MX protocol

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Background :

Molecular detection of arboviruses in the excretions of field-caught mosquitoes offers an innovative, non-invasive approach to monitoring their circulation in a territory. Known as *Molecular Xenomonitoring* (MX), this method has demonstrated its effectiveness in detecting various arboviruses and parasites without requiring the capture or manipulation of vertebrate hosts.

Infected mosquitoes excrete much higher viral loads in their excreta than in their saliva, greatly improving the sensitivity of molecular detection. A single infected female can release between 3 and 5 $\log_{10}$ copies of viral RNA per day (Fontaine et al., 2016) , making excreta a powerful tool for real-time virus tracking. By exploiting these excreta, the MX strategy lies at the interface between entomological and environmental surveillance, making it possible to monitor the emergence and circulation of arboviruses in real time, at low cost while retaining good sensitivity (Ramírez et al., 2018) .

To optimize this approach, we have designed a 3D-printed *MX adapter*, compatible with BG-Sentinel, BG Pro and CDC light commercial traps. This device replaces the collection net and provides a moist environment and food source for captured mosquitoes, prolonging their survival and increasing excreta production. This innovation offers several advantages:

(i) **Extended collection intervals**, reducing the frequency of field interventions.
(ii) **Increased chances of viral detection**, thanks to the accumulation of excreta over several days.
(iii) **Reduced monitoring costs**, as detection remains independent of the number of specimens trapped.

Excreta samples, collected on aluminum foil, can be transported at room temperature for molecular analysis, followed by rapid genomic sequencing of the viruses detected. Unlike conventional methods, the MX strategy enables :

- **Cost-effective monitoring**, with the possibility of screening hundreds of samples at the cost of a few dozen.

- **Efficient detection**, encompassing the entire insect vector community.
- **Simplified logistics**, with easy collection and transport without the need for specialized equipment.
- **Compatibility with complementary analyses**, such as identification of vector species, estimation of infection prevalence and viral isolation.

Thanks to these trap modifications, MX enables continuous viral monitoring with extended collection intervals, improving surveillance efficiency while reducing operational costs. This rapid, scalable and field-adapted approach enhances early detection of emerging viral threats (Bigeard et al., 2024; L'Ambert et al., 2023) .

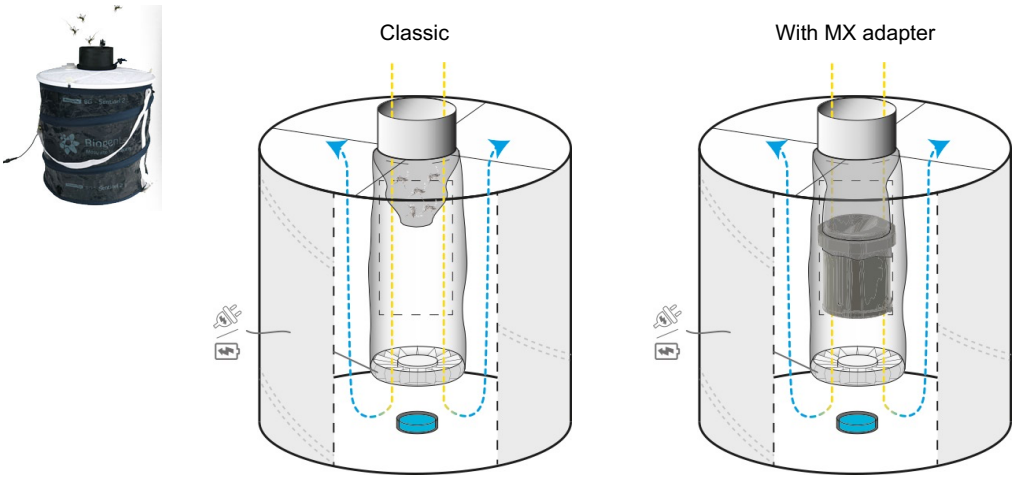


Fig1. View and illustration of the BG-Sentinel trap (Biogents) in its classic configuration and optimized for excretion collection with the MX adapter.

1. Preparing and installing the Mosquito Trap :

1.1 Equipment required in the field :

- BG Pro / BG Sentinel or CDC light Trap.
- 3D-printed adapters (MosquitoBox) for collecting mosquitoes and droppings.
- Sugar diluted to 10% (10g in 100 mL water) to impregnate absorbent cotton to be placed in the feeder inside the adapter.
- Shelter to protect the trap from the elements (wind and rain).
- Pre-cut aluminum foil for excrement collection.
- (Optional) Pressurized carbon dioxide bottles.
- (Optional) 12V batteries for power supply in the absence of mains sockets.

Note to user: It is advisable to prepare the 3D-printed adapter in advance by placing a circle of aluminum foil at the bottom and absorbent cotton soaked in sugar water in the feeder. The

adapter can then be stored at 4°C for several days before use. This saves time in the field, and avoids putting sugar water on the ground, which can attract ants.

1.1 Preparing the MX adapter :

1.1.1 3D printed device presentation

The adapter is a cylinder closed at both ends, with a hollow tube running vertically through it. It is made up of 4 parts:

- Threaded tube (adapter body)
- A bottom (part through which the smallest tube passes and which is closed by a grid)
- A top (part crossed by the longest tube)
- A feeder
- A net (to hold it in place at the mouth of the trap)

All parts are available here with printing instructions.

Fig2. Views of MX adapter with thread.

1.1.2 Net creation

a. Prepare a template with a 16.5 cm circle, with a 4 cm hole in the center. Keep the angle at 165 degrees, and add a 1.5 cm strip on one side for sewing.

b. Use the template to trace the outline of the parts on the fabric (mosquito netting or fabric that lets air through but not insects).

c. Cut sections of polyester braided cord (approx. 50 cm) and burn their ends with a lighter.

d. Use pliers to form a gutter with the cord inside

e. Sew the gutter, removing the clamps, and sew the straight edge to form a cone.

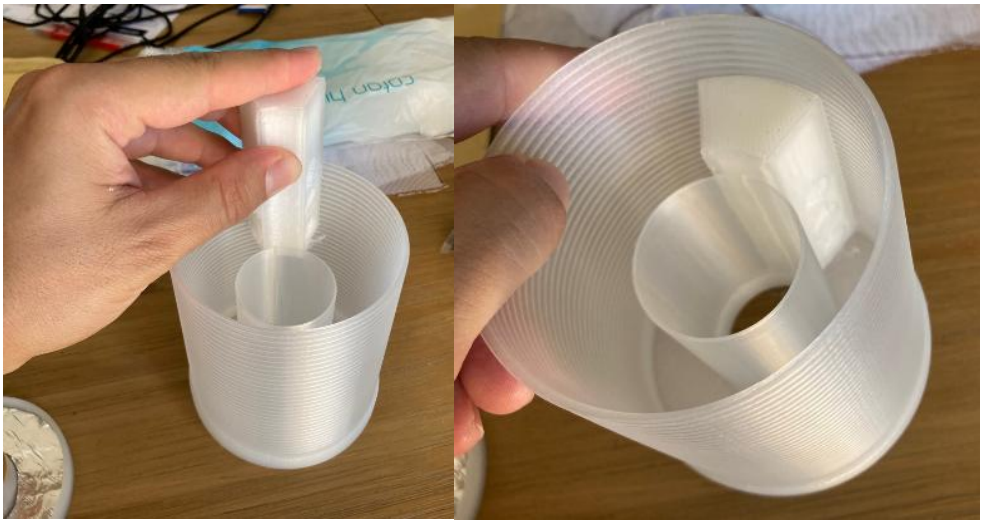
f. The net can be installed on the adapter (unscrew the upper end cap, place it at the base of the net (small hole) and screw the assembly back on.

1.1.3 Food preparation (10% sugar water)

- Start by unscrewing the base to gain access to the interior, and remove the feeder by pulling it downwards.

- Fill the feeder with absorbent cotton soaked in 10% sugar water (10g per 100 mL). This operation can be carried out the day before, in which case keep the feeders at 4°C.

- Slide the feeder back into the adapter, opening upwards, along the wall.



1.1.4 Preparation of aluminum foil dung holders

- Cut the aluminum foil using the doughnut cutting template. You can cut several at a time.
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We're going to create cookie cutters to make this task easier.

- Place a sheet, matt side up, on the bottom of the adapter. Screw the base back onto the cylinder to close the adapter.

1.2 Installing the MX Adapted Trap :

- Place the trap away from wind and rain (under a roof, under a table, etc.). *MX hates rain: it washes away the genetic material deposited on the aluminum foil.*
- Assemble the BG Pro trap according to the instructions supplied.
- Install the MX adapter inside the trap

The adapter can be attached to the trap mouth with the net as shown in the photo. Alternatively, you can first place the adapter inside the trap, open the retaining net and let it spill out around the trap entrance, then attach the trap mouth over it. In short, as long as the mosquitoes get through and the fan is running, you're on your own. ☺

- Install BG lure (Biogents) or (optional) carbon dioxide bottle to attract mosquitoes.
- Check that the trap is working properly and is supplied with power (battery or mains). *Check that the flap opens.*

2. Excrement and Mosquito Collection :

2.1 Collection procedure :

- Capture adult mosquitoes over a period of 3 to 7 consecutive days.

- When collecting, carefully remove the MX adapter from the BG Pro trap while the fan is still running, to prevent escape.
- Carry live mosquitoes in the adapter by folding the net inside the adapter tube to prevent escape.
- Mosquitoes can be kept like this at room temperature for several days.
- Once in the laboratory, place the adapters at -20°C for at least 30 min to kill the captured insects.
- Transfer neutralized mosquitoes to a 50 mL tube. Place at -20°C.
- Remove the excrement-covered aluminum foil and place it in a sealed plastic bag (or between two sheets of paper, which is more environmentally friendly).

In some cases, it may be necessary to remove clumps of insects or snail droppings, dead leaves, etc. with tweezers to loosen the aluminum foil. These photos show what mosquito droppings look like.

- Clearly identify each sample with date and place of collection. *It's important to be able to go back over collections once virus detection has been carried out on excreta.*

3. Sample transport :

- Send the aluminum foil containing the excrement by post to the analysis laboratory, at room temperature. Viral RNA can be stored for several days at room temperature, even in summer.

4. Sample processing in the laboratory :

4.1 Reception and registration :

- On receipt of samples, record each sample in a database with collection details (date, location, etc.).

4.2 Preparing dung-covered aluminum foil :

- Use gloves and personal protective equipment when handling samples.

- Roll the foil into a thick cigarette. Making a coarse pellet works just as well. Slip into a 14mL tube with cap.

4.3 Viral RNA extraction from excreta :

- Add 800 uL of RAV1 lysis buffer (NucleoSpin 96 virus core kit, Macherey-Nagel) and 10 µL of phage MS2 (internal extraction control) to each tube

  Another lysis buffer or PBS may be used at this stage

- Vortex vigorously for 1 min to dissolve excrement in liquid buffer.
- Add 800 uL of 96-100% ethanol, then load onto multi-step NucleoSpin Virus columns to extract RNA/DNA according to the manufacturer's instructions.
- *[OPTIONAL] It is possible to elute in a larger volume, change the lysis buffer or extraction kit, or elute in PBS. Everything works and has already been tested. Adapt to your local protocol. The aim is to elute solid excreta into liquid for extraction. As the doses of viral RNA are high, the volume in which the sample is extracted will not have a major impact on detection sensitivity.*
- Store eluas at 4°C until detection, or at -20°C for long-term storage.

5. Virus detection by RT-PCR :

5.1 RT-PCR preparation :

We use a variety of extraction and detection methods. All our molecular detection methods use Q-RT-PCR with a system of primers and probes from EVAg , the European Virus Archive. Here you'll find all our detection systems, plus a wide choice of internal controls and protocols.

Since 2008, the European Virus Archive (EVA) has been an infrastructure dedicated to the characterization, production and distribution of reference viral resources to support virology research in Europe. It brings together EU and non-EU laboratories specializing in human, animal and plant virology, in line with the "One Virology" concept. Similar to the "One Health" concept, which links human, animal and environmental health, "One Virology" emphasizes the central role of viruses in these systems, making virology a key discipline.

EVA provides constant access to viral strains, diagnostic tools and research materials. This availability enhances epidemic preparedness, stimulates innovation in vaccines, antivirals and diagnostics, and facilitates knowledge transfer. Its action supports public health, global security and strengthens the EU's sovereignty in the face of pandemic threats.

EVAg portal list EVAg |

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