UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

SUBJECT: Developer guidance for arbovirus testing of modified *Aedes* spp. mosquitoes used in autocidal mosquito programs for insecticidal control of *Aedes* spp. populations.

FROM: Wiebke T. Striegel, Ph.D., Senior Scientist
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division (7511P)

WIEBKE STRIEGEL Digitally signed by WIEBKE STRIEGEL Date: 2021.07.29 11:56:27 -04'00'

TO: Matthew Weiner, Risk Manager
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division (7511P)

THRU: Chris A. Wozniak, Ph.D., Biotechnology Specialist **Signed for**
Biopesticides and Pollution Prevention Division (7511P)

WIEBKE STRIEGEL Digitally signed by WIEBKE STRIEGEL Date: 2021.07.29 11:57:22 -04'00'

Cassandra Kirk, Ph.D., Biologist
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division (7511P)

Michael Mendelsohn, Branch Chief **Signed for**
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division (7511P)

WIEBKE STRIEGEL Digitally signed by WIEBKE STRIEGEL Date: 2021.07.29 11:57:58 -04'00'

Aaron C. Brault, Ph.D., Research Microbiologist
Lead, Diagnostics and Reference Team
Division of Vector-Borne Diseases
Centers for Disease Control and Prevention

C. Roxanne Connelly, Ph.D., Chief Entomologist
Lead, Entomology and Ecology Team
Division of Vector-Borne Diseases
Centers for Disease Control and Prevention

C. Ben Beard, Ph.D.,
Deputy Director Division of Vector-Borne Diseases
Centers for Disease Control and Prevention

I. PURPOSE

Because mosquitoes are known disease vectors, the human health risk assessment for mass-reared modified *Aedes* spp. mosquitoes proposed for use in autocidal mosquito programs considers the potential for these mosquitoes to pose human exposure to arboviral (arthropod-borne viruses) diseases. This memorandum evaluates the likelihood for this exposure to occur and provides guidance to developers on the type of information that will help the Agency determine whether arbovirus testing of a mosquito colony should be included in the manufacturing process. In those instances where testing is indicated, the memorandum furthermore provides guidance on determining the viral target, types of diagnostic tests, and sample selection.

II. BACKGROUND

Autocidal mosquito programs (AMPs) are an area-wide, species-specific approach to mosquito control. These programs require mass production of live modified mosquitoes for environmental releases. While it is necessary to produce both modified females and modified males in the insectary, most AMPs aim to only release males as these do not pose a biting hazard to humans and other non-target organisms. Environmental releases are conducted over several months throughout the mosquito season. During this time, modified males outcompete the wild male population in terms of mating frequency with wild females of the same species. The modification introduced into the released males affects the survival of their offspring. In the AMP products that EPA has evaluated to date, mated wild females either produce no viable offspring at all or selectively produce only male offspring (Table 1). In both cases, the resulting reduction of female emergence in the treatment area ultimately leads to the decline of the mosquito population. Four AMP products have been approved by EPA in recent years, all of which target mosquitoes of the genus *Aedes*.

Table 1. Approved modified mosquito products for *Aedes* spp. control

Product	Species	Approach	EPA File Symbol	Type
ZAP Males	*Ae. albopictus*	*W. pipientis*, strain *w*Pip; cytoplasmic incompatibility; no viable offspring	89668-4	Registration
ZAP Males	*Ae. albopictus*	*W. pipientis*, strain *w*Pip; cytoplasmic incompatibility; no viable offspring	89668-EUP-1 89668-EUP-3	Experimental Use Permit
WB1 Males	*Ae. aegypti*	*W. pipientis*, strain *w*AlbB; cytoplasmic incompatibility; no viable offspring	89668-EUP-3; formerly 88877-EUP-2	Experimental Use Permit
OX5034	*Ae. aegypti*	Genetically engineered; lethal gene tTAV-OX5034; male offspring survive	93167-EUP-2	Experimental Use Permit
W. pipientis	*Ae. polynesiensis*	*W. pipientis*; cytoplasmic incompatibility; no viable offspring	88877-EUP-1	Experimental Use Permit

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III. CONCLUSION

Human exposure to arboviruses from released modified *Aedes* mosquitoes used in autocidal mosquito programs is conceivable for arboviruses for which the modified mosquito species is a competent vector. However, the likelihood for this exposure to occur is, generally, very low due to a combination of the controlled mass-rearing conditions and the low frequency of arboviral transmission among mosquitoes. This conclusion is supported even in those instances where the dietary animal blood provided to female mosquitoes is sourced from regions where *Aedes* spp. and *Aedes*-borne diseases are endemic, wild-caught *Aedes* spp. are used for outcrossing, and a small number of females are present in the release batches. To further reduce the likelihood of arboviral exposure to negligible levels, testing for the presence of arboviruses for which the modified mosquito species is a competent vector in both outcrossed wild-caught mosquitoes and the dietary vertebrate blood provided to females is recommended under certain conditions.

IV. ASSESSMENT OF LIKELIHOOD FOR HUMAN EXPOSURE

1. Likelihood of arbovirus introduction into the colony

Mosquitoes are primary vectors of many arboviruses that cause human diseases. Amongst those mosquitoes are several species within the genus *Aedes*, which are competent vectors of arboviruses that cause diseases such as dengue, chikungunya, and yellow fever (Robert et al., 2020). Female mosquitoes are hematophagous and require vertebrate blood to support egg production (Valzania *et al.*, 2019). In the process of blood-feeding they can acquire and subsequently transmit viral particles to humans. Arboviruses are maintained in nature primarily through transmission between the female mosquito and one or several vertebrate reservoirs (WHO, 1985). In contrast, mass-rearing conditions are generally not conducive to the acquisition and maintenance of arboviruses because of the inefficient viral transmission between mosquitoes and because the rearing conditions provide limited opportunity for pathogen exposure if developers of AMP products follow standard arthropod containment guidelines.

There are several biological and non-biological factors ensuring the low likelihood of arbovirus exposure from modified mosquitoes used in AMPs. Mass-rearing facilities commonly follow arthropod containment guidelines, which set standards for the placement of physical barriers to the outside environment and provide guidance for personnel working in these facilities (Scott, 2005; American Committee of Medical Entomology, 2019). For example, mosquito colonies are commonly housed in meshed cages, located in rooms with secured entrances; this controlled access can include double-doors and/or a strong downward airflow above the entrance to the insectary. These barriers all but eliminate any interactions with *Aedes* spp. reared in the same insectary as well as wild *Aedes* spp. that may be present in the area where the facility is located. Thus, chance interaction with other *Aedes* spp. is not seen as a viable source of infection for mass-reared mosquitoes. This leaves two conceivable points of entry for arboviruses into the colony: deliberate outcrossing of the existing colony with wild-caught *Aedes* spp. to improve fitness and the dietary blood provided to females in the rearing facility (Figure 1).

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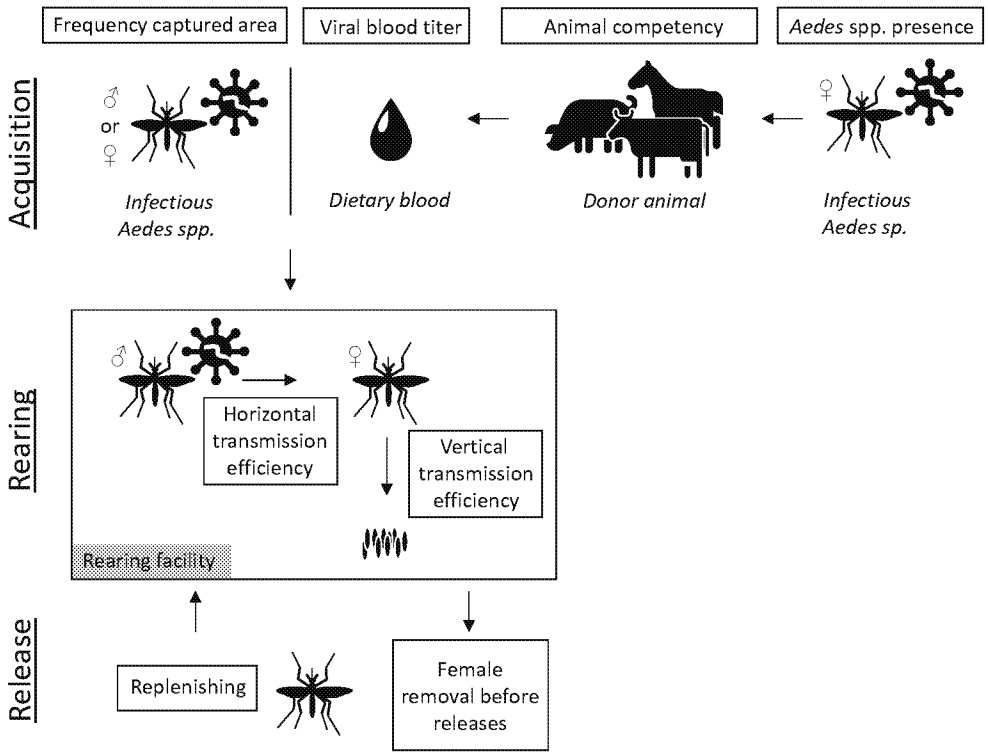

Figure 1. Potential viral movement through *Aedes* spp. colonies and subsequent environmental releases. Top: Viruses could conceivably be introduced into the colony through wild-caught mosquitoes (left) or dietary animal blood (right). If wild males are used for outcrossing, the virus must first be passed from the males to the females horizontally, and subsequently from the infected female to the offspring (depicted here as oval eggs). Similarly, females infected through consumption of the dietary blood must pass the infection vertically to the offspring. The male offspring are then either removed by gender separation for subsequent releases (bottom right) and/or used for replenishing the colony (bottom left). If females are not efficiently separated from males, (or females emerge from the offspring of mating between modified males and wild females) exposure to a virus is possible. Reintroduction of infected offspring into a colony may increase the abundance of infected mosquitoes in that colony. Considerations for each step of viral movement that factor into the likelihood for each step to occur are provided in dashed black boxes along the pathway.

The success of *Aedes* spp. AMPs relies in part on the mating competitiveness of the released males. Therefore, periodic outcrossing of wild-caught mosquitoes into the established mosquito lines may be conducted to counteract inbreeding depression. However, the likelihood of catching infected wild mosquitoes in the environment at any given time, and therefore introduction of that infection into a closed colony, is considered low. This is supported by survey data of wild-caught mosquitoes which suggests that even during an active disease outbreak the frequency of infected mosquitoes in a population is low; ranging from 0.05% to > 10% (Souza-Neto et al., 2019).

The second potential point of entry for arboviruses into the colony is via the dietary vertebrate blood supplied to females to support egg production. However, female arbovirus acquisition is conceivable only if the modified *Aedes* species is a competent vector and the donor animal is a competent host for the same arbovirus (Vector competence is defined as the capacity of a mosquito to acquire the pathogen and support its transmission (Souza-Neto et al., 2019) and host competence is defined as the function of the

magnitude and duration of viremia for a given arbovirus strain (Hamer et al., 2009)). Where and how the donor animals are housed can further reduce the potential for exposure to circulating viruses. For example, animals housed in mosquito-proof areas under veterinary care would be less likely to become infected.

In general, infection of an arbovirus vector occurs when viremia is induced in the bloodmeal host. Only when the viral titer in the host animal's blood is high enough can female acquisition and subsequent passage of the virus to the offspring occur. The lowest titer threshold required for infection through the oral route has been determined for several viruses in wild-caught and laboratory *Aedes* spp. strains (Souza-Neto et al., 2019). For example, the oral threshold for Mayaro virus (MAYV) in a specific strain of *Ae. aegypti* using an artificial blood meal (i.e., sheep blood and PBS at 1:1 ratio) was determined to be between 5.0 and 5.5 $\log_{10}$ PFU/ml, with 0.0 to 24% of mosquitoes becoming infected at doses ranging from 5.1 to 5.6 $\log_{10}$ PFU/ml (Long et al., 2011). Similar results were obtained for several other *Ae. aegypti* strains orally infected with artificial blood meal containing DENV-2, where the median infective dose 50 (MED_{50}) ranged from 5.1 to >6.3 $\log_{10}$ PFU/ml (Gubler et al., 1979). For rearing of mosquitoes in insectaries, common vertebrate blood sources are sheep, goat, chicken, goose, cattle, and horse. Some of these donor animals, such as cattle and chickens, are also used as serological sentinels for a number of mosquito-vectored arboviruses, due to their propensity to be exposed to very high mosquito bite numbers (Shaman et al., 2005; Kim et al., 2014). It is relevant to note that the use as sentinel animals for a specific virus or viruses does not necessarily indicate that they these animals are also a competent host for that virus. In many cases sentinel animals are chosen as they are poor amplification hosts and therefore would not contribute to autochthonous transmission.

2. Likelihood of arbovirus abundance in released mosquitoes

Once an arbovirus has been introduced into the colony of virus competent modified *Aedes* species there is a low likelihood of viral passage from an infected mosquito to their offspring, even though infected adult female *Aedes* spp. were shown to be able to passage arboviruses vertically to their offspring. This process mostly occurs transovarially, but transmission through surface contaminated oocytes has also been hypothesized for some virus/ *Aedes* spp. pairings (Turell et al., 1990; Lumley et al., 2017). However, vertical transmission is inefficient and filial infection rates (FIR; i.e., the percentage of infected offspring from an infected female) are generally very low even under optimal experimental conditions designed to determine the maximum FIR. For example, to determine the FIR for a given virus/ *Aedes* spp. pairing, the viral particles are commonly not administered through the dietary blood, which is how they would be acquired under mass-rearing conditions. Rather, females are inoculated intrathoracically to bypass the midgut escape and replication barriers, allowing for efficient dissemination into secondary tissues, including the ovaries (Stauft et al, 2018; Wu et al., 2018; Danet et al., 2019). Using this approach, FIRs of *Ae. aegypti* have been determined to be in the order of 1:84 - 1:290 for ZIKV (Ciota et al., 2017; Thangamani et al., 2016) or as low as 1:1,300 for DENV-4 (Rosen et al., 1982). These rates are reflective of the FIRs determined for many of the arboviruses in *Aedes* spp. that are relevant to human health (Adams and Boots, 2010; Thangamani et al., 2016; Sanchez-Vargas et al., 2018). Given that the average female *Ae. aegypti* lays up to 500 eggs within her lifetime, the number of infected *Ae. aegypti* offspring, considering the unlikely hatch rate of 100%, would range from 1 - 5 for ZIKV to none for DENV-4 (CDC and University of Florida factsheets on *Ae. aegypti*). The likelihood of filial infection through the introduction of a wild-caught infected male is even lower since the infection of offspring by males would require an additional transmission step (i.e., venereal passage from male to female, followed by vertical passage to the offspring). Venereal transmission of arboviruses from males to females has been observed in *Aedes* spp., however this process is similarly inefficient. For example, under laboratory conditions, venereal transmission from male to female *Ae. aegypti* was reported to be 11% for CHIKV, 31.6% for DENV-2, and 50% for ZIKA (Mavale *et al.*, 2010; Silva Campos *et al.*, 2017; Sánchez-Vargas, *et al.*, 2018).

Taking the considerations outlined in sections 1 and 2 together, the combined likelihood that an infectious modified *Aedes* sp. is released as part of an AMP is negligible for those viruses where the modified *Aedes* species is not a competent vector. For all other viruses, the likelihood is very low.

V. Information requested and testing recommendations

1. Information requested

To determine whether arbovirus testing should be included in the manufacturing process for a given AMP product, every pesticide application should, at a minimum, identify the following:

1) Information on the mosquito containment at the rearing facility, 2) efficiency of gender separation procedures, 3) the arboviruses for which the *Aedes* species used in the AMP is a competent vector, 4) whether any arboviruses for which the *Aedes* species is a competent vector are present in the country where wild mosquitoes are caught and/or the dietary blood is sourced, 5) information on the vertebrate blood donor animal and whether it is a competent host for arboviruses for which the *Aedes* species is a competent vector, 6) source of the dietary vertebrate blood (e.g., vertebrate organism, vendor, information on animal husbandry, blood handling/preparation), and 7) a statement as to whether the developer wishes to conduct outcrossing of the mass-reared strain (note that outcrossing would be assumed for section 3 registrations, but not for Experimental Use Permits). The above information may be presented in the form of a literature-supported scientific rationale and/or empirical data generated by the applicant.

If testing for the presence of arboviruses is indicated (see Units V.2. and V.3. below), the manufacturing process should contain the arbovirus testing protocols, including all applicable quality control and assurance procedures. Similarly, outcrossing protocols with associated quality control and assurance procedures should be provided in the manufacturing process, if applicable.

2. Outcrossing/ wild-caught mosquitoes

a. When testing of mosquitoes is not indicated

If the colony was established from wild-caught mosquitoes and offspring are not used for environmental releases for at least 4 successive generations, arbovirus testing is not indicated. At that point, any infection that may have been present in the parental wild-caught mosquito is expected to be removed from the colony through the mechanisms outlined in Unit IV.2. of this memorandum.

b. Arbovirus testing

- **Choosing a viral target:** Testing should be conducted for arboviruses that meet the following criteria: 1) viruses are known to be vectored by the modified *Aedes* species and 2) that have a historical record of local transmission in the country where the mosquito is caught.
- **Sample:** Testing for viral presence should be conducted on a pooled sample of the field-caught mosquitoes that were used for outcrossing.
- **Diagnostic test:** qRT-PCR or test with appropriate or equivalent sensitivity.

Sensitivity of qRT-PCR is orders of magnitude higher than immuno-based tests and therefore provides an increased likelihood of viral detection. For example, the detection level for West Nile virus (WNV) using a qRT-PCR TaqMan assay is 0.1 PFU/ ml, compared to 100,000 PFU/ml using the immune-based VectorTest (Lanciotti et al., 2000; CDC 2013). Higher sensitivity translates into a lower false-negative

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rate, which is desirable in an assay to determine low viral titers in a small sample size, such as a single mosquito. Several quantitative and semi-quantitative qRT-PCR-based protocols have been developed for the detection of arboviruses (Table 2). The list of arboviruses provided in this table is not exhaustive but provides a starting point from which a developer may utilize as appropriate to their testing needs/requirements. Some of these methods furthermore allow for detection of viral RNA from multiple viruses within the same sample using a multiplex approach. Testing should be conducted using validated tests, such as those that have been published in peer-reviewed journals, that are commercially available, or those that are attached in the Appendix of this memorandum.

Table 2. Examples of qRT-PCR protocols for testing for select arboviruses

Arbovirus	Notes	Reference
ZIKV	Singleplex	Lanciotti et al. 2008 and CDC protocol*
CHIKV	Singleplex	Lanciotti et al. 2007 and CDC protocol*
DENV1-4	Multiplex	Santiago et al. 2013 and CDC protocol*
WEE	Multiplex	Lanciotti and Kerst 2001; Brault et al. 2012; Brault et al. 2015
SLE		
WNV		
WNV	Singleplex	Lanciotti et al. 2000
EEE	Singleplex	Lambert et al. 2003

* *The CDC protocol is appended.*

3. Dietary animal blood

a. When testing of dietary blood is not indicated

Testing of the dietary animal blood is not indicated if species of the genus *Aedes* and the viruses for which they are competent are not endemic in the country where the dietary blood is obtained, **or** there is sufficient evidence to determine that the blood donor is not a competent host for an arbovirus for which the modified *Aedes* species is a competent arbovirus vector, **or** if the blood is treated so that the viral particles are demonstrably inactivated prior to blood feeding **or** if blood is screened by an assay with a limit of detection below the expected oral infection threshold by the supplier of the dietary blood. Any of these considerations would further lower the likelihood of exposure to infectious females in the environment to negligible levels.

b. Arbovirus testing

- **Choosing a viral target:** Testing should be conducted for arboviruses that meet all of the following criteria: 1) the virus is known to be vectored by the modified *Aedes* species, 2) the animal donor is a competent host for that virus (or there is insufficient evidence to determine host competency for that virus), and 3) the virus has a historical record of local transmission in the country where the blood is sourced.
- **Sample:** Testing should be conducted on every lot of the dietary animal blood (serum) before it is offered to the mosquitoes.
- **Diagnostic test:** Tests that reliably detect viral titers required for oral viral acquisition by the mosquito.

The choice of diagnostic test for arboviruses in the dietary animal blood should be based on whether that test is able to reliably detect the low end of the viral titer needed for oral acquisition by female mosquitoes. Again, qRT-PCR is one option as the test provides adequate sensitivity and, if degenerate

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primers are used, the ability to detect multiple viruses, belonging to a family of arboviruses, in a single PCR run (e.g., *Flaviviridae*). We note that qRT-PCR using degenerate primers is not suggested for use on mosquito samples (see testing of wild-caught mosquitoes) as they are more likely to provide false positive results due to the present of insect-specific viruses in the mosquitoes, i.e., not those that are of human health concern. Because of this, it is suggested that the amplicon of every positive result be sequenced to determine the identity of the virus. For bloodmeal screening, other, non-molecular, testing platforms with sufficient sensitivity to detect arboviruses in the range associated with oral infection threshold could be adequate for screening purposes. If proposing to use other tests, such as immuno-based assays in the manufacturing process, supporting information on the limit of detection of that test for use on blood samples in relation to the level of the viral titer needed for oral acquisition by the female mosquitoes should be provided. Examples are information on verified limits of detection of a commercially available test kit or empirical data verifying the sensitivity of the test.

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APPENDIX

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ZIKAV, CHIKV and DENV Real Time RT-PCR Protocols for mosquito pools

Note: We use the kits and protocols described below; however there are several other options for RNA extraction and real-time RT-PCR on the market.

ZIKAV & CHIKV (single-plex assay)

Testing Algorithm: All samples are screened for virus using the first primer/probe set listed below. All positive samples are re-extracted and tested with the second primer/probe set for confirmation. A positive result in any of the negative controls invalidates the entire run. Failure of the positive control to generate a positive result also invalidates the entire run. A sample that is positive with one primer set and negative with the second set is classified as equivocal.

Results Interpretation

We use the following algorithm to evaluate the results.

Positive:	Ct value ≤ 38
Negative:	Ct value > 38

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PCR PLATE SET-UP:

1. Prepare primers and probes according to the following concentrations:
    - Primers: 100 μM in nuclease-free water
    - Probes: 25 μM in TE buffer
2. Real-time RT-PCR Master mix should be prepared in a "clean room" physically separated from all other laboratory activities with dedicated reagents and equipment (i.e. pipettes). Combine the reagents listed below in an RNase free centrifuge tube on ice. Using Qiagen's Quantitect Probe RT-PCR kit (#204443), prepare master mix as follows:

    Per reaction:

    - 25.0 μl master-mix
    - 18.2 μl water* (nuclease-free)
    - 0.5 μl 100μM forward primer
    - 0.5 μl 100μM reverse primer
    - 0.3 μl 25μM probe
    - 0.5 μl RT enzyme
        - Add about 5-10 reactions to your total number of samples (and account for "No template controls" (NTCs), positive controls, and negative extraction controls) and multiply number by volumes above.
        - Ex. You have 20 samples (12 unknown samples, 2 positive controls, 2 negative controls, and 4 NTCs). Make a master mix for 25-30 samples.
        - *Definitions:*
            - NTC = mix ONLY with no sample, to test mix components (*PCR control*)
            - Negative control = extracted water (*extraction control*)
3. Pipette **45 μl of master mix*** into either 0.2 ml optical (specifically for real-time assays; emission fluorescence is read through the cap) PCR tubes or a 96 well optical PCR plate. Use a reservoir and a multichannel pipette for many wells.
4. Pipette **5 μl of RNA*** into each well. Refer to a template to ensure that the proper sample is added to the corresponding well. Do not add anything to NTC samples (master mix only).
    - See RNA extraction notes below

*Note that the volume of RNA added per reaction is typically 5 μl but can be increased (up to 25 μl) with the appropriate adjustment of the water in the master mix. (For example, if you want to test 10 μl RNA, reduce the water per reaction to 13.2 μl, and add 40 μl master mix and 10 μl RNA to each well.)

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Cycling conditions (QIAGEN conditions for Real Time RT-PCR):

1 cycle each:

50° for 30 min

95° for 15 min

45 cycles:

95° for 15 sec

60° for 1 min (data collection step)

Zika primers and probes. There are 2 primer/probe sets available for the detection of Zika virus RNA. Set Zika1087/1108pr/1163c (reference: Lanciotti et. al., *Emerging Infectious Diseases*, Vol. 14, No. 8, August 2008) detects all known genotypes of Zika virus; set Zika4481/4507c-pr/4552c is specific for the Zika virus Asian genotype currently circulating in the Western Hemisphere.

Zika1087	CCGCTGCCCAACACAAG
Zika1163c	CCACTAACGTTCTTTTGCAGACAT
Zika1108pr (FAM)	AGCCTACCTTGACAAGCAGTCAGACACTCAA
Zika4481	CTGTGGCATGAACCCAATAG
Zika4552c	ATCCCATAGAGCACCACTCC
Zika4507c-pr (FAM)	CCACGCTCCAGCTGCAAAGG

Chikungunya primers and probes. One primer/probe set from the EID publication (reference: Lanciotti et. al. Emerging Infectious Diseases, Vol. 13, No. 5, May 2007) has been replaced with a new set that demonstrates greater sensitivity with the Asian genotype CHIK that is currently found in the Caribbean. This new set (3855/3957c/3886 FAM) detects both Asian and East/Central/South/African genotype CHIK viruses. Primer 3957c and Probe 3886 are produced by first reconstituting the individual 3957c (a) and (b) primers to 100µM and the individual 3886-FAM (a) and (b) probes to 25 µM, then mixing (a) and (b) in equal volumes, resulting in the working primer or probe stock used in the assay.

E.g.: 100 µl (3957c-a) + 100 µl (3957c-b) = **200 µl 3957c**, used in the assay at 0.5 µl/reaction;
and 25 µl (3886 pr-a) + 25 µl (3886 pr-b) = **50 µl 3886 pr**, used in the assay at 0.3 µl/reaction.

CHIKV 3855	GAGCATACGGTTACGCAGATAG
CHIKV 3957c	**(a)** TACTGGTGATACATGGTGGTTTC
	(b) TGCTGGTGACACATGGTGGTTTC
CHIKV 3886pr (FAM)	**(a)** ACGAGTAATCTGCGTACTGGGACGTA
	(b) ACGAGTCATCTGCGTATTGGGACGCA

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CHIKV 6856	TCACTCCCTGTTGGACTTGATAGA
CHIKV 6981c	TTGACGAACAGAGTTAGGAACATACC
CHIKV 6919pr (FAM)	AGGTACGCGCTTCAAGTTCGGCG

ZIKAV & CHIKV (duplex assay)

Combine the ZIKA-1108pr set with the CHIKV-3886pr set to screen samples for both viruses in the same reaction. Each probe must be labeled with a different fluorophore (i.e., FAM and HEX) and the real-time machine programmed to detect both fluorophores. Follow the directions above for the single-plex assays, but add both sets of primers and probes to the reaction mix. Re-extract and confirm as described above (in a single-plex assay) any sample that produces a CT value.

DENV 1-4 (multiplex assay)

Reference: Santiago GA, et al. 2013. Analytical and Clinical Performance of the CDC Real Time RT-PCR Assay for Detection and Typing of Dengue Virus. *PLoS Negl Trop Dis* 7(7): e2311.

Testing Algorithm All samples are screened for DENV 1-4 using the primers/probes listed below in a multiplex assay. All positive samples are re-extracted and tested with the individual DENV primer/probe set in a single-plex assay for confirmation. A positive result in any of the negative controls invalidates the entire run. Failure of the positive control to generate a positive result also invalidates the entire run. A sample that is positive in the screening assay and negative in the confirmation assay is classified as equivocal.

Results Interpretation

We use the following algorithm to evaluate the results.

Positive: Ct value ≤ 37

Negative: Ct value >37

PCR PLATE SET-UP:

1. Prepare primers and probes according to the following concentrations:
   - Primers: 50 µM in nuclease-free water
   - Probes: 10 µM in TE buffer
2. Real-time RT-PCR Master mix should be prepared in a "clean room" physically separated from all other laboratory activities with dedicated reagents and equipment (i.e. pipettes). Combine the reagents listed below in an RNase free centrifuge tube on ice. Using Invitrogen's Superscript III Platinum One-step qRTPCR kit (#11732-088), prepare master mix as follows:

   Per reaction:

   - 12.5.0 µl 2x master-mix
   - 2.2 µl water (nuclease-free)
   - 0.5 µl 50 µM **DENV-1** and **DENV 3** forward and reverse primers
   - 0.25 µl 50 µM **DENV-2** and **DENV 4** forward and reverse primers

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- 0.45 µl 10µM **all probes**
- 0.5 µl RT enzyme
    - Add about 5-10 reactions to your total number of samples (and account for "No template controls" (NTCs), positive controls, and negative extraction controls) and multiply number by volumes above.
    - Ex. You have 20 samples (12 unknown samples, 2 positive controls, 2 negative controls, and 4 NTCs). Make a master mix for 25-30 samples.
    - *Definitions:*
        - NTC = mix ONLY with no sample, to test mix components (*PCR control*)
        - Negative control = extracted water (*extraction control*)

3. Pipette **20 µl of master mix** into either 0.2 ml optical (specifically for real-time assays; emission fluorescence is read through the cap) PCR tubes or a 96 well optical PCR plate. Use a reservoir and a multichannel pipette for many wells.
4. Pipette **5 µl of RNA** into each well. Refer to a template to ensure that the proper sample is added to the corresponding well. Do not add anything to NTC samples (master mix only).
    a. See RNA extraction notes below.

Cycling conditions (Invitrogen conditions for Real Time RT-PCR):

1 cycle each:

50° for 30 min

95° for 2 min

45 cycles:

95° for 15 sec

60° for 1 min (data collection step)

Dengue Primers and Probes

DENV-1 F	CAA AAG GAA GTC GYG CAA TA
DENV-1 R	CTG AGT GAA TTC TCT CTG CTR AAC
DENV-1 probe (FAM/BHQ1)	CAT GTG GYT GGG AGC RCG C
DENV-2 F	CAG GCT ATG GCA CYG TCA CGA T
DENV-2 R	CCA TYT GCA GCA RCA CCA TCT C
DENV-2 probe (HEX/BHQ1)	CTC YCC RAG AAC GGG CCT CGA CTT CAA
DENV-3 F	GGA CTR GAC ACA CGC ACC CA
DENV-3 R	CAT GTC TCT ACC TTC TCG ACT TGY CT
DENV-3 probe (TX red/BHQ2)	ACC TGG ATG TCG GCT GAA GGA GCT TG
DENV-4 F	TTG TCC TAA TGA TGC TRG TCG
DENV-4 R	TCC ACC YGA GAC TCC TTC CA

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DENV-4 probe (CY5/BHQ3) TYC CTA CYC CTA CGC ATC GCA TTC CG

I. RNA EXTRACTION (for all real-time RT-PCR applications)

NOTES: Avoid contamination while working with RNA

- Maintain physically separated work areas; one dedicated to **pre-amplification RNA work** (RNA extraction) and the other for **Master mix** production.
- Utilize dedicated/separate equipment within pre and post amplification areas; especially pipettes and centrifuges.
- Always wear gloves; even when handling unopened tubes.
- Open & close tubes quickly and avoid touching any inside portion.
- Use RNase free plastic disposable tubes and pipet tips.
- Use aerosol block pipet tips.
- Use RNase free water.
- Prepare all reagents on ice.

1. Solid phase samples (mosquitoes or tissues) are first homogenized in an isotonic buffer to produce a liquid homogenate. Mosquito specimens are homogenized using copper clad steel bead (BB) grinding technique using a vortexer or mixer mill (i.e., Qiagen Tissuelyser). Homogenates are clarified by centrifugation in a microcentrifuge (i.e. Eppendorf) at maximum speed for 5 minutes to pellet any particulate material.

2. Extract RNA from the clarified supernatant using the QiaAmp viral RNA kit (QIAGEN part #52904). Follow the manufacturer's protocol exactly with the following modification for mosquito specimens: include 1 additional wash/centrifugation step with AW1. Extract at least two negative controls and two positive controls along with the test specimens. The positive controls should differ in the amount of target RNA present (i.e. a pre-determined high positive and a low positive). Note: The volume of sample extracted can be greater or less than the standard volume stated in the QIAGEN protocol (140 µl) with the appropriate adjustments to all other volumes in the protocol. (We typically extract 100 µl.)

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