Standard operating procedures for *Aedes*-borne arbovirus surveillance in mosquitoes

Protocol Handbook 2024

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1. BACKGROUND

Mosquito-borne diseases, particularly dengue, chikungunya and zika transmitted by *Aedes* mosquitoes are rising worldwide and have been a critical public health issue. In urban ecosystems, *Aedes*-borne disease risk is associated with contemporary urbanization practices where city developing structures functions as a catalyst for creating breeding habitats for two epidemiologically important mosquito species - *Aedes aegypti* [= *Stegomyia aegypti*] and *Aedes albopictus* [=*Stegomyia albopicta*][1]. Currently, the mosquito control strategy mainly focuses on removing larval habitats, spraying anti-larval agents. However, entomological surveillance is passive often biased towards areas or houses with disease outbreaks. Identifying and typing dengue viruses (DENV) in both *Aedes* mosquitoes and in clinical specimens is important for epidemiological and clinical management of cases. Monitoring arboviruses in immature and adult mosquitoes is an important strategy for identifying the beginning of the epidemic period and initiating control actions, especially in difficult-to-access areas. Transovarial transmission (TOT) (vertical transmission) of dengue virus in *Aedes* species is considered an important mechanism for maintaining the virus during unfavourable conditions [2,3] and even at low human densities [4,5].

This protocol focuses on *Aedes*-borne arbovirus surveillance using adult mosquitoes or larvae collected in various field settings (Fig.1). Data collected in a vector surveillance study helps us to understand and predict the spread of Dengue, chikungunya and zika viruses in an area. This information can aid researchers and virus/vector control departments to develop efficient disease control strategies.

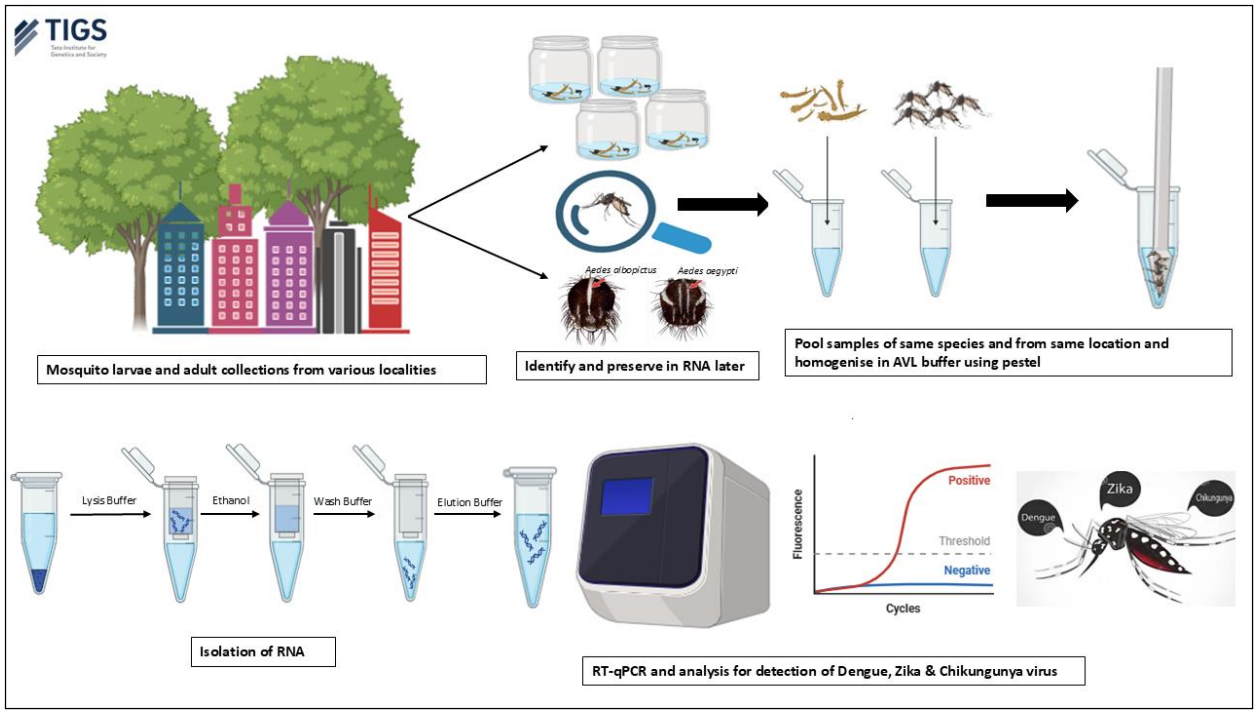

Fig. 1 Schematic representation of mosquito sampling and arbovirus surveillance.

2. SAMPLE COLLECTION

Larval sampling

Aedes species are often associated with a specific type of breeding site, from temporary, ephemeral natural habitats such as water-filled leaf axils, coconut shells, tree holes to manmade artificial habitats such as ground pools, water storing containers, pots, and tyres etc. [6] (Fig. 2).

Cities or semi urban environments provide a diverse gradient of larval habitats such as construction sites [7], leaking connections [8] among other aquatic habitats created by anthropogenic land use modifications (e.g., bromeliads, Colocasia plants, buckets, plastic containers etc.) which are positively associated with the abundance of *Aedes* species.

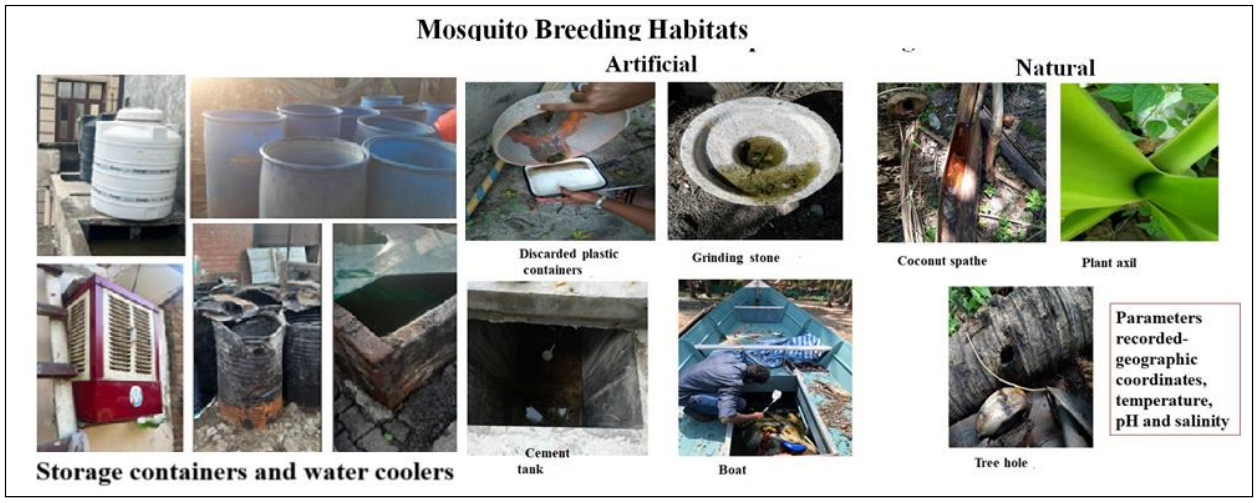

Fig. 2. Examples of mosquito breeding habitats.

Each potential larval habitat should be visually inspected for the presence of mosquito larvae. A microhabitat is determined as 'positive' if a larval presence recorded. For each sampling location, geo-coordinates, microclimate variables such as pH, temperature using HM Digital pH meter and salinity using salinity refractometer can be recorded. Water volume of each habitat can be measured using meter stick (>10L) or by transferring it a graduated beaker. Understanding the linkages between environmental conditions (e.g., hydrology, microclimate), land use, climate change, increasing urbanization are some of the key factors modulating the mosquito life-history traits which influence epidemiologically relevant behaviors and their ability to transmit diseases.

Breeding preference ratio (BPR) for *Aedes* mosquitoes across all breeding sources can be calculated to assess the preference for available breeding habitats. BPR is the ratio of number of containers infested with *Aedes* larvae to the number of water-holding containers examined.

The mosquito larvae samples should be collected in sealed containers with holes in the lids (Fig.1). It is difficult to identify mosquito species in the larval stages (Fig. 3). Therefore, it is recommended to rear the mosquito in an insectary. The larvae reared should be separated by collection source where they are placed in 50-100ml of collection water and provided with fish food. Larvae and pupae are reared to adulthood at 28±2°c and 75± 5% relative humidity and 12:12 hour light-dark photocycle. Once emerged, mosquitoes are frozen at -20°c, sorted by gender and identified to species following Barraud *et al*. [9].

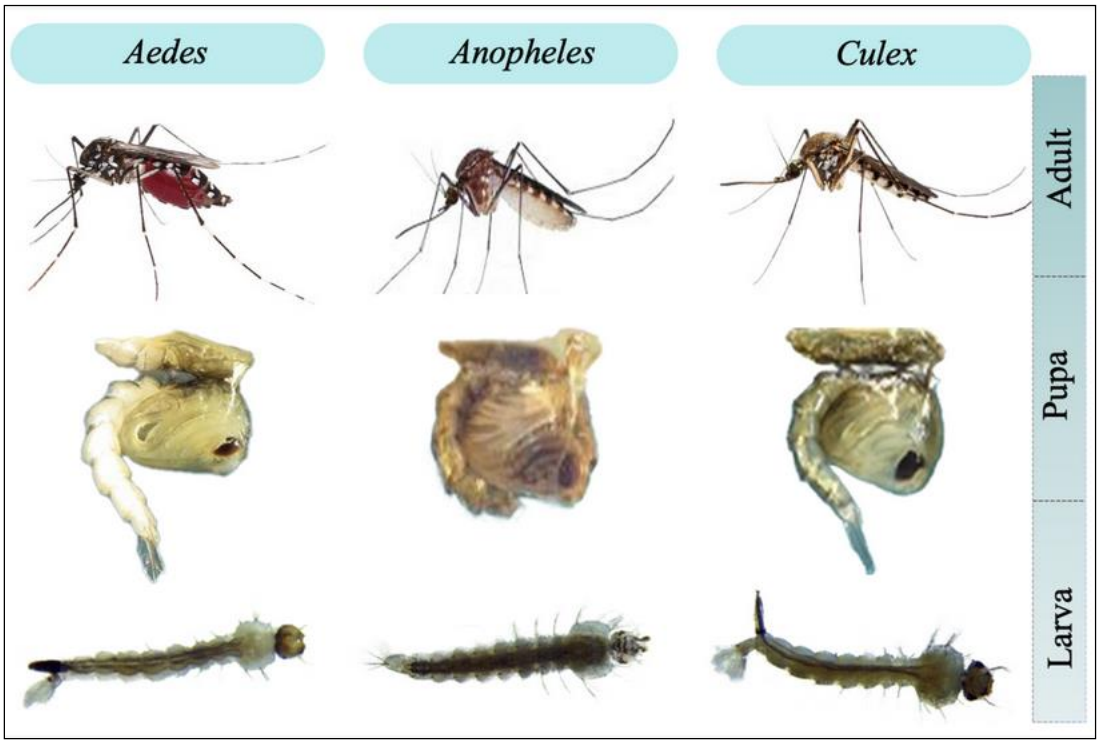

Fig. 3. *Aedes*, *Culex* and *Anopheles* mosquitoes at different stages of their life-cycle.

Adult mosquito sampling

For adult mosquito collection, light traps and the resting collection [10] methods can be used. Because *Aedes albopictus* and *Aedes aegypti* are day-biting mosquitoes, Biogents (BG) sentinel trap using battery operated fan can be deployed for 72 hours for one sampling period. Mosquito traps are baited with a BG-Lure cartridge (Biogents) and CO_2 lure inside the trap. Traps are placed under the cover outside a house premises to increase catch rates. The battery must be changed in the morning for each trap day and catch bags are collected and replaced with a new catch bag to reduce destruction of samples. The traps can be placed at 200 m distance depending on the locality and population density.

For resting collection, mosquitoes are collected using aspirators in randomly selected houses for 15 minutes during day. Mosquito must be sorted by sexes and identified using standard morphological keys and stored in RNA later until further analyses.

TRIZOL Method (Viral inactivation)

Once the *Aedes* breeding site is found, collect the immature stages of mosquito samples, i.e., larval and pupal stages using the plastic sieve from the containers and transfer the samples in collection tubes. Collection tubes are pre-filled with 300-500µL of Trizol reagent [11,12] and should be kept in the ice box or mini-coolers in the field and must be stored at -80°C until further analyses.

Add no more than 10 larvae/pupae samples in a single tube and not more than 5 adults in a single tube containing Trizol.

3. SAMPLE PREPARATION AND RNA EXTRACTION

Pools of mosquito larvae or adult specimens were homogenized keeping on ice using a disposable pestle in a 1.5ml microcentrifuge tube with 140µl of AVL buffer from the QIAamp Viral RNA Mini Kit. Total RNA was extracted from supernatant using QIAamp Viral RNA Mini Kit (QIAGEN) following the manufacturer's recommendations. Finally, extracted RNA was eluted with 60µl of elution buffer and preserved at -80˚C. RNA extraction.

3.1 Protocol: Purification of Viral RNA (Spin Protocol)

Requirements:

- Buffer AVL containing carrier RNA and MS2 phage.
- Ethanol (96–100%)
- Buffer AW1
- Buffer AW2
- Buffer AVE
- 1.5 mL and 2 mL microcentrifuge tubes
- Microcentrifuge

Notes:

- Equilibrate samples and Buffer AVE to room temperature.
- Prepare Buffer AW1 and Buffer AW2 according to instructions.
- Reconstitute carrier RNA in Buffer AVE.
- Read "Important Notes" before starting.

Procedure:

A. Label each tube with the unique ID corresponding to the field ID to maintain sample traceability.
B. Process the sample for RNA extraction following the Qiagen Viral RNA mini kit protocol, with the following steps:
    - Pipet 560 µL of Buffer AVL containing carrier RNA and MS2 phage into a 1.5 mL microcentrifuge tube.
    - Adjust Buffer AVL volume accordingly for larger sample volumes.
    - Add 140 µL of the resuspended sample to the Buffer AVL–carrier RNA in the tube. Pulse-vortex for 15 s.
    - Incubate at room temperature for 10 min.
    - Briefly centrifuge to remove drops from the lid.
    - Add 560 µL of ethanol to the sample, mix by pulse-vortexing for 15 s, and centrifuge briefly.
    - Apply 630 µL of the solution to the QIAamp Mini column without wetting the rim.
    - Centrifuge at 8000 rpm for 1 min. Discard the filtrate tube.
    - Repeat the previous step if necessary for larger sample volumes.
    - Add 500 µL Buffer AW1, centrifuge at 8000 rpm for 1 min, and discard the filtrate tube.
    - Add 500 µL Buffer AW2, centrifuge at full speed (14,000 rpm) for 3 min.
    - Optional: Repeat step 10 to eliminate possible Buffer AW2 carryover.
    - Recommended: Centrifuge the column at full speed (14,000 rpm) for 1 min with a new collection tube.
    - Place the column in a clean 1.5 mL microcentrifuge tube. Add 60 µL Buffer AVE, incubate at room temperature for 1 min, and centrifuge at 8000 rpm for 1 min.
    - Elute RNA in 60 µl elution buffer provided in the Qiagen Viral RNA mini kit.
C. Utilize 30 µl of the eluted RNA for RT-PCR/sequencing analysis.
D. Store the remaining 30 µl of eluted RNA at recommended storage conditions for future use.

Note: Ensure adherence to all safety protocols and manufacturer's instructions during RNA extraction.

For detailed protocols, refer to Qiagen Viral RNA mini kit protocol.

3.2 RNA isolation using TRIZOL method

- Make up the final volume of trizol in sample tubes to 500 µL or 1000 µL.
- Incubate the tubes for 10 minutes at room temperature.
- Centrifuge the tubes at 5000g for 10 minutes at 4°C to remove debris and transfer the supernatant to fresh tubes.
- Add 200µL of chloroform per 1mL of trizol, to each tube and vortex the tubes vigorously for 15 seconds. Incubate the tubes at room temperature for 5 minutes.
- Centrifuge the tubes at 12000g for 15 minutes at 4°C.
- Collect the aqueous phase that appears on the top carefully, and transfer it to a fresh tube.
- Add 500µL of isopropanol per 1mL of trizol, to each tube containing aqueous phase collected in the last step and incubate at room temperature for 15 minutes or at -20°C overnight.
- Centrifuge the tubes at 12000g for 15 minutes.
- Remove the supernatant and wash the pellet twice with 500 µL of 75% ethanol.
- Remove the ethanol completely and air-dry the pellet for 10 minutes.
- Add 10-50 µL of nuclease free water to dissolve the pellet.
- Assess the quality and quantity of the RNA on micro-spectrophotometer.

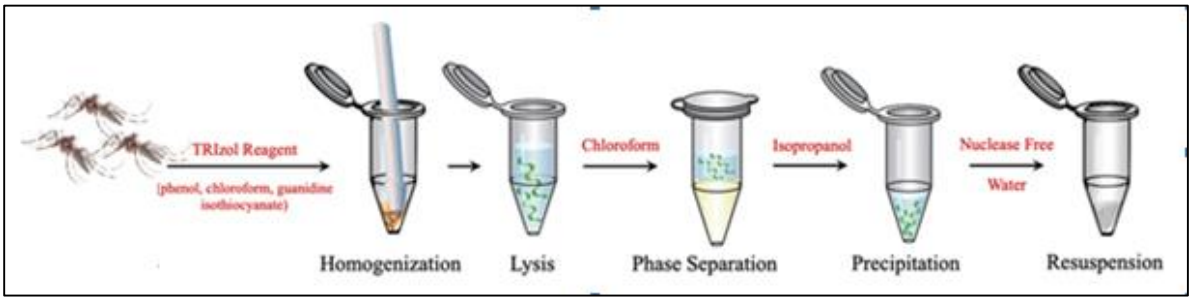

Fig. 4. Schematic representation of RNA isolation protocol [16].

4. RT-qPCR FOR VIRAL AMPLIFICATION

4.1 Multiplex Zika(Z), Chikungunya(C) and Dengue-PAN(D) - ZCD RT-qPCR assay

Materials required

1. 1.5 ml/ 2ml micro centrifuge (MC) tubes
2. 0.2 ml PCR grade tubes with ultra-clear attached cap/ 0.2 ml PCR grade 96 well plates with ultra-clear sealer
3. Well-calibrated micropipettes
4. Sterile filter tips
5. Vortexer
6. Micro centrifuge
7. Mini spin (0.2 ml tube adopter)/ Centrifuge (0.2 ml 96 well plate adopter)
8. qPCR machine (QuantStudio™ 5 Real-Time PCR System)
9. Nuclease free water (Ambion, Catalog no: AM9937, Invitrogen)
10. Luna Probe One-Step RT-qPCR 4X Mix with UDG (NEB, Catalog no: M3019L)
11. Dengue, Chikungunya and Zika primers (Sigma-Aldrich)
12. Dengue probes (Thermo), Chikungunya and Zika probes (Sigma-Aldrich)
13. Extracted RNA samples

Primers and Probe details

Pools of larvae by location and species will be screened by qualitative real-time reverse transcriptase PCR (RT-qPCR) for arboviruses (Chikungunya and Zika) following Waggoner *et al.* [13] and (Dengue –PAN) following Gurukumar *et al.* [14].

Target	Primer and Probe	Sequences
Dengue (PAN)	Forward Primer	5’-GARAGACCAGAGATCCTGCTGTCT-3'
	Reverse Primer	5’-ACCATTCCATTTTCTGGCGTT-3'
	Probe	5’FAM-AGCATCATTCCAGGCAC-NFQMGB-3'
Chikungunya	Forward Primer	5’-CATCTGCACYCAAGTGTACCA-3'
	Reverse Primer	5’-GCGCATTTTGCCTTCGTAATG-3'
	Probe	5’TAMRA-GCGGTGTACACTGCCTGTGACYGC-BHQ2-3'

Zika	Forward Primer	5’-CAGCTGGCATCATGAAGAAYC-3'
	Reverse Primer1	5’-CACTTGTCCCATCTTCTTCTCC-3'
	Reverse Primer2	5’-CACCTGTCCCATCTTTTTCTCC-3'
	Probe	5’HEX-CYGTTGTGGATGGAATAGTGG-BHQ1-3'

Assay Procedure

Procedure Notes

- Wipe the working bench with 70% Ethanol before starting the experiment.
- Wear gloves throughout the entire procedure.
- In case of contact between gloves and sample, change gloves or sterilize with 70 % ethanol immediately to avoid contamination**.**

Reaction Setup procedure

- Thaw Luna Probe One-Step RT-qPCR 4X Mix with UDG (M3019) at room temperature, then place on cold rack at 4°C or ice.
- After thawing completely, briefly mix all components by inversion, pipetting or gentle vortexing.
- Determine the total volume for the appropriate number of reactions, plus 10% overage and prepare assay mix of all components except RNA template, according to the table below:

Component	**Volume per 15 µL reaction**	**Final Concentration**
Luna® Probe One-Step RT-qPCR 4X Mix with UDG (M3019)	3.75 µL	1X
Zika/ Chikungunya/ Dengue-PAN - FP	0.6+0.6+0.6 µL	0.4 µM
Zika (RP1, RP2) /Chikungunya/ Dengue-PAN - RP	0.6+0.6+0.6+0.6 µL	0.4 µM

Zika/ Chikungunya/ Dengue-PAN -Probe	0.3+0.3+0.3 µL	0.2 µM
Template RNA	5 µL	< 1 µg (total RNA)
Nuclease-free Water	1.15 µL	

- Mix thoroughly but gently by pipetting or vertexing. Collect liquid to the bottom of the tube by brief centrifugation
- Aliquot assay mix into qPCR tubes or plate. For best results, ensure accurate and consistent pipetting volumes and minimize bubbles.
- Add RNA template to qPCR tubes or plate along with no template and positive control target. Seal tubes with flat, optically transparent caps or seal plates with optically transparent film. Care should be taken to properly seal plate edges and corners to prevent artifacts caused by evaporation.
- Spin tubes or plates briefly to remove bubbles and collect liquid (1 minute at 1600 rpm).

Cycle conditions

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Carryover Prevention	**25°C**	**30 seconds**	**1**
Reverse Transcription	**55°C**	**10 minutes**	**1**
Initial Denaturation	**95°C**	**1 minute**	**1**
Denaturation	**95°C**	**10seconds**	**45**
Annealing, detection & Extension	**54°C**	**10seconds**	

***Note:** The above conditions and annealing temperature are optimized for mentioned master mix, positive standards and mosquito RNA samples.

Qualitative positive control for Chikungunya and Dengue-PAN – Clinical positive RNA samples were amplified with the above corresponding primers and amplicons were diluted and used.

Qualitative positive control for Zika – Single stranded synthetic DNA oligos of Zika target amplicon sequence to the corresponding primers were diluted and used.

4.2 Multiplex Dengue Serotype specific RT-qPCR assay

MATERIALS REQUIRED

- 1.5 ml/ 2ml micro centrifuge (MC) tubes
- 0.2 ml PCR grade tubes with ultra-clear attached cap/ 0.2 ml PCR grade 96 well plates with ultra-clear sealer
- Well-calibrated micropipettes
- Sterile filter tips
- Vortexer
- Micro centrifuge
- Mini spin (0.2 ml tube adopter)/ Centrifuge (0.2 ml 96 well plate adopter)
- qPCR machine (QuantStudio™ 5 Real-Time PCR System)
- Nuclease free water (Ambion, Catalog no: AM9937, Invitrogen)
- Luna Probe One-Step RT-qPCR 4X Mix with UDG-No Rox (NEB, Catalog no: M3029)
- Dengue serotype specific primers and probes (Sigma-Aldrich)
- Extracted RNA samples

Primers and Probe details

Positive pools of larvae by location and species for Dengue-PAN will be screened further for Dengue serotype specific by qualitative real-time reverse transcriptase PCR (RT-qPCR) assay following primers and probe details from Johnson *et al.* [15].

Target	Primer and Probe	Sequences
Dengue Serotype 1	Forward Primer	5’-CAAAAGGAAGTCGTGCAATA-3'
	Reverse Primer	5’-CTGAGTGAATTCTCTCTACTGAACC-3'
	Probe	5’-FAM-CATGTGGTTGGGAGCACGC-BHQ1-3'
Dengue Serotype 2	Forward Primer	5’-CAGGTTATGGCACTGTCACGAT-3'
	Reverse Primer	5’-CCATCTGCAGCAACACCATCTC-3'

	Probe	5’-HEX-CTCTCCGAGAACAGGCCTCGACTTCAA-BHQ1-3'
Dengue Serotype 3	Forward Primer	5’-GGACTGGACACACGCACTCA-3'
	Reverse Primer	5’-CATGTCTCTACCTTCTCGACTTGTCT-3'
	Probe	5’-ROX-ACCTGGATGTCGGCTGAAGGAGCTTG-BHQ2-3'
Dengue Serotype 4	Forward Primer	5’-TTGTCCTAATGATGCTGGTCG-3'
	Reverse Primer	5’-TCCACCTGAGACTCCTTCCA-3'
	Probe	5’-Cy5-TTCCTACTCCTACGCATCGCATTCCG-BHQ3-3'

Assay Procedure

Procedure Notes

- Wipe the working bench with 70% Ethanol before starting the experiment.
- Wear gloves throughout the entire procedure.
- In case of contact between gloves and sample, change gloves or sterilize with 70 % ethanol immediately to avoid contamination**.**

Reaction Setup procedure

- Thaw Luna Probe One-Step RT-qPCR 4X Mix with UDG –No ROX (M3029) at room temperature, then place on cold rack at 4°C or ice.
- After thawing completely, briefly mix all components by inversion, pipetting or gentle vortexing.
- Determine the total volume for the appropriate number of reactions, plus 10% overage and prepare assay mix of all components except RNA template, according to the table below

Component	**Volume per 20 µL reaction**	**Final Concentration**
Luna® Probe One-Step RT-qPCR 4X Mix with UDG no ROX (M3029)	5 µL	1X
Dengue serotype (1+2+3+ 4) - FP	0.8+0.8+0.8+0.8 µL	0.4 µM
Dengue serotype (1+2+3+ 4) - RP	0.8+0.8+0.8+0.8 µL	0.4 µM
Dengue serotype ((1+2+3+ 4) -Probe	0.4+0.4+0.4+0.4 µL	0.2 µM
Template RNA	5 µL	< 1 µg (total RNA)
Nuclease-free Water	2 µL	

- Mix thoroughly but gently by pipetting or vertexing. Collect liquid to the bottom of the tube by brief centrifugation
- Aliquot assay mix into qPCR tubes or plate. For best results, ensure accurate and consistent pipetting volumes and minimize bubbles.

- Add RNA template to qPCR tubes or plate along with no template and positive control target. Seal tubes with flat, optically transparent caps or seal plates with optically transparent film. Care should be taken to properly seal plate edges and corners to prevent artifacts caused by evaporation.
- Spin tubes or plates briefly to remove bubbles and collect liquid (1 minute at 1600 rpm).

Cycle conditions

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Carryover Prevention	**25°C**	**30 seconds**	**1**
Reverse Transcription	**55°C**	**10 minutes**	**1**
Initial Denaturation	**95°C**	**1 minute**	**1**
Denaturation	**95°C**	**10seconds**	**45**
Annealing, detection & Extension	**56°C**	**10seconds**	

***Note:** The above conditions and annealing temperature are optimized for mentioned master mix, positive standards and mosquito RNA samples.

4.3 Equipment:

- gloves, lab coat
- sieve
- Mosquito adult or larvae samples
- Pestle
- Pipettes (2-20µL, 20-200µL, 100-1000µL)
- Vortex
- 1.5 ml microcentrifuge tube
- 50 ml centrifuge rack
- 50 ml centrifuge tubes
- Stopwatch/Timer
- Disposable Filter Tips (2-20µL, 20-200µL, 100-1000µL)
- 200µL PCR 8 or 12 tube strips or as PCR Plate
- Cryo Box
- Cryo labels
- Reagent reservoir
- Biohazard bag
- Fridge (+4C)
- Freezer (-20C to -80C)
- Nitrile gloves
- RT-qPCR machine (We used QuantStudio 5 version)

- Hot Bead bath
- Parafilm
- Centrifuge (50ml, 2ml & PCR plate bucket)
- Magnetic stand (200µL & 1.5ml)

4.4 Reagents

- Qiagen Viral RNA mini kit (250 Reaction kit) (Qiagen, Catalog No: 52906)
- AVL Lysis buffer (Qiagen) without carrier RNA, 155 mL (Qiagen, Catalog No: 19089) Trizol (Invitrogen 15596026) Chloroform (Merck C2432) Isopropanol (Merck 19516) Ethanol (Merck 107017 or CS)

PRECAUTIONS AND POINTS TO REMEMBER

- **Seek consent from the local authorities or residents prior to mosquito collection visits.**
- Cover your skin completely to avoid mosquito bites while visiting mosquito collection sites.
- Keep ice box or mini-cooler ready reagents before field collections.
- Label the tubes properly at the time of sample collection.
- Wear clean lab coat and gloves while performing RNA isolation.
- Use chemical hood for handling/ aliquoting the chemicals.
- Change gloves frequently and discard in autoclavable bags.
- Discard used pipette tips in 10% bleach/hypochlorite solution.
- Used tubes containing samples should be discarded in autoclavable bags.
- Avoid spillage of samples at the lab. In case of spillage, area should be properly cleaned with 10% bleach and ethanol.
- In each larval breeding site, the following information will be recorded: coordinates of the breeding site (using GPS), date and time of larval collection, weather conditions, type of breeding site.

Checklist for field collection trip

- Clothing that covers the arms, legs, and neck completely to avoid mosquito bites.
- Gloves.
- Trizol/RNA later filled tubes.
- Mini-cooler or Ice box.
- Micro pestles for homogenization.
- Pair of sieves and 50mL tubes for specimen collection.
- GPS enabled phone for recording the location coordinates.
- Markers or premade stickers for labeling the tubes.

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