Field-deployable molecular diagnostic platform for arbovirus and *Wolbachia* detection in *Aedes aegypti*

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Abstract

Background: Surveillance of mosquito infection status is critical for planning and deployment of proper mosquito control initiatives. Concurrently, *Wolbachia* is being widely used as a control method for arboviral transmission. Point-of-care (POC) detection assays are necessary for monitoring the infection prevalence and geographic range of viruses as well as *Wolbachia* in mosquito vector populations. We therefore assessed the novel qPCR bCUBE molecular diagnostic system as a tool for virus and *Wolbachia* detection.

Results: We developed a reliable, specific, and sensitive diagnostic assay for detecting Zika virus and dengue virus serotype 2 using the real-time qPCR platform bCUBE. With bCUBE-based qRT-PCR, both *Wolbachia* bacterium and virus RNA could be reliably detected in individually infected *Ae. aegypti* mosquitoes and in pools of 5, 10, or 15 mosquitoes.

Conclusions: The portable qPCR bCUBE diagnostic platform is capable of detecting Zika and dengue virus as well as *Wolbachia* in mosquitoes and therefore has potential as a practical field-deployable diagnostic test for vector-borne disease surveillance programs.

Keywords: *Aedes aegypti*, Zika, dengue, diagnostics, qPCR

Introduction

Arthropod-borne diseases threaten over two-thirds of the global population and are exhibiting an ongoing expansion of their geographic range and prevalence as a result of climate change, urbanization, and globalization (1-4). According to the World Health Organization, dengue virus infection has increased 30-fold over the past 50 years and now affects nearly 100 million people, primarily in the Americas and Asia (5, 6). Recent outbreaks of Zika virus have also led to widespread concern because of the virus's ability to cause newborn malformations (7, 8).

Dengue and Zika viruses are members of the genus *Flavivirus* that are primarily transmitted by the *Aedes* mosquito (7, 9, 10). Other members of the *Flavivirus* genus are also recognized as vector-borne pathogens of public health significance, including West Nile virus (WNV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), and Chikungunya virus (CHIKV). These viruses result in similar flu-like symptoms with the potential to progress to neuroinvasive outcomes. *Aedes* mosquitoes, with their aggressive blood-feeding behavior, have allowed for efficient human-mosquito transmission of these arboviruses (3, 11). The geographical presence of this mosquito vector has dramatically increased in the last few decades, leading to an expanded transmission of these arboviruses (12-14). The lack of vaccines and treatment against these arboviruses highlights the importance of mosquito control and surveillance strategies (15). Current and future mosquito-targeted control strategies are, and will be, having a significant epidemiological impact but also require mosquito and pathogen surveillance (15). Surveillance of geographical distribution of the vector mosquitoes and the pathogens they carry is an essential component of disease prevention and control.

72 *Wolbachia pipientis* is increasingly being used as a method for limiting arboviral transmission in
73 dengue and Zika virus-endemic countries. This endosymbiotic bacterium is passed from mother
74 to offspring and has been shown to suppress dengue and Zika virus transmission in *Aedes*
75 mosquitoes (16, 17). Field-release trials require continual monitoring of *Wolbachia*-infected *Ae.*
76 *aegypti* in mosquito populations (18-20). Real-time qPCR has been used as the primary method
77 for detecting *Wolbachia* genetic material in mosquitoes (21).
78
79 Accurate, rapid, and cost-effective mosquito and pathogen surveillance is critical for monitoring
80 infection prevalence and thereby mitigating transmission risk. Methods currently used for
81 arboviral detection include viral culture, antibody detection, antigen detection, and RNA
82 detection using quantitative real-time RT-PCR (qPCR). Currently, the qPCR method is the gold
83 standard because of its use of specific molecular markers (22, 23). Many of these assays are
84 laborious and laboratory-based and require expensive and bulky instruments, making them
85 incompatible with low-resource regions (24). Recently, several novel, advanced point-of-care
86 (POC) diagnostic measures have been developed for detecting mosquito-borne viruses in the
87 field, including honey-baited nucleic acid preservation cards (25, 26), loop-mediated isothermal
88 amplification (LAMP) (27), biosensors (28), and adaptations of near-infrared spectrometry
89 techniques (29). However, these techniques have documented limitations, including cross-
90 reactivity with other flaviviruses or requirements for training on specialized equipment, making
91 the adoption of these new diagnostic tools difficult (30, 31). Although the global burden of
92 emerging outbreaks of Zika and dengue is clearly recognized, there is a gap in resources for
93 endemic countries and regions, which are consistently plagued by a lack of equipment and

94 adequate resources to consistently monitor the prevalence and range of infected mosquitoes (5,
95 32, 33).
96
97 Disease surveillance and integrated vector control are essential for curbing disease transmission.
98 Nucleic acid-based testing to detect viral RNA allows for specific and sensitive virus monitoring
99 in mosquito surveillance programs (24). Several conventional and more recent real-time PCR-
100 based assays have been established for mosquitoes and their vectored pathogens, and the ready
101 availability of genome sequences for both vectors and pathogens can support the identification of
102 additional PCR-compatible molecular markers (34-36).
103
104 In the present study, we evaluated a novel and portable real-time PCR platform, bCUBE (Hyris,
105 Ltd), as a PCR-based arboviral detection method with potential for field-deployability. bCUBE
106 makes possible the genetic testing of biological samples in any setting, at any time, with real-
107 time access to results on its dedicated cloud-based software platform. This technology is a
108 portable device, similar in size to a Bluetooth speaker, which is used for biological analysis in
109 several fields, including agricultural pest control. The device can be operated from a laptop,
110 tablet, or cellular device through an easy-to-use gateway and generates centralized data analysis
111 immediately after a reaction. The device is capable of performing thermocycling reactions such
112 as real-time PCR, as well as loop-mediated isothermal amplification (LAMP). The system can be
113 calibrated to distinguish between positive and negative samples in a single reaction with
114 predetermined conditions that have been established ahead of time in the laboratory. This
115 feature allows the bCUBE to be operated by individuals lacking in depth training in qPCR assays
116 and data analysis skills. We have now explored the use of bCUBE technology for detection of

117 both dengue and Zika virus in *Aedes aegypti,* optimizing and standardizing the sample
118 preparation method to be used with a commercially available one-step qRT-PCR assay kit.
119 Finally, we have developed a bCUBE-compatible qPCR diagnostic assay for the surveillance of
120 arboviral pathogens and *Wolbachia* in *Aedes* mosquitoes.
121
122 **Methods**
123 **Ethical Statement.** This study was carried out in strict accordance with the recommendations in
124 the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH).
125 Mice were used according to an animal protocol (permit # MO15H144) approved by the Animal
126 Care and Use Committee of the Johns Hopkins University and were used as the blood source for
127 the maintenance of the mosquito colonies. Commercial anonymous human blood (InterState
128 Blood Bank) was used for Zika and dengue virus infection assays in mosquitoes, and informed
129 consent was therefore not applicable.
130
131 **Mosquito rearing and mosquito infections.** *Ae. aegypti* Liverpool strain LVPB12 mosquitoes
132 were maintained on 10% sucrose solution under standard insectary conditions at 27±0.5°C and
133 75-80% humidity with a 12-h light/dark cycle. Mosquitoes were reared using a standard rearing
134 protocol, and colonies were maintained on Swiss Webster mice (Charles River Laboratories).
135 DENV2 and ZIKV infection of mosquitoes was carried out as previously described (37). Virus
136 supplemented blood was fed to mosquitoes using an artificial membrane glass feeder, and
137 infected mosquitoes were double-caged and incubated in a reach-in incubator under conditions
138 similar to those in the standard insectary chamber described above. *Wolbachia w*AlbB-infected

139 *Ae. aegypti* (WB1) mosquitoes were reared and maintained under the standard insectary
140 conditions described above and in (38).
141
142 **Cell culture and virus propagation.** *Aedes albopictus* C6/36 cells (ATCC CRL-1660) were
143 cultured, and viral stocks were prepared as previously described in (39). In brief, C6/36 cells
144 were cultured in MEM medium (Gibco) supplemented with 10% heat-inactivated fetal bovine
145 serum (FBS), 1% penicillin-streptomycin, and 1% non-essential amino acids and maintained in a
146 tissue culture incubator at 32°C and 5% CO_2. Baby hamster kidney strain 21 (BHK-21, ATCC
147 CCL-10) cells were maintained at 37°C and 5% CO_2 in the DMEM medium (Gibco)
148 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 5µg/ml
149 Plasmocin™. DENV serotype 2 New Guinea C strain (DENV2) and ZIKV strain FSS 13025
150 (ZIKV) were used in the indicated experiments. For viral stock preparation, C6/36 cells grown to
151 80% confluence were infected with ZIKV and DENV2 at a multiplicity of infection (MOI) of 10
152 and incubated at 32°C and 5% CO_2 for 5 days (DENV2) and 6 days (ZIKV). Virus was
153 harvested by three freeze-thaw cycles using dry ice and a water bath (37°C), then centrifuged at
154 2,000 rpm for 10 min at 4°C. The supernatant from this cell lysis was mixed with the original
155 cell culture supernatant to yield the final viral stock. Viral stocks were aliquotted and stored at -
156 80°C for long-term storage. Viral stock titration was done by plaque assay as described below.
157
158 **Primer design for real-time quantitative RT-PCR (qPCR).** The ZIKV envelope (E) protein
159 was chosen as the target for ZIKV detection, and primers were developed based on previously
160 established sequences (40). Dengue virus serotype 2 (DENV2) qPCR was performed using
161 previously developed protocols that employ primers detecting serotype 2 in the 3'UTR region

162 (41) (Table 1). The primers were modified and optimized for bCUBE qPCR (Table 1). Primer
163 sequences were assessed with NCBI Blast to search for potential cross-reactivity against other
164 viruses, bacteria, and vectors. Previously established primers for *w*AlbB detection were used for
165 *Wolbachia*-infected *Ae. aegypti* (42). The sequences of the primers are summarized in Table 1.

Primer	Sequence 5'-3'	Nucleotide position	Amplicon size (bp)	Accession Number	Reference
ZIKV-forward	AGCAACATGGCGGAGGTAAG	1,128-1,147	145	FSS13025	(34)*
ZIKV-reverse	CTGTCCACTAACGTTCTTTTGCAGA	1,249-1,273			
DENV-2-forward	TCCCTTCCAAATCGCAGCAACAATG	10,517-10,541	168	NC_001474.2	(35)*
DENV-2-reverse	CGTTCTGTGCCTGGAATGATG	10,665-10,685			
wAlbB-forward	CCTTACCTCCTGCACAACAA	213,522-213,541	110	CP031221.1	(36)
wAlbB-reverse	GGATTGTCCAGTGGCCTTA	213,394-213,412			
WNV-forward	TTGTGTTGGCTCTCTTGGCGTTCTT	233-257	408	AF196835	(38)
WNV-reverse	CAGCCGACAGCACTGGACATTCATA	640-616			
JEV-forward	GGCAGAAAGCAAAACAAAAGA	390-410	367	AF080251	(39)
JEV-reverse	CGGATCTCCTGCTTCGCTTGG	736-756			
YFV-forward	CACGGCATGGTTCCTTCCA	5,656-5,674	71	MN708497	(37)
YFV-reverse	ACTCTTTCCAGCCTTACGCAAA	5,707-5,728			
CHIKV-forward	TACAGGGCTCATACCGCATC	10,357-10,376	154	NC_004162	(40)
CHIKV-reverse	AAAGGTGTCCAGGCTGAAGA	10,492-10,511			

166

167 **Table 1**. **Primer pairs for viral detection and cross-reactivity panel for real-time qPCR**
168 **using the bCUBE.**
169 *ZIKV and DENV-2 primers were modified and optimized for qRT-PCR. The cross-reactivity
170 panel primer sequences were included to confirm viral RNA obtained from BEI Resources. Four
171 viruses were included in this study: West Nile virus (WNV), Japanese encephalitis virus (JEV),
172 yellow fever virus (YFV), and Chikungunya virus (CHIKV). Primer sequence, nucleotide
173 position, and amplicon size are listed.
174

175 **Cross-reactivity panel of mosquito-borne viruses.** Viral nucleic acid sequences included in the
176 cross-reactivity panel were CHIKV (H20235 ST MARTIN 2013), JEV (India R53567), WNV
177 (CO 1862), and YFV (17D), obtained from BEI Resources. A NanoDrop Spectrophotometer
178 (ThermoFisher Scientific) was used to measure the RNA concentration of these viral samples.

179 Previously established primers were used, as listed in Table 1, for qPCR to confirm the presence
180 of corresponding viral RNA in the samples (43-46).
181
182 **Total RNA preparation for DENV2- and ZIKV-infected whole mosquitoes or tissues**. Total
183 RNA extraction was performed using a squash buffer (10 mM Tris base, 1 mM EDTA, 50 mM
184 NaCl) supplemented with 1:8 part Proteinase K (Qiagen, 15mg/ml). Mosquito abdomens with
185 the midguts and heads with thoraces were individually collected in 50 µl of squash buffer at 7
186 and 14 days, respectively, after an infectious blood meal and stored at -80°C until extracted.
187 Proteinase K was added at 1/8 volume to each sample to give a final concentration of 15 mg/ml
188 and homogenized with a cordless Pellet Pestle Motor (Kontes) for 40-60 s. Samples were
189 incubated at 57°C for 5 min, followed by 95°C for 5 min for enzyme deactivation. The
190 supernatant from this crude RNA extraction was used immediately for qPCR or stored at -80°C
191 until use.
192
193 **gDNA preparation of *Wolbachia*-infected mosquitoes.** Total gDNA from *Wolbachia*-infected
194 total mosquitoes or tissues was prepared as described above (47). These crude gDNA extractions
195 were immediately used for qPCR or stored at -80°C until use.
196
197 **Laboratory standard real-time PCR with Applied Biosystems equipment.** A StepOnePlus
198 Real-Time PCR system (Applied Biosystems) was used for comparative analysis. The mosquito
199 housekeeping gene ribosomal protein S17 (*rps17)* was amplified in *Aedes aegypti* using the two
200 different PCR machines (Applied Biosystems and bCUBE) with the same real-time PCR
201 settings. SYBR Green PCR Master Mix (Applied Biosystems) was used according to

202 manufacturer's instructions. Mosquitoes were collected individually in 50 µl of squash buffer,
203 and crude gDNA was extracted. Identical samples were applied to both the bCUBE- and
204 StepOnePlus-based qPCR systems for comparative evaluation.
205
206 **Hyris bCUBE real-time RT-PCR.** The portable qPCR machine Hyris bCUBE 2.0 thermocycler
207 (Hyris, London, UK) and the 16-well cartridges were kindly provided by Hyris Inc. The Hyris
208 data analysis platform was used for this study. GoTaq 1-Step RT-qPCR kit (Promega) was used
209 at a final volume of 20 µl with: 0.4 µl of kit reverse transcriptase enzyme, 10 µl of RT-PCR
210 buffer, 0.3 µl of 10 µM of forward and reverse primer, 9 µl of DNA/RNA free water, and 1 µl of
211 crude RNA or gDNA sample. Each sample was performed in technical duplicate. Each cartridge
212 run included one negative and one positive control. The following thermocycling settings were
213 used: reverse transcription for 15 min at 37°C, heat-inactivation of reverse transcriptase at 95°C
214 for 10 min, followed by 30 PCR cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s.
215 Melting curve analysis was performed at the end by cooling to 60°C, followed by heating to
216 95°C at 0.05°C/s. Automatic data analysis was generated with the Hyris data analysis platform.
217 Two technical replicates were performed on the platform for each biological replicate. Three
218 biological replicates were included for each assay.
219
220 **Absolute quantification of viral copy numbers through qRT-PCR**. *In vitro*-transcribed RNA
221 of the E gene of ZIKV and the 3'-UTR region of DENV2 were used for absolute quantification
222 and cloned into a TOPO-TA vector. Total RNA was extracted using TRIzol reagent
223 (ThermoFisher Scientific). RNA (2 µg) was used for cDNA synthesis with M-MLV Reverse
224 Transcriptase (Promega). Conventional PCR was used with the primer (Table 1) to amplify a

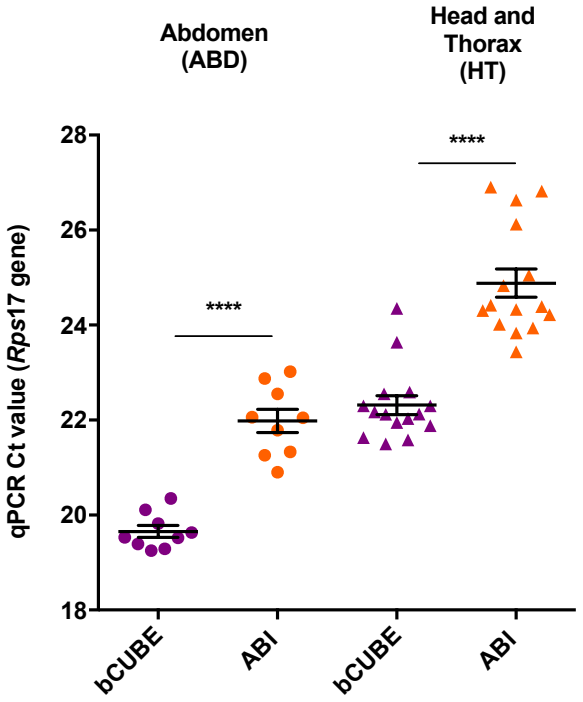
225 DNA fragment of 168-bp for DENV2 and 145-bp for ZIKV. PCR was followed by PCR
226 purification using a QIAquick PCR Purification kit and protocol (Qiagen). About 50 ng of
227 cleaned PCR product of either DENV2 or ZIKV was separately cloned into a TOPO-TA PCR
228 cloning vector (ThermoFisher Scientific) according to the manufacturer's instructions. The
229 ligation product was transformed with TOP10-competent cells (ThermoFisher Scientific), and
230 positive clones grown on LB selection agar plates were screened through colony-PCR, followed
231 by plasmid mini-prep (Qiagen) and sequencing confirmation. The plasmids of the final
232 confirmed-positive clones were purified using the Maxiprep kit (Qiagen) according to the
233 manufacturer's instructions. Plasmid DNA was used to prepare absolute standards. A HiScribe
234 T7 High Yield RNA Synthesis Kit (NEB) was used for RNA synthesis, and the concentration of
235 the RNA was checked with a NanoDrop Spectrophotometer (ThermoFisher Scientific).
236 Molecular weights were converted to copy numbers using the New England BioLabs Calculator
237 (https://nebiocalculator.neb.com/#!/ssrnaamt). The concentration of RNA was adjusted to 10^{10}
238 copies/µl and serially diluted 10 times for the standard curve on the bCUBE. The standard curve
239 was generated using GoTaq 1-Step RT-qPCR (Promega).
240
241 **Viral titration by plaque assay.** The titers of ZIKV and DENV2 in the original viral stocks and
242 in the infected mosquitoes were determined by plaque assays in BHK-21 cells. Each experiment
243 was performed with at least three biological replicates. Whole mosquito or mosquito tissue
244 samples were collected at 7 and 14 days post-infectious blood meal in 150 µl of complete
245 DMEM medium with glass beads. Tissue samples were homogenized with a Bullet Blender
246 (Next Advance) and serially diluted with DMEM complete medium. One or two days before
247 plaque assay, the BHK-21 cells were split at a 1:10 dilution and grown on 24-well plates to 80%

248 confluency. Serially diluted mosquito or viral samples (100 µl each) were added to the BHK-21
249 cells, followed by incubation at room temperature for 15 min on a rocking shaker (VWR) and
250 subsequent incubation at 37°C with 5% CO_2 in a cell incubator (ThermoScientific) for another
251 45 min. The 24-well plates with infected BHK-21 cells were overlaid with 1 ml of 0.8%
252 methylcellulose in complete DMEM medium with 2% FBS and incubated for 5 to 6 days in the
253 cell culture incubator (37°C and 5% CO_2). Plaques were fixed and developed with staining
254 reagent (1% crystal violet in 1:1 methanol/acetone solution) at room temperature for
255 approximately 30 min. Plates were rinsed with DI water and air-dried, and plaques were counted
256 and multiplied by the dilution factor to calculate the plaque forming units (PFUs) per sample.
257
258 **DENV2 and ZIKV mosquito pools.** Preliminary pooled sample experiments involved a total of
259 300 *Aedes aegypti*: 276 uninfected, 12 infected with DENV2 and 12 infected with ZIKV. First,
260 12 mosquitoes were infected with ZIKV and 12 with DENV2. Following confirmation of
261 infection by RT-qPCR of individual infected mosquitoes, each was placed into a pool of
262 uninfected mosquitoes. Four different pools were used to measure the sensitivity of the infection
263 detection for pools of 5, 10, 15, and 20. The squash buffer volumes used were 250, 450, 700, and
264 950 µl, respectively, followed by the addition of 1:8 part Proteinase K.
265
266 **Statistical Analysis.** Graphs were generated using GraphPad Prism Software version 8. The
267 estimation of several diagnostic parameters for leg samples, including sensitivity, specificity,
268 accuracy, and positive and negative predictive values was calculated using the web-based
269 software MedCalc Diagnostic Test Evaluation Calculator.
270

271 **Results**

272 **The reliability of the portable bCUBE qPCR machine is equal to that of a laboratory-**
273 **standard qPCR system**

274 The reliability of the novel bCUBE real-time PCR system was validated by comparing the
275 system to a standard qPCR instrument (Applied Biosystems StepOnePlus, ABI). Our proof-of-
276 principle studies used mosquito tissue homogenates (abdomen with the midgut, and head with
277 the thorax) in squash buffer that were tested with the mosquito housekeeping gene (ribosomal
278 protein S17, *rps*17). *Aedes aegypti* were dissected into crude compartments, including the
279 abdomen and head plus thorax. Identical samples were processed using both qPCR machines,
280 identical settings, and identical SYBR Green Master Mix and instructions (Applied Biosystems).
281 As shown in Fig. 1, the housekeeping *Rps*17 gene was detected in all 24 samples by both
282 machines. The cycle threshold (Ct) values for the *Rps*17 gene in *Ae. aegypti* were detected
283 significantly earlier in the bCUBE system than in the laboratory standard procedure, with a mean
284 difference of 2.40 Ct value (paired t-test, p-value < 0.0001). This result suggests that the bCUBE
285 qPCR is reliable in detecting a mosquito gene from a single mosquito tissue using a crude
286 sample-preparation method.



287

288 **Fig. 1. Comparison of *Ae. aegypti Rps*17 gene detection in abdomen (ABD) and head plus**
289 **thorax (HT) samples using bCUBE and Applied Biosystems real-time PCR.** *Ae. aegypti*
290 abdomens and head plus thoraces were collected, and gDNA was extracted using the squash
291 buffer method. The housekeeping gene *Rps*17 was amplified using the bCUBE and Applied
292 Biosystems real-time PCR systems. Cycle threshold (Ct) values are plotted. Statistical
293 significance was determined by Mann-Whitney test (****, $p < 0.0001$; Mann-Whitney).
294

295 **bCUBE-based one-step qRT-PCR is sufficiently sensitive for DENV2 and ZIKV detection**

296 We performed sensitivity studies on the bCUBE-based qPCR system based on absolute
297 quantification standard curve analysis. Cloned fragments of DENV2 and ZIKV were quantitated
298 for RNA copy number and serially diluted to establish a qPCR standard curve. End-point
299 limitations were set to < 30 for Zika virus, whereas dengue end-point limitations were set to < 29
300 based on standard curve (Supplementary Fig. 1). Correlation coefficient (R^2) values were 0.99
301 for DENV2 and 0.98 for ZIKV. Based on the standard curve analyses, the limit of detection
302 range was 10 viral RNA copy numbers for DENV2 and ZIKV. Amplification above these values
303 was attributed to non-specific amplification.

304

305 **bCUBE-based qRT-PCR is specific for DENV2 and ZIKV detection**

306 The specificity of the ZIKV and DENV2 assays was then determined using a cross-reactivity
307 panel. To address challenges involving primer cross-reactivity with other arboviruses (46, 48) as
308 well as the tendency toward false-positive amplification in negative samples, we tested the
309 specificity of the assay against a panel of *Flaviviruses* and one *Alphavirus*. The frequently co-
310 circulating arboviral RNA samples, obtained from BEI resources (Table 2) for this study, were:
311 CHIKV (H20235 ST MARTIN 2013), JEV (India R53567), WNV (CO 1862), and YFV (17D).

312 The ZIKV and DENV2 primer pairs showed a high degree of specificity for amplification of

313 their respective virus RNAs (Table 2), since no amplification of other virus RNAs occurred.

Family	Genus	Species	Strain	BEI No.	Result for ZIKV qPCR Assay	Result for DENV-2 qPCR Assay
Flaviviridae	*Flavivirus*	Zika virus			+	-
		Dengue virus serotype 2			-	+
		Japanese Encephalitis virus	India	NR-9592	-	-
		West Nile virus	CO 1862	NR-50434	-	-
		Yellow Fever virus	17D	NR-2869	-	-
Togaviridae	*Alphavirus*	Chikungunya virus	St Martin 2013	NR-50130	-	-

314

315 **Table 2. Evaluation of the specificity of bCUBE-based qRT-PCR for DENV2 and ZIKV**
316 **detection**
317 A cross-reactivity panel of frequently co-circulating viruses was used to evaluate the specificity
318 of bCUBE-based qRT-PCR for DENV2 and ZIKV. Arboviral RNA samples were obtained from
319 BEI Resources. The presence of all cross-reactivity panel RNA samples was quantified on
320 NanoDrop for molecular weight and amplified on qRT-PCR with their own primer sequences.
321

322

323 **bCUBE-based qRT-PCR is as accurate as plaque assay in detecting infectious viral RNA**

324 PCR detection of non-infectious viral RNA is possible when viral RNA is not actively

325 replicating (49). Therefore, the viral RNA copy number and viral infectious RNA load can differ

326 in mosquitoes. We thus sought to evaluate the infection prevalence using bCUBE qRT-PCR and

327 plaque assays as opposed to titer comparisons. Three biological replicates of *Ae. aegypti* were

328 infected with ZIKV and DENV-2. Each replicate of infected mosquitoes was collected and

329 separated into two halves to compare infectious prevalence as determined by plaque assay and

330 bCUBE qPCR analysis. In each of the three replicates, no-significant differences were seen in

331 infection prevalence values for both ZIKV and DENV2 between the two methods. These results

332 suggest that bCUBE qRT-PCR analysis can accurately detect infectious ZIKV AND DENV2

333 (Fig. 2).

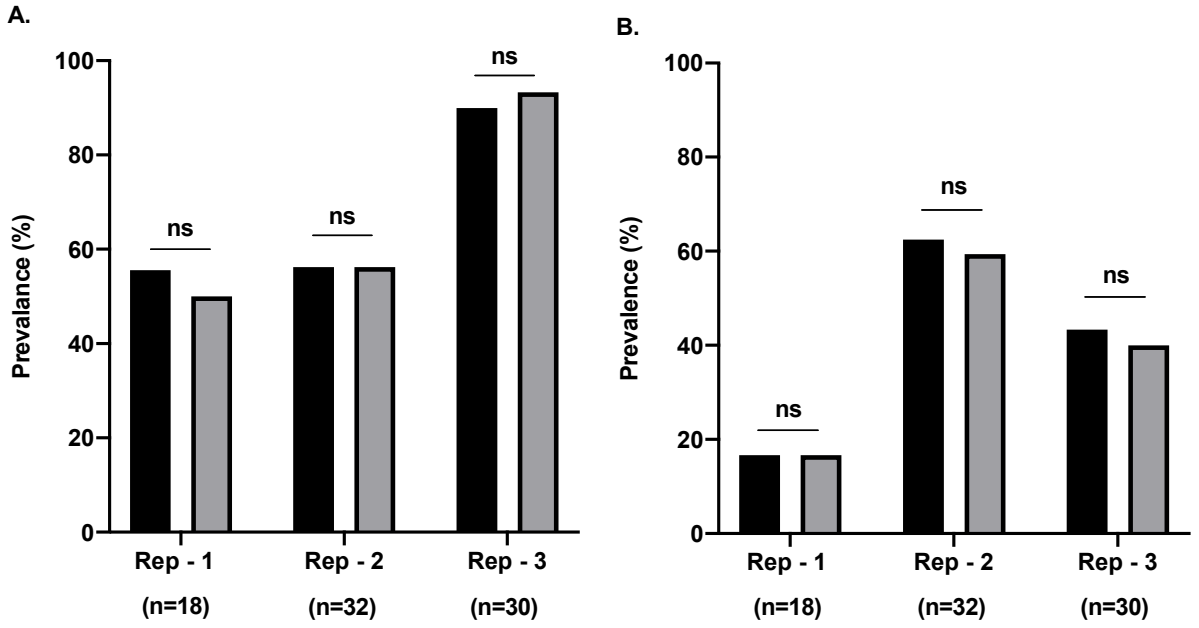


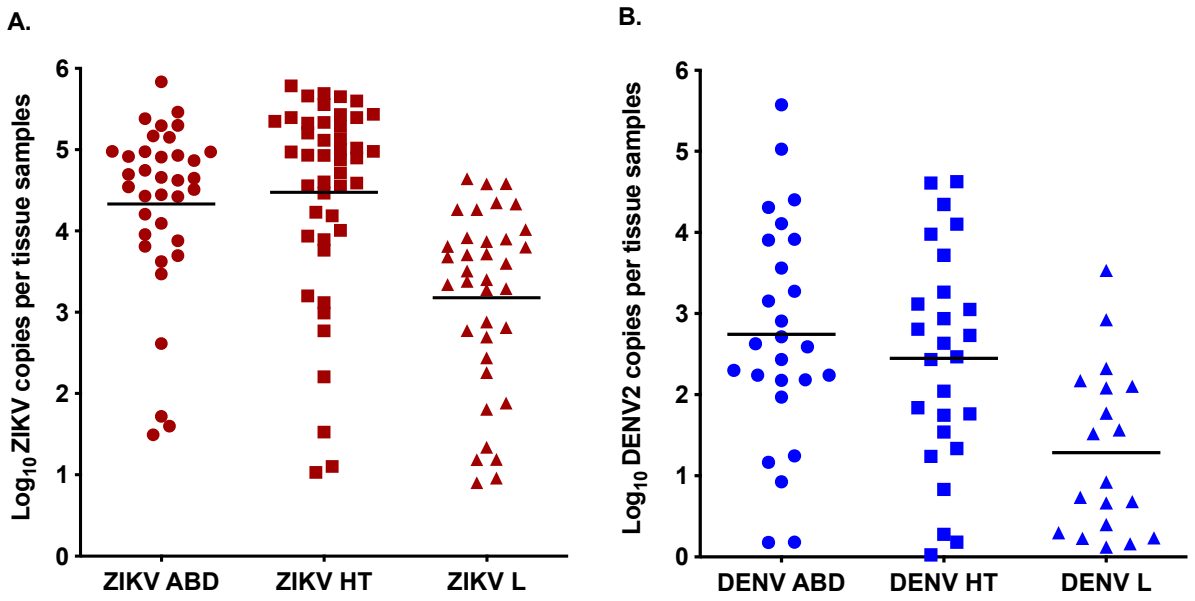
Fig 2. ZIKV AND DENV2 infection prevalence in *Ae. aegypti* as detected in the bCUBE versus plaque assay. *Ae. aegypti* were infected with Zika (A) and dengue (B) virus via an artificial blood meal. Each biological replicate was split into two groups and analyzed using the bCUBE assay (black bars), and the other half was used for the plaque assay (grey bars). No significant difference was detected between the plaque assay and bCUBE qPCR in terms of infectious prevalence (ns: not significant; Fisher's exact test).

ZIKV and DENV-2 RNA can be detected and quantified in individual mosquito samples by bCUBE assay

Ae. aegypti mosquitoes were experimentally infected with ZIKV (n=112) and DENV2 (n=112) through an artificial blood feeder containing anonymous human blood supplemented with virus, and samples from the mosquitoes were evaluated for infection status with the bCUBE platform at 7 and 14 days post-blood meal. Samples with Ct values < 30 were considered positive for ZIKV and < 29 for DENV2 infection. Each sample was run in duplicate to confirm infection. Both ZIKV and DENV2 viral RNA were detected from an individually infected mosquito using the squash buffer extraction method and the bCUBE platform. Viral RNA could be detected and

351 quantitated in samples containing single abdomen with midguts and head with thoraces of

352 infected mosquitoes (Fig. 3).



353

354 **Fig 3. Viral RNA concentrations of individual *Ae. aegypti* tissues collected at various time**
355 **points.**
356 Individual *Ae. aegypti* that were infected with Zika (A) and dengue virus (B) were collected at 7
357 or 14 days post-infectious blood meal. Abdomens with midgut samples (ABD) were collected on
358 day 7 post-infectious blood meal, and head with thorax samples (HT) were collected on day 14.
359 Corresponding leg (L) samples were also collected on day 14. Viral RNA extraction was
360 performed using the squash method, followed by amplification using 1-step qPCR on the
361 bCUBE. Uninfected mosquitoes were not included.
362

363 Next we investigated detection of virus in leg samples of *Ae. aegypti* (Fig. 3). Forty-six leg

364 samples were collected from mosquitoes that were also analyzed for infection in the heads and

365 thoraces to confirm infectious status. We found significant differences between the Ct values for

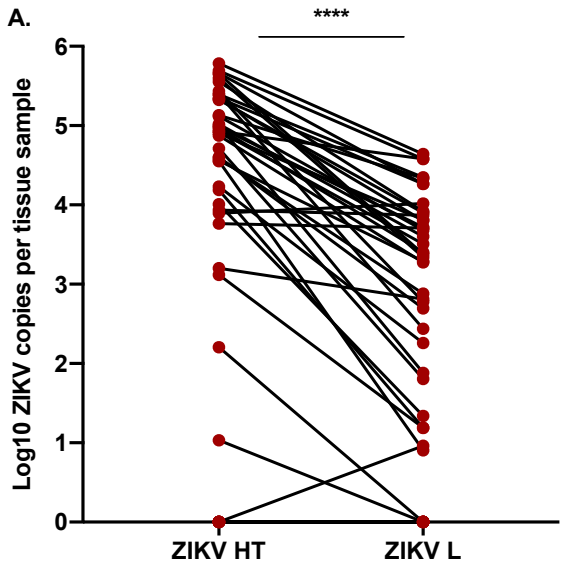
366 the head with thorax samples versus the leg samples. For the leg samples, six false negative

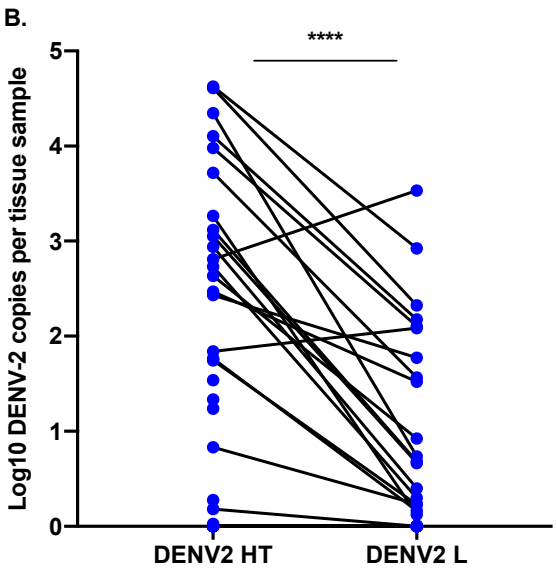
367 sample results were obtained for DENV2, and two false negative results were obtained for

368 ZIKV. One false positive result was detected for ZIKV (Fig. 4). These results indicate that

369 analysis of infection using leg samples is not reliable. No amplification occurred in uninfected

370 *Aedes aegypti* used as a negative control. Because of the false negative results, the DENV2
371 sensitivity of leg samples was 76% (95% CI, 54.87% to 90.64%) and the diagnostic specificity of
372 leg samples was 100% (95% CI, 83.89% to 100.00%). The sensitivity for ZIKV of leg samples
373 was 94.59% (95% CI, 81.81% to 99.34%), and the specificity was 88.89% (95% CI, 51.75% to
374 99.72%). The overall accuracy of the bCUBE qPCR platform for detecting leg samples was
375 93.48% for ZIKV (95% CI, 82.10 to 98.63%) and 86.96% for DENV2 (95% CI, 73.75% to
376 95.06%) (Fig. 4).





Statistic for ZIKV	Value	95% CI
Sensitivity	94.59%	81.81%-99.34%
Specificity	88.89%	51.75%-99.72%
Positive Likelihood Ratio	8.51	1.34-54.12
Negative Likelihood Ratio	0.06	0.02-0.24
Disease prevalence	80.43%	66.09%-90.64%
Positive Predictive Value	97.22%	84.63%-99.55%
Negative Predictive Value	80.00%	50.47%-94.01%
Accuracy	93.48%	82.10%-98.63%

Statistic for DENV2	Value	95% CI
Sensitivity	76.00%	54.87%-90.64%
Specificity	100.00%	83.89%-100.00%
Positive Likelihood Ratio	-	-
Negative Likelihood Ratio	0.24	0.12-0.48
Disease prevalence	54.35%	39.01%-69.10%
Positive Predictive Value	100.00%	-
Negative Predictiv Value	77.78%	63.53%-87.55%
Accuracy	86.96%	73.74%-95.06%

377

378 **Fig. 4.** Infected *Ae. aegypti* (n=46) were collected, and identical samples of head with thorax and
379 legs were amplified for Zika virus (A) and DENV2 (B) using bCUBE-based qRT-PCR. Infection
380 status was compared (****, $p<0.0001$; paired two-tailed t-test).
381

382

383 **bCUBE-based qRT-PCR detects ZIKV and DENV-2 viral RNA in pooled *Ae. aegypti***
384 **samples**
385 Surveillance programs monitoring ZIKV and DENV2 transmission will typically assay the
386 presence of virus in pooled samples ranging from 5 to 50 mosquitoes (50). We pooled individual
387 ZIKV- or DENV2-infected mosquitoes with 5, 10, 15, or 20 uninfected *Aedes* in order to assess
388 the assay's sensitivity with pooled samples. The infection status of individual infected
389 mosquitoes was confirmed by viral RNA extraction using squash buffer followed by bCUBE-
390 based qRT-PCR. Following confirmation of infection, positive mosquitoes were placed into four
391 different pools for further viral detection. ZIKV could be detected in all pooled samples. DENV2
392 could be detected in pools of 5, 10, and 15 mosquitoes, but DENV2 viral RNA could not be
393 detected in one of the three biological replicates of the 20-pooled mosquitoes. We found
394 significant differences between the individual infected samples and pooled samples; however,
395 the detection in pooled samples was still within the range for a positive identification
396 (Supplementary Table 1).
397
398 **bCUBE-based qRT-PCR detects *Wolbachia* in individual *Ae. aegypti***
399 *Wolbachia w*AlbB was amplified from individual mosquitoes with qRT-PCR using the bCUBE
400 platform (n=35) (Supplementary Table 2). Uninfected *Ae. aegypti* (n=10) were used as negative
401 controls. All *Wolbachia* -infected mosquitoes were positive for *Wolbachia* infection, and the
402 negative controls did not amplify. These data suggest that bCUBE qPCR can be used for
403 *Wolbachia* detection employing previously established primers.
404
405 **Discussion**

406 Arboviral pathogens represent an expanding threat across the world. Monitoring the vector and
407 its infectious cargo is essential for planning adequate mosquito control to reduce transmission.
408 Endemic countries frequently lack surveillance resources for monitoring pathogen and vector
409 distribution. Therefore, cost-effective field-deployable pathogen detection methods can greatly
410 facilitate surveillance and control efforts. We sought to develop a method for detecting viral
411 pathogens in their *Aedes aegypti* vector with the potential for field deployment. Currently,
412 pathogen detection in the field is limited by expensive lab-based equipment and reactions and the
413 necessity for highly trained personnel to process samples. Real-time qPCR has long been
414 considered the gold standard for viral detection; however, inadequate access to sample
415 preparation methods and the requirement for bulky equipment render this method incompatible
416 with fieldwork.
417
418 As an alternative to laboratory PCR, we have now explored the use of bCUBE, a novel qPCR
419 platform, with a simple nucleic acid extraction method for detection of arboviruses and
420 *Wolbachia* in mosquito vectors. Actions were taken to limit the cost needed for analysis. We
421 used DNA dye binding qPCR commercial kits as an alternative to hydrolysis probe-based qPCR
422 kits. Furthermore, we extracted DNA/RNA using the squash buffer methodology as opposed to
423 expensive extraction kits. It is important to note that current bCUBE cartridges are restricted to
424 16-wells thereby limiting the number of samples run during each experiment. Hyris bCUBE
425 software can be programmed to display results as positive or negative result as opposed to
426 interpreting cycle threshold and melting curve values. This minimizes training for remote
427 personnel.
428

429 We confirmed the reliability of the bCUBE by comparing its performance against laboratory
430 standards. Therefore, bCUBE is capable of performing real-time qPCR while overcoming the
431 barriers presented by the need for bulky equipment. We then employed a crude extraction
432 method using squash buffer to extract viral DENV2 and ZIKV RNA from mosquitoes for use in
433 the bCUBE system. To address challenges involving primer cross-reactivity with other
434 arboviruses (46, 48) as well as the tendency toward false-positive amplification in negative
435 samples, we tested the specificity of the assay against a panel of frequently co-circulating
436 arboviruses. The results confirmed the assay's specificity for DENV2 and ZIKV RNA, with the
437 potential to apply these primer pairs for field conditions. To negate any non-specific
438 amplification of the host's genetic material, we used a standard curve to determine the limit of
439 detection and subsequently, the cut off values for detecting ZIKV and DENV2. This allowed for
440 positive identification of viral RNA from infected mosquito samples while limiting the
441 amplification of false positive samples as a result of background noise. Following primer
442 optimization, we compared the bCUBE assay to plaque assays for determining infection
443 prevalence. Because qPCR is generally capable of detecting low RNA copy numbers in
444 individual samples, it is important to evaluate the assay's potential for detecting infectious viral
445 RNA rather than viral RNA that is not replicating (49, 51). No significant differences were seen
446 between the bCUBE and plaque assay for three biological replicates, suggesting that the bCUBE
447 assay is able to detect infectious viral RNA.
448
449 We evaluated the use of *Ae. aegypti* leg samples for viral detection. Some infected leg samples
450 turned out to be false negatives suggesting this sample type is unreliable for assaying virus.
451 However, mosquito surveillance agencies typically evaluate arboviral infection status in pooled

452 samples as opposed to assaying individual mosquitoes. ZIKV RNA was detected in pools of 5,
453 10, 15, and 20 with uninfected mosquitoes in three biological replicates. DENV RNA was not
454 detected in one of three biological replicates of 20-mosquito pools, suggesting possible
455 limitations for DENV detection. This highlights the bCUBE platform's potential for pool testing
456 in the field.
457
458 Given RNA's inherent instability and frequency of mutation, it is likely for sequence variability
459 to occur under field conditions. Therefore, it is important to vigilantly monitor the viral strains
460 that are co-circulating in a region to accurately and specifically detect viral RNA in mosquitoes
461 when applying this methodology in the field. It is critical to perform absolute quantification
462 analyses and determine end-point limitation values during optimization to avoid amplification of
463 false positive samples as a result of qPCR background noise. Potential future studies involve
464 bringing the portable platform into the field to analyze its feasibility for performing qPCR under
465 true field conditions. The versatility of the assay can be applied to future studies involving
466 *Plasmodium falciparum* detection in *Anopheles* species and monitoring of various species of the
467 *Anopheles* vector as well as detecting insecticide resistance in field-caught mosquitoes.
468
469 **Conclusion**
470 We have developed a highly sensitive qPCR method for detection and quantitation of DENV2
471 and ZIKV in single mosquitoes using the portable qPCR Hyris bCUBE platform. Our assay
472 allowed for sensitive and reliable DENV2 and ZIKV detection that make it a potential tool for
473 mosquito surveillance programs in endemic countries facing arboviral outbreaks. The detection
474 of *Wolbachia* in *Ae. aegypti* further demonstrates the platform's ability to be used as a multi-

475 detection assay. By overcoming the challenges of costs associated with reagents and equipment,
476 the bCUBE qPCR platform provides a potential field-deployable laboratory resources for
477 mosquito surveillance agencies in remote regions.
478

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486

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