VERSION 3

FEB 04, 2020

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.bb7airie

Protocol Citation: Michael Povelones, Abigail Mccrea 2020. *Dirofilaria immitis* and *Brugia malayi* emergence assay for individual or pools of mosquitoes. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.bb7airie>

MANUSCRIPT CITATION: Edgerton EB, McCrea AR, Berry CT, Kwok JY, Thompson LK, Watson B, et al. Activation of mosquito immunity blocks the development of transmission-stage filarial nematodes. *Proc Natl Acad Sci U S A*. 2020. Epub 2020/02/06. doi: 10.1073/pnas.1909369117. PubMed PMID: 32015105. <https://pubmed.ncbi.nlm.nih.gov/32015105>

McCrea AR, Edgerton EB, Oliver GT, O'Neill FM, Nolan TJ, Lok JB, et al. A novel assay to measure the emergence of third-stage filarial nematodes in individual mosquitoes. *bioRxiv*. 2020. doi: <https://doi.org/10.1101/2020.02.04.934653>. <https://www.biorxiv.org/content/10.1101/2020.02.04.934653v1>

Dirofilaria immitis and *Brugia malayi* emergence assay for individual or pools of mosquitoes V.3

Michael Povelones¹, Abigail Mccrea²

¹University of Pennsylvania School of Veterinary Medicine;

²University of Pennsylvania

povelab

Tech. support email: mpove@vet.upenn.edu

Michael Povelones

University of Pennsylvania School of Veterinary Medicine

ABSTRACT

This assay is for determining the number of infective third-stage (L3) larvae capable of emerging from individual mosquitoes. These are referred to as emerging L3 or eL3. This assay can be used to quantitatively measure how mosquito manipulations or filaria manipulations affect eL3. This assay can be performed on groups of individual mosquitoes in a 96-well plate, or en masse in a large petri dish. The latter format is useful for producing large numbers of eL3 for assays, such as motility, molting, transformation, or for infection of a naive host animal. In addition, since the assay can be performed on large number of input mosquitoes with little increase in effort or cost, it offers a favorable low-tech alternative to other mosquito infection diagnostic methods, such as PCR or dissection when live-caught mosquitoes are available.

This protocol assumes that you are starting with a population of infected mosquitoes by a filarial nematode. We have tested the protocol with [Aedes aegypti \(Blackeye strain, cat number NR-48921\)](#) obtained from [BEI Resources](#) via the [Filariasis Research Reagent Resource Center](#) infected with *Dirofilaria immitis* and maintained at 27 °C with 80% relative humidity. Emerging L3 are produced as early as day 12. The last day that we have checked is 21 days post infection, but it is very likely eL3 can be produced for the lifespan of the infected mosquitoes. We saw maximum yields at day 13 and beyond. The same *Ae. aegypti* strain infected with *Brugia malayi* was not tested with as many time points, however, we found robust and similar yields at days 12-14, but did not establish the minimum incubation time. We also tested a strain of *Ae. albopictus* ([ATM-NJ95 strain, cat number NR-48979](#)) following *D. immitis* infection and found that it also is capable of producing eL3.

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working
We use this protocol in our group with both parasites and different mosquito species and it is working.

Created: Feb 04, 2020

Last Modified: Mar 22, 2023

PROTOCOL integer ID:
32706

Keywords: L3, emerging L3, eL3, Filaria, *Dirofilaria immitis*, *Brugia malayi*, Mosquito, Aedes, host-pathogen interactions, Assay

GUIDELINES

Filariae are human and animal pathogens so all mosquito husbandry and infections should be performed in accordance to local safety and containment guidelines that meet or exceed published guidelines. We have performed this assay with *Brugia malayi* (a human pathogen) and *Dirofilaria immitis* (an animal pathogen) under the appropriate containment level.

American Committee Of Medical Entomology American Society Of Tropical Medicine And Hygiene. Arthropod Containment Guidelines, Version 3.2. *Vector Borne Zoonotic Dis.* 2019 Mar;19(3):152-173. doi: 10.1089/vbz.2018.2431. Epub 2019 Jan 29. PMID: 30694736; PMCID: PMC6396570.

MATERIALS

MATERIALS

☒ 70% Ethanol **Contributed by users**

☒ DMEM with L-glutamine; high glucose with sodium pyruvate **Fisher Scientific Catalog #MT10-013-CM**

☒ Phosphate Buffered Saline (PBS) **Corning Catalog #MT21-031-CV**

STEP MATERIALS

☒ DMEM with L-glutamine; high glucose with sodium pyruvate **Fisher Scientific Catalog #MT10-013-CM**

☒ Ethanol 70% **Contributed by users**

☒ Phosphate Buffered Saline (PBS) **Corning Catalog #MT21-031-CV**

Standard microscope slides and 22 mm square cover slips

Any standard 96 well plates

SAFETY WARNINGS

Brugia malayi is a human pathogen
Dirofilaria immitis is an animal pathogen

BEFORE START INSTRUCTIONS

Secure all approvals. Establish standard protocols. Assemble all materials.

1

As stated in the Abstract for this protocol, it is assumed that you already have groups of infected mosquitoes to examine. First, count and record the number of live and dead mosquitoes in each group and remove the dead ones. Count the total number of mosquitoes that will be assayed.

Note

Ideal group sizes will depend on the experiment and the infection level. We typically start with mosquitoes that are infected and treated with dsRNA. The dsRNA treatment group sizes are approximately 65 mosquitoes. We house them in 473 mL paper soup cups with a mesh cover, which are stored in a secondary container (large Bugdorm mosquito cage) in our containment suite. We aim for an average uptake per mosquito of approximately 15 microfilariae and this results in about 30-40 mosquitoes per group on day 14 post infection.

Equipment

16 oz paper soup cup	NAME
Cup	TYPE
Choice	BRAND
760SOUP16WPA	SKU
https://www.webstaurantstore.com/choice-16-oz-double-poly-coated-white-paper-soup-hot-food-cup-with-vented-paper-lid-case/760SOUP16WPA.html	LINK

Equipment

Mosquito No-see-um netting

NAME

Netting

TYPE

Mosquito no-see-um

BRAND

MNZ11

SKU

<https://www.scs-mall.com/Mosquito-No-see-um-sold-by-the-running-yard/productinfo/MNZ11/>

LINK

- 2 Fill wells of 96-well plates with **200 μ L** of DMEM as needed. A multichannel pipette is great to have for this! If you are only performing a single emergence assay to get the eL3 number per mosquito, then you will need one well for each mosquito. If you are planning to assay heads and carcasses after the initial whole-body emergence assay, make two additional wells to accommodate those parts (three wells total per mosquito). We typically do whole mosquitoes in one plate, heads in another plate, and carcasses in another plate using the same order for each so data can be tracked back to the same mosquito. Plates can be made in advance and stored at **4 °C**

Note

Instead of filling wells of a 96-well plate, a 60 or 100 mm petri dish could be used to process groups of mosquitoes en masse. In this case, fill the assay plate with **5 mL** for a 60 mm plate and **10 mL** for a 100 mm plate.

DMEM with L-glutamine; high glucose with sodium pyruvate **Fisher Scientific Catalog**
#MT10-013-CM

Equipment	
96 well plate	NAME
96 well plate	TYPE
Costar	BRAND
M0812	SKU
https://www.sigmaaldrich.com/catalog/product/sigma/m0812	LINK

3

Anesthetize the mosquitoes one group at a time with carbon dioxide flowing through a flypad facing down on top of the cup. Once the mosquitoes are anesthetized, remove the netting and dump them into a netted basket placed in a petri dish containing approximately 10 mL of **[M] 70 % volume** ethanol in water. The exact volume is not important, but it should be sufficient for the mosquitoes to submerge in the basket. The ethanol will wet the cuticle and the mosquitoes will immediately submerge, but the ethanol will not penetrate into the body. This step is performed at ** Room temperature** .

Note

As alternatives to carbon dioxide, ice is a commonly used way to anesthetize mosquitoes. We have not tested this. Another alternative is to flood the cup with **[M] 70 % volume** ethanol and pour the contents into the netted basket. Finally, mosquitoes caught with an aspirator can be directly placed into ethanol. Expelling them against the liquid immediately causes them to submerge.

Equipment	
Flypad	NAME
CO2 anesthetizing apparatus	TYPE
Flystuff	BRAND
59-114	SKU
https://geneseesci.com/shop-online/product-details/59-114	LINK

Equipment	
Plastic tea strainer	NAME
Basket	TYPE
Generic	BRAND
Generic	SKU

Equipment

Tissue Culture Dish, Non-Treated, Sterilized

NAME

Petri dish

TYPE

Corning

BRAND

10861-594

SKU

<https://us.vwr.com/store/product?keyword=10861-594>

LINK

10 cm

SPECIFICATIONS

Ethanol 70% **Contributed by users**

- 4 After 00:02:00 in ethanol, pick up the basket, wipe the outside of the basket with a tissue wipe or paper towel to remove excess ethanol. Dip the basket into two changes of 20 mL distilled water in a 100 mm petri dish. Leave the basket in the second water wash. The mosquitoes will be floating at the surface. Performed at Room temperature .
- 5 Pick up individual mosquitoes with fine forceps and place them head down in the buffer (if possible). You can manipulate them a little, but care must be taken to not disrupt their bodies. Gently touch them with the forceps. It is fine to grab the wings, wing hinge region or and legs roughly, but not the proboscis. Alternatively, if individual mosquito data is not needed then put the mosquitoes into a 60 or 100 mm petri dish with 5 mL or 10 mL DMEM, respectively. The plate is set up at Room temperature . Mosquitoes treated this way do not recover.

1m

Individual *Ae. aegypti* mosquitoes in wells of a 96-well plate containing DMEM.

Equipment

Fine Forceps

Forceps

Dumont

11251-10

<https://www.finescience.com/en-US/Products/Forceps-Hemostats/Dumont-Forceps/Dumont-5-Forceps/11251-10>

NAME

TYPE

BRAND

SKU

LINK

Equipment	
Dissection Microscope	NAME
Microscope	TYPE
Zeiss	BRAND
Stemi 305	SKU

- 6 Mark the lid plate with a marker so you know where each group begins and ends.

- 7 Put the plate or dish into a **🔥 37 °C** incubator and incubate for **🕒 01:00:00** with the lid on. 1h
 We use a standard **[M] 5 %** carbon dioxide incubator used for culture of mammalian cell lines. Carbon dioxide buffering is probably not necessary, however, plates incubated for very long times (i.e. **🕒 Overnight**) are likely to become contaminated and this will interfere with scoring of the plate.

Emergence

Cartoon showing a cross-section of a mosquito in a 96 well plate emergence assay with eL3 emerging and sinking to the bottom of the well containing DMEM.

- 8 The worms can be counted with a dissecting scope or on an inverted microscope. Tip: if you use an inverted microscope, you do not need to remove the mosquito floating near the surface. If you use a dissecting scope, you will need to remove the mosquito to see the emerged worms at the bottom.

D. immitis eL3 imaged at the bottom of a 96 well plate following emergence assay.

Equipment	
Dissection Microscope	NAME
Microscope	TYPE
Zeiss	BRAND
Stemi 305	SKU

Equipment	
Zeiss Primo Vert	NAME
Inverted phase contrast microscope	TYPE
Zeiss	BRAND
Primo Vert	SKU

- 9 Following the emergence assay, mosquitoes can be dissected to assay for L3 present in the head or carcass as well as to determine the number, and developmental stage of larvae present in the Malpighian tubules (in the case of *D. immitis* infections).

Cartoon of workflow to process head, carcass and Malpighian tubules following initial emergence assay.

- 10 Using a concavity microscope slide filled with PBS and a dissecting microscope, remove the head

with fine forceps and place into a well of a new 96 well plate containing 200 μL μl DMEM.

Equipment

Concavity Microscope Slide

NAME

Microscope slide for dissection

TYPE

ThermoFisher

BRAND

1527-006

SKU

<https://www.thermofisher.com/order/catalog/product/1518006>

LINK

Phosphate Buffered Saline (PBS) **Corning Catalog #MT21-031-CV**

- 11** Remove the Malpighian tubules and place them in a 15 μL drop of PBS on a standard microscope slide. Cover with a 22 mm² coverslip. Take the remaining carcass pieces and place them into a well of a new 96 well plate containing 200 μL DMEM.

Note

Note: it is common to find L3 larvae moving around the drop of PBS used for dissection. We record these as coming from the carcass sample.

Supplemental figure from McCrea et al. (A) mosquito in emergence assay. (B) Dissected head and (C) carcass after dissection. (D) Mosquito with head removed in PBS on depression slide. The head was placed into a well of 96 well plate containing DMEM. (E) Malpighian tubules removed from the abdomen (white arrow). The tubules were mounted on a slide in PBS and the carcass was placed into a well of a 96 well plate. The scale bars in A-C and D-E are 500 μm and 1000 μm , respectively.

- 12 Discard PBS used for dissection and read the Malpighian tubules slide for the number and developmental stage (if desired).

Note

Note: this works best with two people. One to dissect and the other to read the Malpighian tubule slides. Even so, It takes approximately 01:00:00 to process about 50 samples. Typically, we process a subset of the total used for the initial emergence assay.

Larvae (white arrows) in live Malpighian tubules are scored by microscopy. Scale bar is 50 μm .

- 13 Once the desired number of heads, carcass, and Malpighian tubule samples are processed, the head and carcass plates are placed in the 37°C incubator for 01:00:00 and scored as described above for whole mosquitoes.