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Adaptation of a microbial detection array as a monitoring tool revealed the presence of mosquito-borne viruses and insect-specific viruses in field-collected mosquitoes

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Running title: A microarray to screen mosquitoes for pathogens in Texas

Abstract

Several mosquito-borne diseases affecting humans are emerging or re-emerging in the United States. The early detection of pathogens in mosquito populations is essential to prevent and control the spread of these diseases. In this study, we tested the potential applicability of the Lawrence Livermore Microbial Detection Array (LLMDA) to enhance bio-surveillance by detecting microbes present in *Aedes aegypti, Aedes albopictus* and *Culex* mosquitoes that are major vector species globally, including in Texas. The sensitivity and reproducibility of the LLMDA was tested in mosquito samples spiked with different concentrations of dengue virus (DENV) revealing a detection limit of >100 but <1000 pfu/mL. Additionally, field-collected mosquitoes from Chicago, Illinois and College Station, Texas of known infection status (West Nile virus (WNV) and Culex flavivirus (CxFLAV) positive) were tested on the LLMDA to confirm its efficiency. Mosquito field samples of unknown infection status, collected in San Antonio, TX and the Lower Rio Grande Valley (LRGV), TX were run on the LLMDA and further confirmed by PCR or qPCR. The analysis of the field samples with the LLMDA revealed the presence of cell fusing agent virus (CFAV) in *Ae. aegypti* populations. *Wolbachia* was also detected in several of the field samples (*Ae. albopictus* and *Culex* spp.*)* by the LLMDA. Our findings demonstrated that the LLMDA can be used to detect multiple arboviruses of public health importance including viruses that belong to the Flavivirus, Alphavirus and Orthobunyavirus genera. Additionally, insect-specific viruses and bacteria were also detected from field-collected mosquitoes. Another strength of this array is its ability to detect multiple viruses in the same mosquito pool allowing for the detection of co-circulating pathogens in an area, and the identification of potential

ecological associations between different viruses. This array can aid in the bio-surveillance of mosquito borne viruses circulating in specific geographical areas.

Importance

Viruses associated with mosquitoes have made a large impact on public and veterinary health. In the US, several viruses including WNV, DENV and chikungunya virus (CHIKV) are responsible for human disease. From 2015-2018, imported Zika cases were reported in the US and in 2016-2017, local Zika transmission occurred in the states of Texas and Florida. With globalization and a changing climate, the frequency of outbreaks linked to arboviruses will increase, revealing a need to better detect viruses in vector populations. With its capacity to detect viruses, bacteria and fungi, this study highlights the ability of the LLMDA to broadly screen field-collected mosquitoes and contribute to the surveillance and management of arboviral diseases.

Introduction

Mosquito-borne viruses emerge and re-emerge at accelerating rates, causing significant morbidity and mortality in humans and animals (1). Due to globalization, mosquito vectors and associated arboviruses have been introduced into new geographic regions (2-5). One noteworthy example was the introduction of WNV into the New World. The virus was first detected in New York in 1999 and then spread throughout the US (6) using several *Culex* species as vectors. The yellow fever mosquito, *Aedes aegypti*, and the Asian tiger mosquito, *Aedes albopictus*, are

70 invasive mosquito species widespread in urban environments of tropical,
71 subtropical, and temperate regions and are responsible for the emergence or re-
72 emergence of multiple mosquito-borne diseases caused by different viral agents
73 including DENV (7-9), CHIKV and, more recently, Zika virus (ZIKV). Since its
74 introduction in Brazil in 2014, ZIKV has spread to the rest of South America, moving
75 north to Central and North America, resulting in the local transmission of the virus
76 in Florida and Texas in 2016-2017 (10-12).

77 These mosquito-borne viruses have proven difficult to manage and control
78 despite considerable attention and the ability to broadly screen mosquitoes for
79 microbes has appeal on many fronts. Microarrays have the ability to detect multiple
80 targets that would be missed by other more specific or targeted assays and could
81 reveal important components of the mosquito microbiome relevant to the
82 transmission of viruses of public and veterinary health importance. Typically,
83 microbial diversity associated with mosquitoes has been studied using both culture-
84 dependent and -independent approaches (13-16). While culture-dependent
85 approaches are time consuming, molecular techniques such as reverse transcription
86 polymerase chain reaction (RT-PCR) (17-19) and quantitative real-time PCR (qRT-
87 PCR) (20-22) are typically designed to be specific at the species or family level. More
88 recently, many new forms of next generation sequencing (NGS) (23, 24) have
89 proven effective to characterize the mosquito microbiome but require the depletion
90 of host derived nucleic acid in order to sensitively detect viruses (25, 26). For
91 bacterial discovery, 16S rRNA sequencing is usually performed (27, 28) but only
92 detects conserved regions of the 16S rRNA gene of bacteria and does not allow for

the detection of viruses and other microbes in the sample. Shotgun metagenomic sequencing provides the highest resolution to detect different kinds of microbes in a sample (29) but remains expensive, time consuming and requires extensive bioinformatic expertise.

Accordingly, this study utilizes the LLMDA, which has been designed to screen diverse samples for thousands of bacteria, viruses, fungi, and protozoa (30, 31). The LLMDA version used in this study detects 10,261 species of microbes including 4,219 viruses, 5,367 bacteria, 293 archaebacteria, 265 fungi, and 117 protozoa (32). The LLMDA has been previously used to detect viral and bacterial pathogens from clinical and archeological samples (30, 33). We conducted a pilot study to evaluate the utility of the LLMDA to screen mosquito pools collected from multiple regions of Texas from 2016 to 2017 for mosquito-borne viruses. The LLMDA was able to detect and identify DENV-2, Rift Valley fever virus (RVFV), Mayaro virus (MAYV) in spiked mosquito samples, and WNV, CxFLAV and CFAV from field-collected mosquitoes. LLMDA results from field-collected mosquitoes were further confirmed using standard and/or quantitative PCR methods, and the co-infection of multiple viruses was detected from spiked and field collected mosquitoes. Viruses were detected from pools of mosquitoes of varying size and tissues including midguts and salivary glands. Additionally, *Wolbachia* was detected from field-collected *Aedes aegypti* and *Culex* mosquitoes.

Results

115 In total, we analyzed 39 mosquito pools representing 512 individual mosquitoes
116 (Table S1). Ten pools were field-collected *Ae. aegypti* (n=116), eight pools were
117 colony-raised *Ae. aegypti* Liverpool (n=80), eight pools were field-collected *Ae.*
118 *albopictus* (n=49), four pools were field-collected *Culex* spp. (n=86), and six pools
119 were field-collected *Cx. quinquefasciatus* (n=138). One pool was colony-raised *Cx.*
120 *quinquefasciatus* (n=10) and one pool was an equal mixture of colony-raised *Ae.*
121 *aegypti* and *Cx. quinquefasciatus* (n=20) to serve as a negative control. To
122 understand the compartmentalization of bacteria within *Ae. aegypti* and *Culex* spp.
123 mosquitoes, four additional pools were analyzed: one pool of 23 midguts (MG) and
124 one pool of 23 salivary glands (SG) for each mosquito species (*Ae. aegypti* and *Culex*
125 spp.).

126 **LLMDA sensitivity and reproducibility**

127 In order to test the LLMDA sensitivity and reproducibility, we spiked known
128 amounts of DENV serotype 2 (DENV-2) in *Ae. aegypti* Liverpool mosquito pools
129 each containing 10 female mosquitoes. Duplicate pools were spiked with 10^2 plaque
130 forming units (pfu/mL) of virus or 10^3 pfu/mL, and two other pools respectively
131 with 10^4 pfu/mL or 10^5 pfu/mL (Table 1). According to our results, the limit of
132 detection or minimum amount of virus required to determine its presence or
133 absence in the sample is equal or less than 10^3 pfu/mL and above 10^2 pfu/mL. The
134 DENV-2 dilutions (10^3 pfu/mL, 10^4 pfu/mL and 10^5 pfu/mL) were all detected using
135 the array, with positive probes hybridizing to different regions of the DENV-2
136 genome (Figure 1A). Because positive signals from more than 20% of the probes for
137 DENV-2 were detected and, in several regions of the genome, these DENV-2 spiked

138 samples are considered DENV positive. As seen on Table 2, the number of positive
139 probes was close to matching the total number of probes present on the array for
140 this target especially for the samples spiked with the highest amount of virus.
141 Additionally, the log CL ratio (ratio between the likelihood of the observed probe
142 signal assuming the target is present in the sample and the likelihood assuming no
143 target is present) was above 0 and therefore considered DENV positive. An increase
144 in the log CL ratio was observed ranging from 56.7 to 224.6 correlating with the
145 increase in amount of spiked virus. The reproducibility of the LLMDA was tested for
146 two of the dilutions in duplicates (10^2 pfu/mL and 10^3 pfu/mL) and showed
147 consistency. For the 10^2 pfu/mL duplicates, no signal was recovered and for the 10^3
148 pfu/mL duplicates the log CI ratio were similar with a respective value of 56.7 and
149 60.7.
150 Samples spiked with the highest amount of DENV (10^5 pfu/mL and 10^4 pfu/mL)
151 were co-infected with a known amount of Mayaro virus (MAYV) (10^4 pfu/mL). Both
152 viruses were successfully detected by the LLMDA (Figure 1A and 1B),
153 demonstrating the ability of the LLMDA to detect viruses from different families if
154 present in the same mosquito sample pool. Additionally, *Cx. quinquefasciatus* spiked
155 with known amount of Rift Valley fever virus **(**RVFV) (10^4 pfu/mL) also resulted in a
156 positive signal, highlighting the ability of the LLMDA to detect other arboviruses of
157 medical and veterinary importance (Figure 1C). The *Ae. aegypti* homogenates spiked
158 with ZIKV tested negative by the LLMDA. First, as seen on Figure 1D, only 3 probes
159 out of the 27 designed to detect ZIKV had a positive signal (the percentage of

positive probes was therefore below the default threshold of 20. Second, the 3 high-intensity probes cover only a specific region of the genome instead of spanning across the genome. And third, the log CL ratio was equal to zero. These spiked samples were confirmed to be ZIKV positive using a qPCR assay with *Ct* values of 20.63 and 28.96 for the samples spiked with 10^4 and 10^2 pfu/mL, respectively. In addition, densoviruses were detected in all of the DENV-2 and MAYV spiked *Ae. aegypti* samples but were further tested by PCR for confirmation (supplemental Table 4).

Application of LLMDA to detection of viruses from field-collected mosquitoes of known infection status

In order to test the ability of the LLMDA to detect natural virus loads within mosquito pools, WNV and CxFLAV naturally infected mosquitoes previously collected in Chicago and College Station were used. Of the two WNV positive mosquito pools previously detected using qPCR (*Ct* values: 15.16 and 19.95), only one was successfully identified as WNV by the LLMDA (Figure 1E). In this particular case, 58 out of the 79 probes that characterized WNV were positive and a log CL score of 115.3 was observed. Interestingly, of these two pools, one was found positive for Culex flavivirus (CxFLAV) by the microarray. In this sample, 19 out of 19 probes were positive (74.4 log CL ratio), revealing the ability of the microarray to detect co-infections from naturally infected mosquito pools (Figure 1F). The two CxFLAV positive controls from College Station (*Ct* values of 18.24 and 30.31) were not detected using the microarray.

Application of the LLMDA to detection of microbes from field-collected mosquitoes of unknown infection status

LLMDA viral analysis

Several viruses were detected in the field-collected mosquito pools (Figure 1). *Ae. aegypti* from LRGV (n=2) and San Antonio (n=1) were found positive for cell fusing agent virus (CFAV), an insect-specific flavivirus (Figure 1G). All the 21 probes designed for that virus on the array were positives (log CL ratio=77). *Aedes aegypti* SGs and MGs pools were also positive for CFAV (log CL ratio=77; positive probe /all target probe =21/21). Interestingly, one *Ae. aegypti* pool from the LRGV was found positive for the avian endogenous retrovirus (23 out of 23 expected probes, log CL ratio=74.9) (Figure 1H). None of the field-collected *Ae. albopictus* or *Culex* spp. tested positive for viruses with the exception of the *Culex* population from Chicago (as described in the previous paragraph). To assess the accuracy of the LLMDA to detect the presence of insect-specific viruses, all samples were tested using conventional PCR methods with gene-specific primers designed for CFAV and CxFLAV (see Table 3 and Table 4). CFAV strain TX AR 11-1022 and CxFLAV strain M23873 obtained from the University of Texas Medical Branch (UTMB) World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) were used as positive controls for the conventional PCR assay. Samples resulting in an amplicon were Sanger sequenced. The CFAV PCR assay confirmed the 5 microarray CFAV positive pools and allowed the detection of 3 additional CFAV positive pools. The CFAV strains detected in the *Ae. aegypti* pools from the LRGV showed 97.7% identity to CFAV strain from Puerto Rico (Accession number: GQ165810) while the CFAV

205 strains from the *Ae. aegypti* population from San Antonio share 100% homology to a
206 CFAV strain from Mexico (Accession number: KJ476731). *Aedes aegypti* SGs and MGs
207 were both confirmed positive for CFAV (Table 4). For CxFLAV, only one of the two
208 positive pools from Chicago identified by the microarray was confirmed positive by
209 conventional PCR. While the microarray was not able to detect any CxFLAV positive
210 in the pools from College Station, these 2 pools were detected as CxFLAV positive by
211 PCR (Table 3). CxFLAV strains from *Cx. quinquefasciatus* (College Station, TX) and
212 *Culex* spp. (from Chicago) show 100% identity to CxFLAV strain isolated from *Culex*
213 *pipiens* in the US (Accession number: KX512322).

214 **LLMDA bacterial analysis**

215 Several *Ae. albopictus* and *Culex* spp. mosquito pools from Texas and Chicago, were
216 found to be naturally infected with *Wolbachia* (*w*) (Figure 2). *Ae. albopictus* from
217 LRGV and San Antonio were infected with the *Wolbachia pipientis Aedes albopictus*
218 strain from the supergroup B (*wAlbB*) (log CL ratio=199.7; positive probe /all target
219 probe =55/59) (Figure 2A). *Culex* spp. mosquitoes from Chicago and Texas (LRGV)
220 were infected with the *Wolbachia pipientis Culex pipiens* strain from supergroup B
221 (*wPip*) (log CL ratio=95.5; positive probe /all target probe =42/58) (Figure 2B). In
222 the San Antonio collection, one pool of *Culex* was found to be infected with *wAlbB*
223 (log CL ratio=199.7; probe detected/expected=55/59) and one pool of *Ae. albopictus*
224 was infected with *Wolbachia pipientis Nasonia vitripennis* from subgroup B (*wVitB*)
225 (log CL ratio=169.6; probe detected/expected=50/56) (Figure 2C). A few other
226 bacteria including *Pseudomonas*, *Klebsiella*, *Erwinia* were detected in various
227 samples (Supplementary Table S4). All mosquito pools identified as positive for

228 *Wolbachia* using the microarray were subject to a *Wolbachia* surface protein (*wsp*)
229 qPCR assay (Table 4). *Ae. albopictus* from the LRGV and San Antonio were
230 confirmed to be harbor *wspB*. Additionally, these samples were found to be positive
231 for the *wspA* gene. Whereas, 2 *Ae. albopictus* pools from San Antonio were found
232 positives with the LLDA, only one was confirm using the for *wsp* qPCR assay. The
233 *Culex* spp. from San Antonio, TX, Chicago, IL and the LRGV were all confirmed
234 positive for the *wspB* gene , with *Ct* values of 23.47, 29.77 and 19.99.
235
236 **Discussion**
237 **Viruses**

238 The LLMDA version used in the study (v7) was developed in 2014 and can
239 detect 4,219 viruses, 5,367 bacteria, 293 archaebacteria, 265 fungi, and 117
240 protozoa. We utilized this platform to evaluate its ability to screen mosquito pools
241 for viruses and other microbes. Our study demonstrates that the LLMDA is a broad
242 screening tool that can be used to detect introduced or emerging pathogens in
243 mosquito populations as well as the presence of other insect-specific viruses and
244 bacteria. The LLMDA is able to generate a comprehensive analysis of microbes
245 circulating in mosquito populations of a specific area that could be used to
246 implement future vector control programs. Because it is highly multiplexed and is
247 based on random amplification, the LLMDA presents advantages over single and
248 multiplexed PCR assays, and a cost and time advantages over next generation
249 sequencing. First, the sensitivity of the array was determined to be above 10^2 and

250 below 10^3 pfu/mL using serial dilution of DENV-2, a virus of major public health
251 importance around the world. The array probes were designed to detect both
252 conserved and unique regions of DENV using whole genome sequences from 3097
253 DENV genomes from all four serotypes of which 403 were specific to DENV-2. The
254 limit of detection of this virus in our array is within the range of viral detection from
255 previous studies using the LLMDA (31, 34) and of other microarrays (35, 36). An
256 interesting feature of the LLMDA is its ability to detect multiple infections from a
257 single sample pool that would normally be missed if a gene-specific PCR approach is
258 used. For example, the LLMDA detected both MAYV and DENV from mosquito pools
259 co-infected with known amounts of both viruses. The LLMDA also successfully
260 detected several viruses in field-collected mosquitoes of known (Table 2) and
261 unknown (Table 3) infection status. For instance, in our study, one *Culex* spp. pool
262 from Chicago, IL, was found to be dually infected with WNV and CxFLAV, which
263 confirms prior studies documenting the co-circulation of these two viruses (37, 38).
264 The presence of several viruses in a mosquito pool does not necessarily mean co-
265 infection in a single mosquito but co-infection of these two viruses has been
266 previously reported (38, 39). Additionally, CxFLAV has been shown to interact with
267 WNV transmission in *Culex* mosquitoes (40). This highlights the ability of the
268 LLMDA to detect and identify two closely related viruses, and viruses from different
269 families within a sample if present.

270 LLMDA and PCR assays both detected the presence of CFAV and CxFLAV in
271 several mosquito pools. When the LLMDAv7 array was designed in 2014, 22

272 CxFLAV sequences and one CFAV genome were publicly available. CxFLAV was
273 detected from *Culex* spp. mosquito pools collected in Chicago, IL, but not in *Cx.*
274 *quinquefasciatus* pools from College Station, TX. The inconsistency of the microarray
275 to detect CxFLAV could be due to the variation in sequence between CxFLAV strains
276 from different geographic origin or from different host species. Here the portion of
277 the NS5 gene sequenced shows a 100% homology to the *Cx. pipiens* strain KX512322
278 but full genome analysis of CxFLAV strains from different localities and different
279 mosquito species have been shown to cluster in two different clades (clade 1 and 2)
280 with all the *Cx. quinquefasciatus* related strains clustering together in clade 2 (41)

281 Additionally, the inconsistency of the results could be due to the difference in
282 sensitivity between the two techniques and the fact that while the conventional PCR
283 relies on the use of gene-specific primers, the microarray relies on the use of
284 random primers during the amplification process. All *Aedes* spp. pools were found
285 to be negative for CxFLAV.

286 CFAV was detected in *Ae. aegypti* from San Antonio, TX and the LRGV, TX. Once
287 again, the conventional PCR allowed the detection of CFAV in two additional
288 samples probably due to the difference in sensitivity between the two techniques.
289 The tissue dissection revealed the presence of CFAV in both the MG and the SG, the
290 two main barriers of arbovirus replication within the mosquito. This tropism
291 suggests its potential for interaction with other viruses present within the mosquito.
292 The ability of CFAV to transmit from one generation to the next (42) as well as its
293 ability to interact with DENV in *Ae. aegypti* cell line (43) makes it a promising

294 candidate for paratransgenesis. *Culex* spp. pools were found to be negative for CFAV.
295 The ability of the LLMDA to detect insect-specific viruses is of interest because it
296 allows the characterization of ecological associations between insect-specific
297 viruses and human pathogens that occur in nature. These could in turn be
298 investigated for the impact of the insect specific virus on the transmission of the
299 human pathogen and serve as potential future vector control strategies.

300 The ZIKV strain PRVABC59 used in this study belongs to the Asian lineage and was
301 not detected using the LLMDA. The LLMDA was designed in 2014, when the only
302 ZIKV sequence available was the MR-766 African lineage strain (accession number:
303 NC_012532.1). Both viral strains share only 87-90% homology (44, 45). Thus, it is
304 likely that the genetic diversity of the PRVABC59 ZIKV strain compared to the MR-
305 766 African strain, did not allow for an efficient detection by the Zika probes present
306 on the LLMDA. This result specifically highlights the need to design additional
307 probes capable of recognizing the more contemporary Asian lineage of ZIKV and
308 more broadly the perpetual need to update the microarray as new viruses or viral
309 strains are discovered or emerge.

310 Overall, this study was able to detect several viral symbionts. In the *Ae.*
311 *aegypti* samples spiked with DENV-2 and/or MAYV, densoviruses were detected but
312 not in the non-spiked sample. This reflects the presence of the densoviruses in the
313 C6/36 cells used to grow the different viruses (46-49). Surprisingly, endogenous
314 avian retrovirus (EAV) was found in one pool of female *Ae. aegypti* collected from an
315 autocidal gravid ovitrap (AGO) from the LRGV. EAV are non-infectious ancient

316 elements of virus that integrated into their host genome and are found in all species
317 of the genus *Gallus* (50, 51). Many homeowners in the communities where mosquito
318 trapping was done have chickens and this result suggests that *Ae. aegypti* had
319 previously fed on chickens or chicken DNA had contaminated the mosquitoes.
320 However, no human pathogen was detected using the LLMDA, presumably, due to
321 our limited set of field samples. In Texas, a total of 381 imported human Zika cases
322 and 10 locally acquired ZIKV cases in the LRGV with 6 cases in 2016 and 4 cases in
323 2017 (11, 12). In this context, the probability of detecting ZIKV infected mosquitoes
324 was low, especially because these mosquitoes were not being collected from or
325 around the homes of human ZIKV cases. The use of the LLMDA for virus detection
326 should be further tested using mosquitoes collected from regions with active
327 arbovirus transmission areas and, if possible, from confirmed or probable human
328 cases households.

329 Although the number of viral species detected in our field samples is low, our results
330 are comparable to other studies using microarrays to determine the virome of field-
331 collected mosquitoes. For example, the study of 10 mosquito pools collected in
332 Thailand revealed the presence of three different viruses: CxFLAV in *Culex*
333 *quinquefasciatus* (n=1), DENV-3 in *Aedes aegypti* (n=1) and Japanese encephalitis
334 virus (JEV) in two pool of *Cx. tritaeniorhyncus* containing respectively 24 and 25
335 mosquitoes (35). Authors using pan viral family primers coupled with conventional
336 PCR also report low numbers of virus positive pools. For example, in a study
337 performed in Puerto Rico, 528 pools representing 1584 mosquitoes lead to the

identification of one insect-specific virus: CFAV in 67 pools (52). Other authors using cell culture (observation of CPE) followed by conventional PCR using pan viral family primers to detect viruses in mosquito samples have rarely detected extensive number of viral species. For example, in a study done in Brazil, researchers collected 950 adult female mosquitoes representing 16 species. From these only two pools tested positive for flavivirus and later identified as Nhumirim virus and Ilheus virus (53, 54).

The LLMDA is able to detect a wide variety of viruses including mosquito-borne RNA viruses and insect-specific RNA viruses, and is able to detect co-infection in mosquito pools, making it an efficient tool for surveillance of known pathogens in under-studied areas such as the LRGV. Given the recent interest of using bacteria or insect-specific viruses as a bio-control tool and the role of co-infection on pathogen transmission, this tool can contribute to better understanding of disease dynamics in a particular region. However periodic updates of probe sequences using genome data from more contemporary strains is necessary to enable detection of emergent RNA virus genomes due to their high mutation rates.

Bacteria

The LLMDA results show the presence of *Wolbachia* in several mosquito pools which was confirmed with qRT-PCR assay targeting the surface protein *wsp. Ae. aegypti,* the primary vector of dengue, Zika, and chikungunya, was found to be negative for the presence of *Wolbachia,* which confirms previous observations (55).

359 The secondary vector of these viruses, *Ae. albopictus,* was found to be infected with
360 *Wolbachia* in 60% of the pools tested. The presence of *Wolbachia* in natural
361 populations of *Ae. albopictus* has been previously reported (56) and *Ae. albopictus* is
362 often found infected with group A (wAlbA) and B strains (wAlbB) as suggested by
363 our results. Additionally, report of superinfection with the two strains has been
364 published (55). *Wolbachia* have been shown to limit DENV transmission (57) and
365 modulate CHIKV replication (58) in *Ae. albopictus*. The current study also detected
366 *Wolbachia* in *Culex* populations from Chicago, San Antonio and the LRGV, confirming
367 previous studies in *Cx. quinquefasciatus* from Australia (O'Neil et al 1992), Brazil and
368 Argentina (59) and other *Culex* spp. in the USA such as *Culex pipiens* (56, 60, 61). The
369 presence of these endosymbionts in field populations in Texas is significant since
370 *w*Pip (Group B) has been reported to induce resistance to WNV in *Cx.*
371 *quinquefasciatus* mosquitoes (62, 63). Because of its impact on transmission of
372 human pathogens, and on the mosquito reproduction, lifespan and resistance to
373 insecticides, knowledge of *Wolbachia* strains circulating in specific areas are needed
374 if *Wolbachia*-based vector control strategies are to be implemented.

375 Overall, the number of bacterial hits in the mosquito pools was lower than
376 expected which might be explained by the lack of sufficient genomic sequences
377 specific to insect related bacterial species available during the array probe design,
378 the low concentration of bacterial species in the samples or the genetic divergence
379 of the bacterial strains present in our samples when compare to bacterial genomes
380 used to develop the microarray. Additionally, the LLMDA was designed using only

full genomes sequences and if at that time only partial bacterial sequences related to the mosquito microbiome were available, they would not have been included on the microarray. Since the development of this array many studies have shown the importance of bacteria (64-66), viruses (67-69) and fungi (70, 71) in the epidemiology of mosquito-borne diseases, demonstrating the need to better characterize the mosquito microbiome. Updating the microarray with probes designed to detect the major components of insects' microbiome could help alleviate the low number of bacterial hits detected in this study. In this study, we wanted to test the LLMDA's ability to detect microbes present in mosquito samples without the need of a targeted enrichment. The LLMDA was successful at identifying viral pathogens without a baited approach, but is not adequate to detect the whole bacterial community. Instead the LLMDA seems to be efficient at detecting dominant bacterial species. *Wolbachia* has been reported to be the dominant member of *Ae. albopictus* and *Culex* mosquitoes (56) and has been successfully detected with the LLMDA. Other bacteria including *Pseudomonas*, *Klebsiella* and *Erwinia* have been detected from *Culex* spp. and *Ae. aegypti* in our samples (Supplemental Table 4) which have already been reported in mosquitoes and their breeding sites (16, 29, 72-75). We also encountered issues related to non-specific probe binding in our samples, mostly to conserved regions of bacteria such as 23S or 16S might also explain the low number of bacterial species. Because we used a stringent threshold of determining a positive signal, i.e., at least 20% of probes were detected for a target sequence, and the criteria that probes should cover various regions of the genome, these non-specific hits were not reported. In our case after removal of non-

specific bacterial hits, *Wolbachia* was the most significant bacterial species confirmed to be present in the mosquito pools. Such challenges have been reported previously in low biomass samples (76). Other approaches, such as shotgun metagenomic sequencing, would be alternative methods to characterize the microbiome.

In summary, to explore the potential usefulness of the LLMDA for bio-surveillance, we took advantage of an on-going mosquito surveillance program along the Texas-Mexico border in the LRGV where ZIKV circulated in 2016-2017 resulting in 10 of local transmission, involving *Ae. aegypti* as the vector (12). A subset of the mosquito collections was tested using the LLMDA and although no pools tested positive for ZIKV, the microarray was able to detect CFAV in *Ae. aegypti* populations from the LRGV and San Antonio that could have an impact on the epidemiology of *Aedes*-vectored viral diseases. Similarly, CxFLAV was observed in several *Culex* populations. *Wolbachia* was detected in high frequency in *Ae. albopictus* and *Culex* spp. mosquitoes but not found in *Ae. aegypti*. Further characterization of the presence and strain types of locally occurring insect-specific viruses and *Wolbachia* is important (77, 78) for possible biological-based control interventions (66, 79, 80). The study presents the broad detection capability, sensitivity and ease of use of the LLMDA approach for surveillance of mosquito-borne diseases of medical importance. This detection array could also aid in the surveillance of pathogens transmitted by other arthropods vectors, such as ticks. The study also demonstrated some limitations of the LLMDA and the need to

develop an improved array including updated viral and bacterial full genomic sequences deposited in GenBank since 2014 for more up to date bio-surveillance studies.

Material and methods

Mosquito samples

Mosquitoes were collected in several locations in Texas (San Antonio and the LRGV) using three trapping methods. Autocidal gravid ovitraps (AGO; SpringStar Inc.), BG sentinel traps (Biogents), and Prokopack aspirators (John W. Hock Co) were used (supplemental Table 1). Whole female mosquitoes were pooled by trap and species with a maximum size of 50 individuals per pool. Additionally, MG and SG of *Ae. aegypti* and *Culex* spp. were obtained by dissection of a subset of mosquitoes from the LRGV and pooled. These specimens were first surface sterilized (5 minutes in 70% ethanol) and rinsed twice in a sterile Phosphate buffered saline (PBS) solution and then individual MG and SG were dissected under a dissecting microscope and rinsed in PBS.

LLMDA sensitivity and reproducibility

Four different viruses were used in this assay; one alphavirus: Mayaro virus (MAYV) strain INHRR11a-10, two flaviviruses: DENV-2 strain INH125271 and ZIKV strain PRVABC59, and one bunyavirus: Rift Valley fever virus (RVFV) strain MP-12. For dengue virus, 100 µL of a 10-fold serial dilution (10^5 pfu/mL -10^2 pfu/mL) of the virus was spiked into *Ae. aegypti* Liverpool strain mosquito homogenate. The dilutions corresponding to 10^2 pfu/mL and 10^3 pfu/mL were done in duplicate to

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assess reproducibility. Additionally, 100 µL of a 10^4 pfu/mL the of MAYV virus was spiked into the mosquito homogenates containing 100 µL of 10^4 pfu/mL and 100 µL of 10^5 pfu/mL of DENV-2. 100 µL of 10^4 pfu/mL of RVFV was spiked into *Cx. quinquefasciatus* pool. One pool of *Ae. aegypti* and *Cx. quinquefasciatus* was used as a negative control. For ZIKV, two dilutions were tested, 10^4 pfu/mL and 10^2 pfu/mL. The ZIKV spiked mosquito pools were tested by the ZIKV reverse transcription quantitative real time PCR assay targeting the non-structural protein 5 (NS5) gene (81, 82) to verify for the presence/absence of infection (Table S2).

LLMDA validation using field-collected sample of known status

WNV positive field-collected mosquitoes from Chicago, IL (2010) and CxFLAV positive field-collected mosquitoes from College Station, TX (2013) were assessed on the LLMDA. These pools had been previously tested positive in other studies using qRT-PCR targeting the envelope genes of WNV and CxFLAV (20, 39).

Mosquito sample preparation and nucleic acid extraction

Three sample preparation methods were tested to evaluate different processing protocols that would optimize recovery of nucleic acid, retain the ability to isolate viruses, and remove surface exogenous nucleic acid. In method 1, mosquitoes were directly homogenized in TRIzol. In method 2, mosquitoes were homogenized in Hank's balanced salt solution (HBSS, Thermo). In method 3, mosquitoes were washed in 70% ethanol for 5 minutes followed by 2 PBS washes. Each mosquito pool was homogenized in a 2 mL microcentrifuge tube containing a single 2.8 mm stainless steel bead. Mosquitoes used for the MG and SG dissection were prepared following the procedure from method 3. Tubes were then centrifuged for 5 minutes

at 15,000 g. Nucleic acids were extracted from 100 µL of the homogenate supernatant using a RNA and DNA TRIzol extraction method.

LLMDA analysis

The LLMDA v7 4x180K microarray consists of probes that targets both conserved and unique genomic regions of sequenced microbial species and has multiple probes per microbial genomic sequence to serve as an internal validation mechanism (34). All samples were analyzed using the LLMDA as described previously (30, 32). Briefly, RNA was reverse-transcribed to cDNA using the phosphorylated random hexamer/SuperScriptIII (P-N6/SSIII) method, which uses the Superscript III Reverse Transcription kit (Invitrogen) and 5′-phosphorylated random hexamers (P-N6) (Eurofins MWG Operon) followed by the Qiagen QuantiTech Whole Transcriptome kit (30, 32). Each sample was loaded onto the LLMDA and allowed to hybridize for 40 h at 55°C in a rotator oven. After hybridization, the microarray was washed following standard manufacturer's protocols with CGH wash buffers (Agilent) and further cleaned using a nitrogen gas stream to remove any particulates from the array surface. The microarray was then scanned and the data analyzed using a statistical method previously described (34). Briefly, the intensity of each probe is transformed into a positive or negative signal. A positive signal is obtained when the intensity of the probe exceeds an intensity threshold set to the 95th percentile of negative controls (33). In other words, if the probe intensity is above the 95th percentile of the sum of the intensity of the random control probes on the array, then that probe is considered to have a positive signal. Given the different parameters used to validate our results, there is still a 5% chance

for a false positive probe signal (100% - 95%). A sample was assigned to a species when at least 20% of all the probes present for this particular species had a positive signal. Since we set a 20% threshold of all probes to assign a species as positive, there is still a certain probability that even with 20% of the probes lighting up, the sample would be a false positive detection.

We then used a likelihood maximization algorithm to identify the target that explains the largest portion of the observed positive probes signal while minimizing the number of negative probe signal. The log likelihood for each of the possible targets was estimated from the BLAST similarity scores of the array feature and target sequences, together with the feature sequence complexity and other covariates derived from the BLAST results as described previously (34).

PCR assay to confirm microarray results

Confirmation of the viral species detected in the field samples from San Antonio and the LRGV was performed by conventional PCR using gene-specific primers amplifying a 206 bp region of NS5 of CxFLAV (39) and a 340bp fragment of CFAV E gene (42). Additionally, presence of *Wolbachia* in the mosquito samples was confirmed using quantitative PCR targeting the *Wolbachia* outer surface protein *wspA* and *wspB* genes (58) (Supplemental Table 3).

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Conflict of Interest

No conflict of interest declared

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814 **Tables**

815 **Table 1:** LLMDA limit of detection and reproducibility in spiked mosquito pools

Virus	Pfu/mL	LLMDA detection	Log Cl ratio	Probes positive/total	Mosquito species
DENV-2	10^2	NEG	-	-	*Ae. aegypti*
	10^2	NEG	-	-	*Ae. aegypti*
	10^3	POS	56.7	20/27	*Ae. aegypti*
	10^3	POS	60.7	23/33	*Ae. aegypti*
DENV-2 + MAYV	10^4; 10^4	POS	197.1; 78.5	46/47; 20/25	*Ae. aegypti*
	10^5;10^4	POS	224.6; 122.3	53/54 ; 25/25	*Ae. aegypti*
RVFV	10^4	POS	52.8	16/19	*Cx. quinq.*
ZIKV	10^4	NEG	0	3/27	*Ae. aegypti*
	10^2	NEG	0	3/27	*Ae. aegypti*

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818 **Table 2:** Comparison of LLMDA and qPCR results in naturally infected mosquito pools

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Virus	qPCR detection	Observed Ct values	LLMDA detection	Log Cl ratio	Probes positive/total	Mosquito species	Additional LLMDA virus detected	Log Cl ratio	Probes positive/total
WNV	POS	15.16	POS	115.3	58/79	*Culex spp.*	CxFLAV	74.4	19/19
	POS	19.95	NEG	-	0/79	*Culex spp.*	CxFLAV	-	0/19
CxFLAV	POS	18.24	NEG	-	0/75	*Cx. quinq.*	-	-	-
	POS	30.31	NEG	-	0/75	*Cx. quinq.*	-	-	-

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822 **Table 3**: LLMDA and conventional PCR detection of field-collected samples

Locality	Mosquito species	Sample size	Virus	LLMDA detection*	PCR detection*	Sanger sequencing %

						identity
LRGV	*Ae. aegypti*	96	CFAV	2(9)	3(9)	97.7% GQ165810
San Antonio	*Ae. aegypti*	33	CFAV	1(2)	2(2)	100% KJ476731
Colony	*Ae. aegypti*	40	CFAV	0(4)	0 (4)	-
LRGV	*Ae. albopictus*	4	CFAV	0(3)	0(3)	-
San Antonio	*Ae. albopictus*	36	CFAV	0 (2)	0(2)	-
College Station	*Ae. albopictus*	9	CFAV	0(3)	0(3)	-
LRGV	*Cx. quinq.*	25	CxFLAV	0(2)	0(2)	-
San Antonio	*Cx. quinq.*	13	CxFLAV	0(2)	0(2)	-
College Station	*Cx. quinq.*	100	CxFLAV	0(2)	2(2)	100% KX512322
Chicago	*Culex spp.*	70	CxFLAV	2(2)	1(2)	100% KX512322
LRGV	*Culex spp*	16	CxFLAV	0(2)	0(2)	-

823 *x(x): number of positive pools out of total number of pools tested

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825 **Table 4:** LLMDA and conventional PCR detection of insect-specific viruses in mosquito midgut and salivary glands

Locality	Mosquito Species	Tissue	n	Virus detected	LLMDA detection*	PCR detection*
LRGV	*Ae. aegypti*	Midgut	23	CFAV	POS (1/1)	POS (1/1)
LRGV	*Ae. aegypti*	Salivary glands	23	CFAV	POS (1/1)	POS (1/1)
LRGV	*Culex* spp.	Midgut	23	CxFLAV	NEG (0/1)	NEG (0/1)
LRGV	*Culex* spp.	Salivary glands	23	CxFLAV	NEG (0/1)	NEG (0/1)

826 (x/x): number of positive pools out of total number of pools tested

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829 **Table 5:** *Wolbachia* detection in field mosquito sample from Texas and Chicago using LLMDA and qPCR using the *wsp* gene.

	LLMDA	qPCR

Locality	Mosquito species	n	Bacteria	detection*	Strain	detection*	*wsp*	Ct values
LRGV	*Ae. albopictus*	4	*Wolbachia*	1(3)	*wAlbB*	1(3)	A+B	25.02; 24.34
San Antonio	*Ae. albopictus*	36	*Wolbachia*	2(2)	*wAlbB,*	1(1)	A+B	19.37; 21.70
					wVitB	1(1)	B	19.99
San Antonio	*Cx. quinq.*	13	*Wolbachia*	1(2)	*wAlbB*	1(2)	B	23.47
Chicago	*Culex* spp.	70	*Wolbachia*	1(2)	*wpip*	1(2)	B	29.77
LRGV	*Culex* spp.	41	*Wolbachia*	1(4)	*wpip*	1(4)	B	19.99

830 *(x/x): number of positive pools out of total number of pools tested

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834 **Figure legends**

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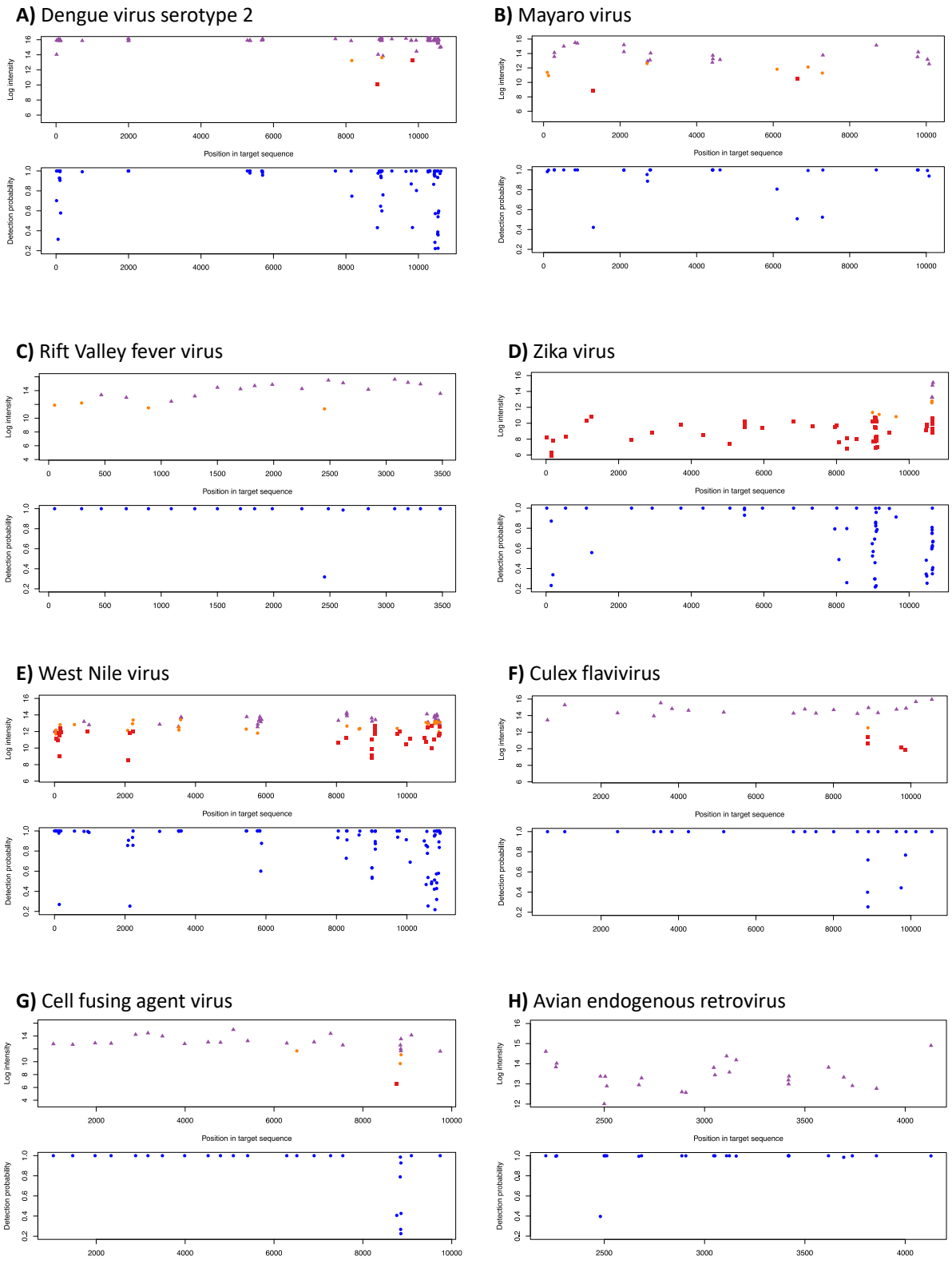
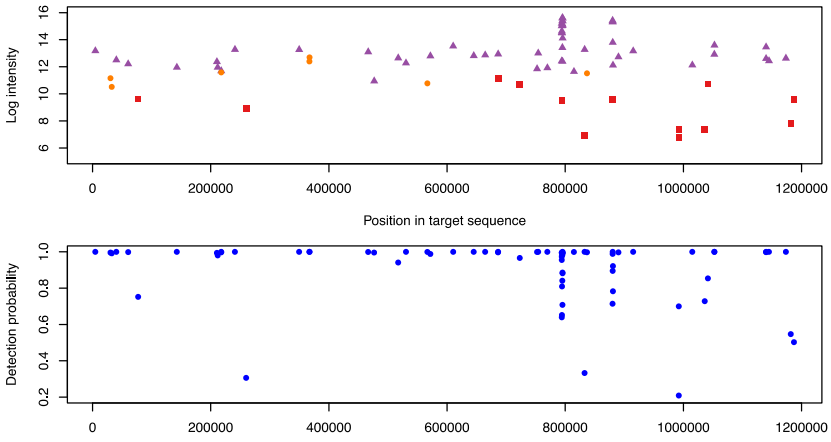
836 **Figure 1**: LLMDA probe detection for A) DENV-2, B) MAYV , C) RVFV, D) ZIKV, E)
837 WNV, F) CxFLAV, G) CFAV, H) Avian endogenous retrovirus. For each virus, two
838 graphs are available, the upper panel represents the intensity of the probes
839 according to the position of the target in the genome; the lower panel represents the
840 probability of detection according to the genome region. Sample for which i)
841 intensity was higher than the 99th percentile of the control probes are shown in
842 purple, ii) intensity is comprised between the 99th and 95th percentile are shown in
843 orange and iii) the probes for which the intensity was below the 95th percentile of
844 the control probes are in red.

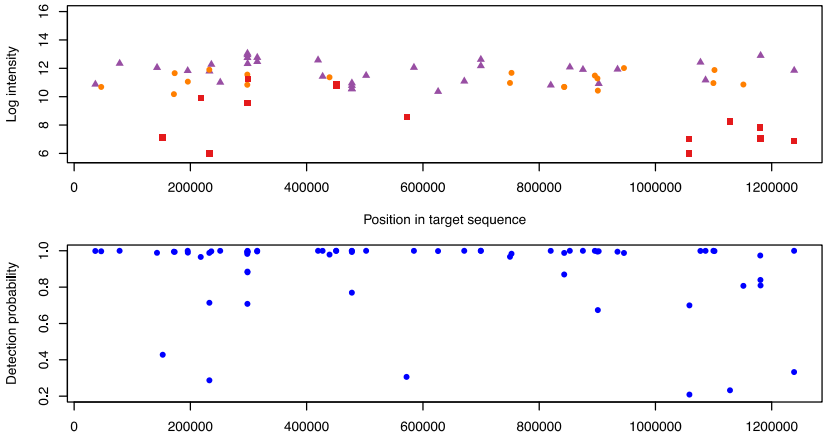
845 **Figure 2**: LLMDA probe detection of *Wolbachia* strains. A) *Wolbachia pipientis*
846 *wAlbB*, B) *Wolbachia endosymbiont wVitB,* C) *Wolbachia* endosymbiont of *Culex*
847 *quinquefasciatus*. For each bacteria, the upper panel represents the intensity of the
848 probes according to the position of the target in the genome (< 99th percentile of
849 control in purple, [95th-99th] in orange, > 95th in red. The lower panel represents the
850 probability of detection according to the genome region
851
852



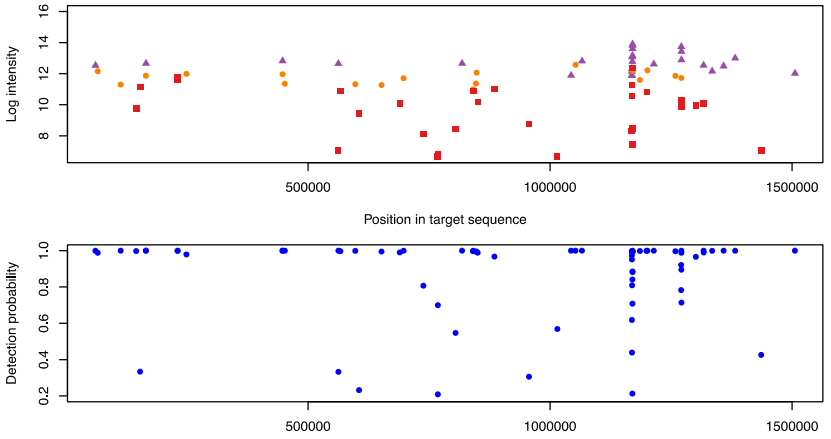
A) *Wolbachia pipientis wAlbB*



B) *Wolbachia pipientis* of *Culex quinquefasciatus*



C) *Wolbachia wVitB* of *Nasonia vitripennis*



Included **Excluded from analysis**

- Intensity above 99th percentile of random controls
- Intensity between 95th and 99th percentiles
- Intensity below 95th percentile