the females to emerge in separate cages in groups of 10 to 15 and add an equal number of age-matched wild type males **[1]**.

4.2.1. Talent adding males to females.

4.3. Allow adults to mate for 4 to 5 days **[1]** and provide females with a blood meal **[2]**. Collect the eggs and rear emerging next generation G_1s *(pronounce 'G-ones')* **[3]**.

4.3.1. Adult mosquitoes. Videographer's NOTE for 4.3.1: audio slate. action from 00:50

4.3.2. Talent providing a blood meal.

4.3.3. Talent collecting eggs.

4.4. Collect G_1 L_3 and L_4 larvae in a Petri dish lined with wet filter paper or on a microscope slide **[1]** and screen them using a fluorescent stereoscope for the presence of the marker introduced with the *attB*-tagged cargo **[2]**. *Videographer: This step is important!*

4.4.1. Talent collecting larvae in a Petri dish or on a slide.

4.4.2. Talent screening the larvae with the fluorescent microscope.

Videographer's NOTE for 4.2.2: audio slate. action starts at 00:20

4.5. For single-*attB* designs, screen for the presence of the new and pre-existing marker **[1]**. For double-*attB* designs for cassette exchange, screen for the presence of the new marker and the loss of the pre-existing one **[2]**.

4.5.1. LAB MEDIA: Figure 4 B.

4.5.2. LAB MEDIA: Figure 5 C.

Videographer's NOTE for 4.5.1: No slate. action at 00:40 or 01:10. Would it be possible to clean up the noise and lift the fluorescence in the larvae and show the footage to the author? Author wasn't happy with the footage. Author might like it when footage will be cleaned up and edited in to the film, rather than static lab media.

Author's NOTE for 4.5.1 and 4.5.2: Authors had issues with the lighting in the room and were not able to capture adequate fluorescent images. Please use Lab Media Figures.

4.6. Sort transformed G_1 pupae by sex and cross them *en masse* with opposite-sex age-matched wild type individuals **[1]**. Allow adults to mate for 4 to 5 days and provide a blood meal **[2]**.

4.6.1. Talent sorting the pupae.

4.6.2. Talent providing a blood meal.

4.7. For single integration experiments, collect eggs directly from the *en masse* cross **[1]**. For cassette exchange experiments, collect eggs from single females and maintain progeny separate until molecular assessment is complete due to the potential presence of two alternative cassette orientations **[2]**. *Videographer: This step is important!*

4.7.1. Talent collecting eggs *en masse*. Videographer's NOTE for 4.7.1: board marked as take 2.

4.7.2. Talent collecting eggs from single females. Videographer's NOTE for 4.7.1: action begins at 00:23.

4.8. Screen the G_2 progeny for the presence of the fluorescent marker **[1]** and set aside a subset of G_2 positive individuals for molecular analysis. Rear the rest to adulthood **[2]**.

4.8.1. Talent observing the progeny under a microscope. Videographer's NOTE for 4.8.1: Reuse 4.4.2.

4.8.2. Talent setting aside a subset of G_2 positive individuals.

4.9. Perform molecular validation of the insertion sites with PCR as described in the text manuscript **[1]**. For single integration, ensure that the predicted insertion site carries the original docking construct plus the whole sequence of the donor plasmid between the two hybrid sites *attL* and *attR* **[2]**.

4.9.1. Talent programming a PCR machine.

4.9.2. LAB MEDIA: Figure 3 A.

4.10. For cassette exchange, ensure that the predicted insertion site is identical to that of the docking line where hybrid inverted *attL* sites replace the original inverted *attP* sites and the exchange template replaces the cassette originally present between them **[1]**.

4.10.1. LAB MEDIA: Figure 3 B.

Results

5. Results: Validation of *φC31* Single Integration and RMCE in *An. stephensi* Larvae

5.1. The protocol was used to generate a stable *Anopheles* transgenic line in approximately 10 weeks. Phenotypic validation of transformation was performed by screening for fluorescent markers regulated by the *3xP3 (pronounce '3-X-P-3')* promoter are shown here **[1]**.

5.1.1. LAB MEDIA: Figure 4.

5.2. A new *Anopheles stephensi* line was obtained by insertion of a DsRed-marked cassette into a docking line marked with CFP **[1]**, which resulted in G_1 progeny expressing both markers as indicated by the red and blue fluorescence in the eyes **[2]**.

5.2.1. LAB MEDIA: Table 1. *Video Editor: Emphasize the 3rd Anopheles stephensi row, line named 80.9.*

5.2.2. LAB MEDIA: Figure 4.

5.3. Cassette exchange designs result in the replacement of the marker originally inserted into the docking line with that of the donor plasmid **[1]**. This marker exchange was demonstrated in an *Anopheles gambiae* docking line, where the CFP marker was lost and the YFP marker acquired resulting in yellow eye and nerve cord fluorescence **[2]**

5.3.1. LAB MEDIA: Figure 5 A and B.

5.3.2. LAB MEDIA: Figure 5 B and Table 1. *Video Editor: Emphasize the last row, line named A11.*

5.4. Occasionally, RMCE can result in a single integration event instead of the exchange of the desired transgenic cassette, where a larva is marked with both the original CFP and the new YFP markers **[1]**.

5.4.1. LAB MEDIA: Figure 5 C.

5.5. When screening for the presence of a fluorescent marker, it is crucial to distinguish its signal from possible background autofluorescence **[1]**. Increasing the magnification and focusing on the tissues and organs where fluorescence is expected to be driven by the promoter is necessary to identify true CFP-positive individuals **[2]**.

5.5.1. LAB MEDIA: Figure 6 A.

5.5.2. LAB MEDIA: Figure 6 B.

5.6. Individual transformants were also assessed molecularly via PCR to confirm the expected insertion site. PCR validation in individuals from an exchange *Anopheles gambiae* line is shown here **[1]**.

5.6.1. LAB MEDIA: Figure 7.

Conclusion

6. Conclusion Interview Statements

6.1. **Adriana Adolfi:** Given appropriate microinjection technique, the accurate design and preparation of donor and helper plasmids and following the appropriate mosquito husbandry scheme are key for obtaining transgenic individuals.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2 for microinjection, 2.1.2 – 2.1.3 for plasmid design, 2.3.1 for plasmid prep, and 4.1 – 4.2 for mosquito husbandry*

Videographer's NOTE for 6.1.1: Adriana Adolfi head shot.

6.2. **Amy Lynd:** This procedure can be used to insert elements causing gene overexpression or silencing, for example using the GAL4/UAS system, as well as gene drive elements and anti-pathogen molecules for mosquito genetic control.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer's NOTE for 6.2.1: Amy Lynd head shot.