Video Article

Protocol for RNAi Assays in Adult Mosquitoes (A. gambiae)

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Abstract

Reverse genetic approaches have proven extremely useful for determining which genes underly resistance to vector pathogens in mosquitoes. This video protocol illustrates a method used by the Dimopoulos lab to inject dsRNA into Anopheles gambiae mosquitoes, which harbor the malaria parasite. The technique manipulating the injection setup and injecting dsRNA into the thorax is illustrated.

Video Link

The video component of this article can be found at https://www.jove.com/video/230/

Protocol

RNAi-mediated gene silencing in mosquitoes requires prior preparation and purification of specific dsRNAs that target the gene of interest. For dsRNA synthesis, we recommend the Ambion Megascript kit which makes use of a T7 RNA polymerase-mediated *in vitro* transcription reaction. For dsRNA purification, we recommend the Qiagen RNeasy kit. Samples of dsRNA should be quantified and adjusted to 3 µg/µL in water. Other materials you will need include: fine-tipped forceps, glass slide, glass microcapillary needles, microinjector (we use Drummond's Nanoject II), a light microscope mounted above a cold block, a covered glass Petri dish, paper towels and a bucket of ice. You will also need a way to collect mosquitoes and suitable container for them along with a food source such as cotton ball soaked in 10% sucrose.

1. ***Collect your mosquitoes***. Using a battery-powered aspirator, collect the mosquitoes you wish to inject. Plug the nozzle with a paper towel or cotton so no mosquitoes can escape and remove the reservoir. Since cold temperatures anesthetize mosquitoes, bury the reservoir in a bucket of ice and wait 5-10 minutes for the mosquitoes to stop moving. Anesthetization can also be achieved by placing the reservoir in a cold room or refrigerator or by using CO_2. During these 5-10 minutes, continue to the next step.
2. ***Prepare your work area***. Set a flat surface cold block to 2°C and place a glass slide on it. This is where the mosquitoes will rest as you inject them. Attach a glass needle onto the microinjector and use your forceps to break the tip of the needle to the desired width. It is important that the width is large enough to allow enough room for liquid to flow through at a reasonable rate but is narrow enough to inflict a minimal wound during injection. A good needle tip will be narrow enough to bend slightly but not break. Set your injection equipment to the desired output volume (for RNAi, we use 69 nL). Submerge the tip of your needle into your sample and draw your liquid sample into the needle using the manufacturer's instructions. The liquid sample can be a dsRNA solution, bacterial suspension, or other; the injection process is the same for all. Embed a glass Petri dish in the ice bucket so that the bottom of the dish is completely contacting the ice. This is where you will put mosquitoes until you are ready to inject them.
3. ***Prepare your mosquitoes***. Transfer 50-100 mosquitoes from the reservoir to the glass Petri dish. Use your forceps to thin them to a single layer, making sure most have contact with the cold bottom of the dish to keep them anesthetized. Cover these mosquitoes with the dish cover when not in use. Return the reservoir to the ice bucket to keep the rest of the mosquitoes anesthetized.
4. ***Mount mosquitoes on cold block***. Use your fine-tipped forceps to transfer mosquitoes one at a time from the dish to the glass slide mounted on the cold block under the light microscope. Handle the mosquitoes gently, preferably by the legs. Line up as many as 30 mosquitoes side-by-side on the slide, making sure you can get to each one with the microinjector and making sure each one makes contact with the cold slide. Make sure your mosquitoes are lined up orderly so that as you go along you know which have and which have not been injected. Leave other mosquitoes covered in the chilled Petri dish.
5. ***Inject your first group of mosquitoes***. Hold your fine-tipped tipped forceps in one hand and hold the microinjector in the other hand like a pencil. Use the forceps to move the mosquito as you need (handle them by the legs to avoid injury) and to keep the mosquito steady as you inject. This works best if you use the forceps to support one side of the mosquito as you inject from the other side. Carefully insert just the needle tip into mosquito's thorax. It is best to inject into a taut yet thin part of the cuticle to minimize injury. Also, avoid injecting too deeply; you want just the tip to be inside the thorax; deeper injections cause greater wounds which will compromise survival. Once the needle tip is in position, press and release the foot pedal of the microinjector apparatus which will deliver your pre-set volume of dsRNA. Wait a second or two and remove the needle from the mosquito. If a drop of liquid appears outside the wound site, wait a few seconds for it to be absorbed into the wound. If it does not absorb within a few seconds, discard that mosquito and try again. Continue the injection process for all mosquitoes on your glass slide.
6. ***Store your mosquitoes.*** When you are finished, place your injected mosquitoes inside labeled containers (we prefer 1 pint wax-lined cardboard cups covered by mesh netting and secured with rubber bands and a cardboard lid).

7. ***Continue injections until finished***. Continue to place mosquitoes on the slide, inject and transfer to cups until you have the sample size you desire. Account for some deaths-beginners will have many mosquitoes die from the trauma of injection. This will improve with practice until almost 100% of your mosquitoes survive injection.
8. When finished, place a folded, wet paper towel over the cup along with a food source and cover the cups with something to retain moisture such as plastic wrap or a plastic Petri lid. Injected mosquitoes are prone to drying out so the moisture provided by the wet towel and retained by the plastic will help prevent this.
9. Place your mosquitoes in an environmentally controlled chamber for incubation.

Note: Efficiency of gene silencing is highly variable and depends on a number of factors including transcript and protein turn-over rates, and dsRNA uptake efficiency by cells and organs. We have found that silencing begins as early as 1 day post injection, and can last up to 6 days post injection. Use transcript and protein detection methods such as quantitative PCR and Western blotting to validate silencing.

Note: There are several variations on several steps of the mosquito injection and RNAi-mediated gene silencing technique. Some variations are described by: Boisson, *et al.* (2006) and Blandin, *et al.*(2002).

Discussion

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References

1. Boisson, et al. (2006)

2. Blandin, et al. (2002).

