Primary Reviewer: Cassandra Kirk, Ph.D., Biologist, Emerging Technologies Branch **Date:** Date: 2020.04.16 10:48:11 -04'00'

Secondary Reviewer: Chris A. Wozniak, Ph.D., Biotechnology Special Assistant, OPP/BPPD **Date:**

DATA EVALUATION RECORD

REQUIREMENT: EPA OCSPP

TEST MATERIAL (PURITY): *Aedes aegypti* OX5034

SYNONYMS: OX5034

CITATION: Arbovirus testing in OX5034 *Aedes aegypti female* adults, Volume 26, EUP Submission; MRID 50889425; July 16, 2019

SPONSOR: Oxitec Ltd, 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX United Kingdom

AUTHOR: Oxitec Ltd.

TEST SITE: Milton Park, Abingdon, Oxfordshire, UK.

COMPLIANCE: Good Laboratory Practice Standards, 40 CFR Part 160, are not applicable to this report. However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

This DER does not contain FIFRA CBI.

EXECUTIVE SUMMARY:

The developer of the male-selective *Aedes aegypti* OX5034 mosquito, Oxitec Ltd., used commercially available test kits (VecTOR Test Systems Inc.) to demonstrate the absence of arbovirus infections in the OX5034 production colony. OX5034 mosquitoes were tested for the detection of arboviruses including Dengue Virus (DENV), Chikungunya Virus (CHIKV), West Nile Virus (WNV), Saint Louis encephalitis (SLE), Eastern Equine Encephalitis (EEE), Western Equine Encephalitis (WEE), Venezuelan Equine Encephalitis (VEE), Mayaro (MAY) and Sindbis (SIN) viruses. Tests were carried out on mixed pools of 50 individuals (20 pools in total) per assay. None of the pools of adult OX5034 mosquitoes tested were positive for any of the arboviruses tested.

The reviewer disagrees with the study author's conclusion that the results of the VectorTest® Systems Inc. arbovirus detection kits used for detection of DENV, CHIKV, WNV, SLE, EEE,

WEE, VEE, MAY and SIN viruses confirms that the OX5034 production colony is free of arbovirus infection, due to uncertainties regarding the sensitivity and specificity of the assays utilized for arbovirus testing. The VectorTest® was developed as a screening tool for the surveillance and rapid detection of arbovirus in mosquitoes under field conditions where laboratory equipment and resources are limited. This type of test should ideally be followed up with a confirmatory assay.

STUDY PURPOSE AND BACKGROUND:

Oxitec submitted the study in order to demonstrate the absence of arbovirus infections in the OX5034 production colony. OX5034 mosquitoes were tested for arboviruses including DENV, CHIKV, WNV, SLE, EEE, WEE, VEE, MAY and SIN viruses. The current review focusses on the diagnostic validity of the Vector Test® dipstick assay. An evaluation of the appropriate selection of arboviruses for which to screen the source colony located in the UK, is addressed in MRID 51094403.

It is of note that for the EUP, the colony will be maintained at Oxitec's insectary in Milton Park, UK and OX5034 eggs will be shipped to rearing facilities in the US for deployment to release sites. Briefly, some of the considerations discussed later pertain to the presence in the UK of *Aedes aegypti* populations and the arboviruses for which it is a vector. The UK currently has no known established populations of any invasive *Aedes* spp. mosquitoes (Medlock et al., 2019). In addition, none of the arboviruses (DENV, CHIKV, WNV, YFV, and Zika Virus) for which *Aedes aegypti* is a major vector occur naturally in the UK. The most recent data in the European Centre for Disease Prevention and Control's (ECDPC) Surveillance Atlas for Infectious Disease show no locally acquired cases of these viruses in the UK (ECDPC, 2017).

CLASSIFICATION: SUPPLEMENTAL due to the above described uncertainties.

I. MATERIALS AND METHODS:

A. GUIDELINE FOLLOWED: Non-Guideline

Deviations from guideline: Not Applicable

B. MATERIALS:

1. Test Material: VecTOR® Test Systems Inc. commercially available test kits (Table

Table. 1 Details of the kits used in this study for arbovirus detection in whole mosquitoes.

Cat. Number	Lot Number	Arbovirus
DEN-K050	DEN-010518	Dengue Virus (all four serotypes)
WSE-K050	WSE-010518	West Nile Virus Saint Louis Encephalitis Virus Eastern Equine Encephalitis Virus
ALP-K050	ALP-010518	Alphaviruses including: Chikungunya Virus Eastern Equine Encephalitis Virus Western Equine Encephalitis Virus Venezuelan Equine Encephalitis Virus Mayaro Virus Sindbis Virus

Assays are based on an immuno-chromatographic wicking assay method, whereby antibodies detecting virus-specific antigens have been immobilized on a wicking test strip. A dual monoclonal antibody "sandwich" principle is used in the assay. Viral antigens present in a mosquito homogenate are bound by a gold-conjugated antibody and migrate through the wicking strip. Test zones on the strip contain the second monoclonal antibody and form a "sandwich" (first antibody-antigen-second antibody) and can be visualized as a red line on the strip if the antigen is present. Unbound antibodies are captured in a control zone and form a second red line, confirming that the test has been carried out correctly (Hinson et al., 2015; Ryan et al., 2003; Wanja et al., 2014).

Control Substance: None

2. **Test Organism:**

Species (common and scientific names): Yellow Fever Mosquito (*Ae. aegypti* OX5034-tTAV / DsRed2-OX5034)

Age at study initiation: Adult

Number of test individuals /Sex:
OX5034 were reared on doxycycline according to standard operating procedures (TD-SOP-00069 and QD-R-00065) at the OX5034 mosquito UK production facility in April 2019. The pupae were collected and kept in cages to eclose in the Quality Control Laboratory. Dead adults were removed from the cages 6-8 days post-eclosion and the remaining live adults frozen at -20°C. 1,000 adults were tested for arbovirus presence. Tests were carried out on mixed pools 50 individuals per 20 pools of non blood-fed OX5034 adults.

Test Methods:
Briefly, OX5034 were reared on doxycycline to standard operating procedures (TD-SOP-00069 and QD-R- 00065) at the OX5034 mosquito UK production facility in April 2019. The pupae were collected and kept in cages to eclose in the Quality Control Laboratory. Dead adults were removed from the cages 6-8 days post-eclosion and the remaining live adults frozen at -20°C. 1,000 adults were tested for arbovirus presence. Tests were carried out on mixed pools of 50 individuals (20 pools in total). Insects were homogenized in 2.5 ml of the grinding buffer and 4 copper-coated beads provided with the kit. The capped tubes were vortexed at high speed for 1-2 minutes. For each assay, 250 µL of the homogenate from each of these 20 pools was tested. The homogenate was probed with the wicking strips specific to the relevant antigen; the assays were allowed to develop for 20-30 minutes before reading, according to the manufacturer's protocols.

RESULTS:
Results are summarized in Table 2. None of the pools of adult OX5034 mosquitoes tested were positive for any of the arboviruses tested. No kits are commercially available for detecting Zika virus infection in mosquito samples. However, VectorTest® has a Zika virus detection kit under development (ZIK-K050, http://www.vectortest.com/products.html) and once available, Oxitec plans to add this test to the testing protocol for arbovirus screening of OX5034.

Study Author's Conclusion:
The study author concludes that these results confirm that the OX5034 production colony is free of arbovirus infection, as determined by the VecTOR® Test Systems Inc. arbovirus detection kits used here for detection of CHIKV, DNV, WNV, SLE, EEE, WEE, VEE, MAY and SIN viruses.

Table 2. Summary for the arbovirus testing on OX5034 adult females.

Arbovirus test kit	Number of OX5034 adult mosquito pools tested (50 mosquitoes/pool)	Number of OX5034 adult mosquito pools positive for arbovirus
Dengue Virus (all four serotypes)	20	0/20
West Nile Virus Saint Louis Encephalitis Virus Eastern Equine Encephalitis Virus	20	0/20
Chikungunya Virus Eastern Equine Encephalitis Virus Western Equine Encephalitis Virus Venezuelan Equine Encephalitis Virus Mayaro Virus Sindbis Virus	20	0/20

Reviewer's Comments:

1) Outstanding questions on the sensitivity and specificity of the three VectorTests
The company website does list citations for peer reviewed journal articles, however, only one pertains specifically to an assay that Oxitec plans to use to screen the OX5034 production colony. Wanja et al. (2014) performed an assessment of the DENV VectorTest® dipstick assay and determined that the assay is accurate and practical for laboratory and field use, however, they noted additional studies are needed to confirm that this assay will work under field conditions and that exotic mosquitoes and the presence of exotic animal blood in these mosquitoes will not affect the functionality of the assay. During initial studies, they found that the DENV dipstick was able to detect ≥75% of positive samples when read within 20-25 minutes, with accurate detection rates of 73, 80, 100, and 53% for DENV 1, 2,3, 4, respectively. However, when the dipsticks were allowed to wick for 26-30 minutes, they detected all positive samples for each serotype. No false positives were found following either development time. Wanja et al. (2014) concluded that this simple and robust assay could enable public health workers to detect DENV in infected mosquitoes rapidly without the need for specialized equipment, expertise, or training, making virus surveillance more expedient.

Hinson et al., determined that, similar to the DENV 1-4 test, increasing the wicking time for the Chikungunya virus specific assay increased the ability to detect positive samples from ≥60% to 100%, however this is not the same assay that will be used by Oxitec. Oxitec plans to use VectorTest® Alphavirus Antigen Assay (Cat No. ALP K050), which screens for Chikungunya Virus, Eastern Equine Encephalitis Virus, Western Equine Encephalitis Virus, Venezuelan Eastern Equine Encephalitis Virus, Mayaro Virus, and Sindbis Virus simultaneously (i.e. not Chikungunya Virus alone). Whether or not increasing the development time for the broader-specificity kit detecting a range of alphaviruses would increase background is unknown.

In summary, Oxitec only provided information addressing the sensitivity of the DENV VectorTest® dipstick assay (Wanja et al., 2014). As a result, uncertainty remains regarding the sensitivity of the remaining VectorTest® dipstick assays.

2) Relative sensitivity of tests routinely used for arbovirus surveillance
A variety of methods have been utilized for detection of arboviruses in captured mosquitoes for mosquito surveillance programs in the field for public health protection. Plaque assays are recognized as a standard method for detecting and quantifying arboviruses in mosquitoes which allow the direct quantification of infectious virions and antiviral substances through the counting of discrete plaques (infectious units and cellular dead zones) in cell culture (Beaty et al; 1989). These assays are sensitive and detect a wide variety of arboviruses; however, they are costly, laborious, and may require BSL2/3 containment. Alternative methods of arbovirus detection in mosquitoes include enzyme-linked immunosorbent assay (ELISA) and reverse-transcriptase polymerase chain reaction (RT-PCR) assays. The ELISA procedure requires laboratory expertise and is expensive. Nucleic acid detection using RT-PCR has become one of the most popular methods of virus detection and has potentially displaced virus isolation as the new gold standard. Real time quantitative RT-PCR (qRT-PCR) platforms, such as TaqMan®, are ideal for routine testing of mosquitoes, since they reduce processing time significantly (sometimes to less than an hour), allowing for high throughput screening (Kauffman et al., 2003; Pyke et al., 2004) Since

these assays detect both infectious virus and RNA, they have comparable or better sensitivity than virus isolation (Lanciotti, 2003). RT-PCR-based molecular methods are standard for arbovirus detection and identification during outbreak investigations (Nawrocki et al. 1996, Lanciotti et al; 2000, Hadfield al; 2001), and are used in laboratories throughout the United States and in arbovirus surveillance programs (Ramírez et al., 2018). Although rapid, RT-PCR methods are costly and more complex than ELISA protocols. The VectorTest® dipstick assay is a simple, one-step wicking assay that comes in a kit that can be stored at ambient temperatures, requires no specialized equipment or highly trained personnel to obtain results, and provides results quickly as compared to other common detection methods. It was developed to provide real-time critical information on the presence of arboviruses in mosquitoes to public health personnel. Table 3. compares the relative advantages and disadvantages of these three common arbovirus surveillance methods (Ramírez et al; 2018). The VectorTest® dipstick assay falls in the "virus detection in pools of mosquitoes using rapid antigen detection assays" class, which has a lower sensitivity than molecular methods but offers lower cost, rapid results and is ideal for routine surveillance in low resource settings.

Table 3. Comparison of common arbovirus surveillance methods (modified after Ramírez *et al.*, 2018)

Method	Advantages	Disadvantages	Application
Virus isolation from pools of mosquitoes	Increases virus titer allowing for genotypic and phenotypic characterization	Time consuming. Requires special infrastructure (biological containment). Requires a cold chain	Routine surveillance, virus identification, inform control strategies
Virus detection in pools of mosquitoes using molecular assays	Allows high throughput screening. High sensitivity	Will only detect RNA from viruses that the assays were designed to detect. Requires special infrastructure	Routine surveillance, research, inform control strategies
Virus detection in pools of mosquitoes using rapid antigen detection assays	Rapid. Does not require specialized equipment. Lower cost	Lower sensitivity than molecular methods	Routine surveillance in low resource settings

During an outbreak, when infected specimens are relatively abundant, a test with high specificity and relatively low sensitivity can be very useful for detecting areas that need special attention for intervention rapidly. Assays such as these, including the VectorTest® dipstick assay, are first-tier screening tool that should be followed-up with a second-tier confirmatory assay such as RT-PCR. To avoid false negatives, negative pools of mosquitoes should be evaluated with another procedure to determine what proportion of positives, if any, may be missed by the dipsticks. This is particularly important because information regarding the specificity and sensitivity of the VectorTest® is not provided by the VecTOR Test Systems, Inc., or by Oxitec. Use of a surveillance assay with low sensitivity designed for obtaining rapid results in a field setting is not an appropriate tool for screening the laboratory-maintained colony from which OX5034 males will be sourced for release.

The reviewer disagrees with the study author's conclusion that the results of the VectorTest® Systems Inc. arbovirus detection kits used for detection of CHIKV, DENV, WNV, SLE, EEE, WEE, VEE, MAY and SIN viruses confirms that the OX5034 production colony is free of

arbovirus infection, due to uncertainties regarding the sensitivity and specificity of the assays utilized for arbovirus testing. The VectorTest® was developed as a screening tool for the surveillance and rapid detection of arbovirus in mosquitoes under field conditions where laboratory equipment and resources are limited. This type of test should ideally be followed up with a confirmatory assay.

CONCLUSION: SUPPLEMENTAL

Reviewers Conclusions and Recommendations:

In order to address the uncertainty regarding the diagnostic validity of the VectorTest® dipstick assay, EPA issued a 75-day letter requesting that Oxitec submit arbovirus testing data utilizing an RT-PCR method with demonstrate diagnostic validation, quantitative measures of analytical specificity and sensitivity, in addition to appropriate controls and sample size that effectively demonstrate that laboratory colonies are arbovirus free. The complete data evaluation of Oxitec's response can be found in MRID 51094403.

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