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

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1 Works for me dx.doi.org/10.17504/protocols.io.bb7airie

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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.

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

NAME	CATALOG #	VENDOR
70% Ethanol		
DMEM with L-glutamine; high glucose with sodium pyruvate	MT10-013-CM	Fisher Scientific
Phosphate Buffered Saline (PBS)	MT21-031-CV	Corning

STEPS MATERIALS

NAME	CATALOG #	VENDOR
DMEM with L-glutamine; high glucose with sodium pyruvate	MT10-013-CM	Fisher Scientific

NAME	CATALOG #	VENDOR
Ethanol 70%		
Phosphate Buffered Saline (PBS)	MT21-031-CV	Corning

MATERIALS TEXT

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 STARTING

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.

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.

16 oz paper soup cup
Cup

Choice 760SOUP16WPA

Mosquito No-see-um netting
Netting

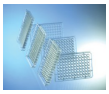
Mosquito no-see-um MNZ11

2 Fill wells of 96-well plates with **200 µl** 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 at **4 °C**

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
by Fisher Scientific
Catalog #: MT10-013-CM

96 well plate
96 well plate
Costar M0812



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 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** .

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.

Flypad
CO2 anesthetizing apparatus
Flystuff 59-114

Plastic tea strainer
Basket
Generic Generic

Tissue Culture Dish, Non-Treated, Sterilized
Petri dish
Corning 10861-594
10 cm

Ethanol 70%

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** . 1m

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.

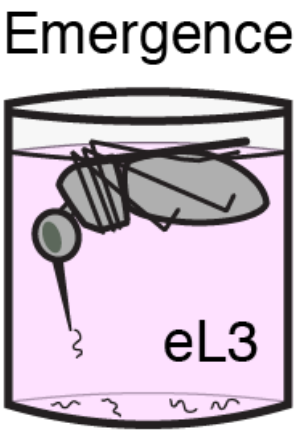
Individual *Ae. aegypti* mosquitoes in wells of a 96-well plate containing DMEM.

Fine Forceps
Forceps
Dumont 11251-10

Dissection Microscope
Microscope
Zeiss Stemi 305

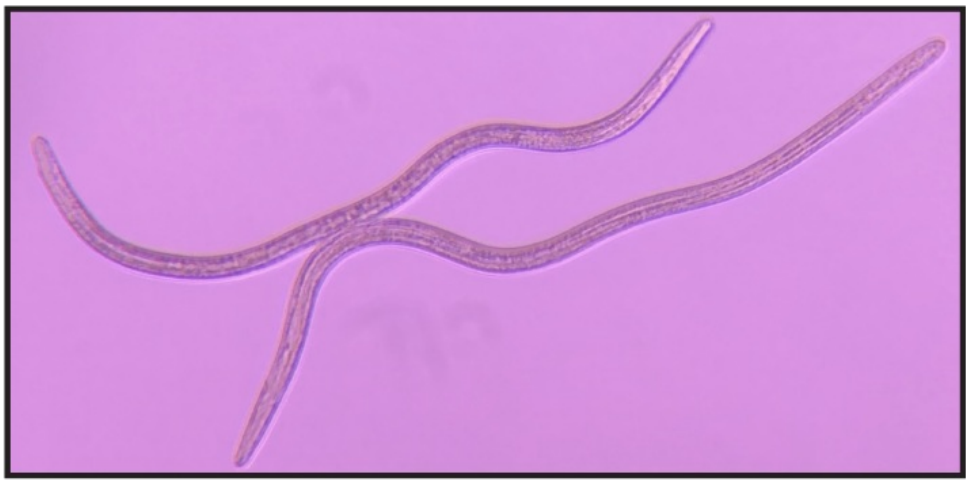
6 Mark the lid plate with a marker so you know where each group begins and ends.

7 Put the plate or dish into a **37 °C** incubator and incubate for **01:00:00** with the lid on. We use a standard **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. 1h



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.

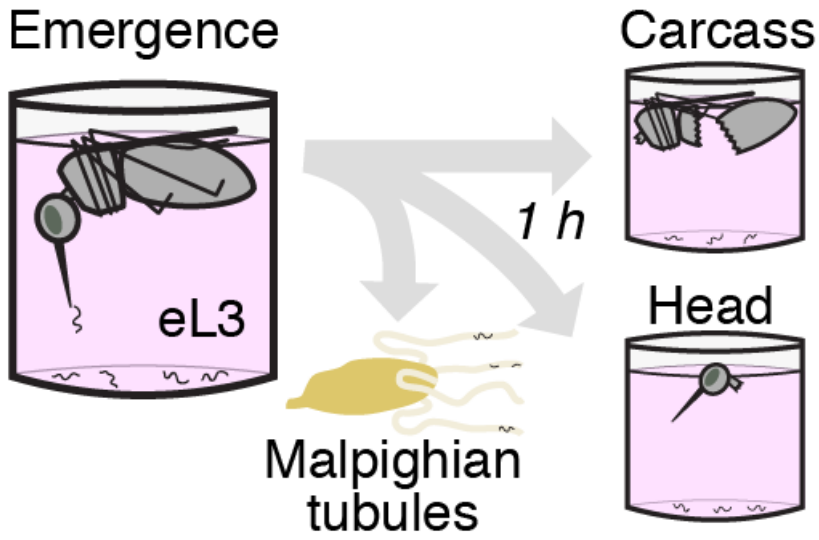


D. immitis eL3 imaged at the bottom of a 96 well plate following emergence assay.

Dissection Microscope
Microscope
Zeiss Stemi 305

Zeiss Primo Vert
Inverted phase contrast microscope
Zeiss Primo Vert

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.

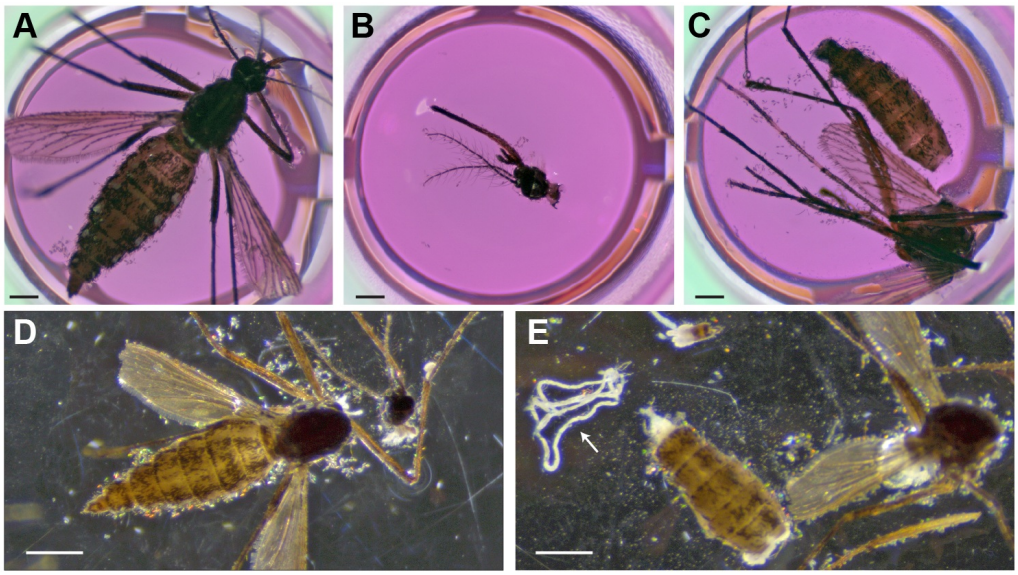
10 Using a concavity microscope slide filled with PBS and a dissecting microscope, remove the head with fine forceps and place into a well of a new 96 well plate containing **200 µl** µl DMEM.

Concavity Microscope Slide
Microscope slide for dissection
ThermoFisher 1527-006

Phosphate Buffered Saline (PBS)
by 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 mm^2 coverslip. Take the remaining carcass pieces and place them into a well of a new 96 well plate containing **200 µl** DMEM.

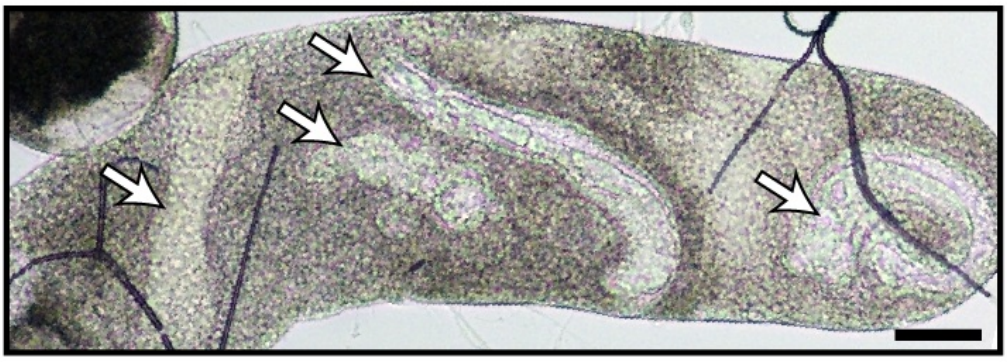
Note: it is common to find L3 larvae moving around the drop of PBS used for dissection. We record these as coming from the carcass sample.



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: 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.