

# One-step pentaplex real-time polymerase chain reaction assay for detection of zika, dengue, chikungunya, West Nile viruses and a human housekeeping gene

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## ABSTRACT

**Background:** Recent emergence of Zika virus (ZIKV), and the global spread of dengue (DENV), chikungunya (CHIKV) and West Nile viruses (WNV) raised urgent need of accurate and affordable molecular diagnosis of these clinically indistinguishable arboviral infections.

**Objectives:** We established a pentaplex real-time reverse transcription PCR (rRT-PCR) assay (CII-ArboViroPlex rRT-PCR) for specific and sensitive detection of the African and American genotypes of ZIKV, all four serotypes of DENV, CHIKV, WNV and a housekeeping gene as internal control in single reaction.

**Study design:** Specific primers and probe sets were designed for ZIKV, DENV, CHIKV, WNV and RNase P (housekeeping gene) and tested for *in-vitro* transcribed RNA standards, virus cultures, clinical samples positive for ZIKV, DENV, CHIKV and WNV and limit of detection (LOD) were determined for each. Results Using ten-fold serially diluted *in-vitro* transcribed RNA, CII-ArboViroPlex rRT-PCR assay has LOD of 100 RNA copies/reaction (Rn) for ZIKV in serum or urine, 100 RNA copies/Rn for DENV in serum, and 10 RNA copies/Rn for CHIKV and WNV in serum. LODs from sera spiked with quantitated viral stocks were  $2.6 \times 10^2$  GEQ/Rn for ZIKV,  $2.2 \times 10^1$  GEQ/Rn for DENV-1,  $9.4 \times 10^0$  GEQ/Rn for DENV-2,  $2.3 \times 10^2$  GEQ/Rn for DENV-3,  $1.4 \times 10^3$  GEQ/Rn for DENV-4,  $2.7 \times 10^2$  GEQ/Rn for CHIKV, and  $1.05 \times 10^1$  GEQ/Rn for WNV.

**Conclusions:** The CII-ArboViroPlex rRT-PCR assay is a quantitative one-step pentaplex rRT-PCR assay for the molecular detection and differential diagnosis of ZIKV, DENV, CHIKV, WNV and a human housekeeping gene control in a single-PCR reaction.

## 1. Background

ZIKV infection is typically asymptomatic or associated with a mild febrile illness but may result in fetal abnormalities during pregnancy or Guillain-Barré syndrome in adults [1, 2]. Infection with DENV results in a broad spectrum of symptoms that range from mild fever to dengue hemorrhagic fever and dengue shock syndrome [3]. Although the

majority of WNV infections are asymptomatic, approximately 1% of cases result in neuroinvasive disease that manifests as aseptic meningitis, encephalitis or flaccid paralysis [4, 5]. CHIKV frequently causes a febrile illness associated with arthralgia and skin rash. Like ZIKV, CHIKV has also been associated with Guillain-Barré syndrome [6, 7].

Through January 2019, more than two million cases of Zika virus

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(ZIKV) infection have been reported globally, including the Americas, Asia, and Oceania [8]. Recent outbreaks in Central and South America, particularly in Brazil, have been associated with neurodevelopmental disorders, including microcephaly in infants born to mothers with a history of gestational infection, as well as Guillain Barre syndrome in infected adults [1,2]. Regions where ZIKV infections are reported are frequently endemic for dengue virus (DENV), chikungunya virus (CHIKV), or West Nile virus (WNV). ZIKV, DENV, and CHIKV are carried by same mosquito vector, *Aedes aegypti* and *Aedes albopictus* [9]. Early clinical manifestations of infection with these viruses are similar and typically comprise a mild febrile illness. Thus, differential diagnosis requires molecular or serological assays. During the acute phase of infection when patients are viremic, real time reverse transcription PCR (rRT-PCR) assays are the mainstay of molecular diagnostics. Many singleplex to triplex PCR assays have been developed in the last few years for detection of ZIKV, DENV, CHIKV and WNV [10–13] but to our knowledge none of the current molecular assays can simultaneously detect both African and American ZIKV strains, all four DENV serotypes, CHIKV, WNV and a human housekeeping control gene for assay validity.

## 2. Objectives

Our main objective focused to establish an affordable assay to differentially diagnose both African and American ZIKV strains, all four DENV serotypes, CHIKV, WNV and also detect a human housekeeping control simultaneously in a single PCR reaction.

## 3. Study design

### 3.1. Primer and probe selection

We aligned 40 complete genomic sequences of ZIKV (available at the time of study) from Africa, Asia and Americas, 37 representative complete genomic sequences of DENV (representing all four serotypes), 20 complete genomic sequences of CHIKV from different geographical locations, and 32 complete genomic sequences of WNV from GenBank using Geneious 10.1.2 software (<https://www.geneious.com>, Biomatters, Auckland, New Zealand) (Supplementary Table 1). Primers and probes for specific detection of viral RNAs were designed and synthesized (Biosearch Technologies, Novato, CA) in the 3' UTR regions of ZIKV and DENV, NSP2 region of CHIKV and NS5 region of WNV genomes (Table 1). Primers and a probe for the detection of a human housekeeping gene, RNase P, were also selected.

**Table 1**

. Primer and probe sets, linked reporter fluorophores and quenchers used for detection of ZIKV, DENV, CHIKV, WNV and RNase P in the CII-ArboViroPlex rRT-PCR assay.

Prime and Probe name	Gene target	"Sequence (5' to 3')	Length (bp)	Probe modifications	
				5'-modification (dye)	3'-modification (quencher)
ZIKA.3 P.QF	3' UTR	ARTGTTGTCAGGCCTGCTAG	20		
ZIKA.3 P.QR	3' UTR	CTTGRTTTCAGGCKTCTCT	21		
ZIKA.3 P.Probe.R610	3' UTR	GGGAAAGCTGTGCAGCCTGT	22	CAL Fluor Red 610	BHQ-2
DENV.3 P.QF	3' UTR	ACTAGAGGTTAGAGGAGACCCCC	23		
DENV.3 P.QRA	3' UTR	GGCGCTCTGTGCCTGGATT	19		
DENV.3 P.QRB	3' UTR	TGGCGTTCTGTGCCTGGAAT	20		
DENV.3 P.Probe.O560	3' UTR	CCCAGCGTCAATATGCTGT	19	CAL Fluor Orange 560	BHQ-1 plus
CHIK.NSP2.NQF	NSP2	CATCTGCACYCAAGTGTACCA	21		
CHIK.NSP2.NQR	NSP2	GCGCATTTTGCCTTCGTAATG	21		
CHIK.NSP2.Probe.FAM	NSP2	AAAAGTATCTCCAGGCGG	18	FAM	BHQ-1 plus
WNV.NS5.NQF	NS5	CATTTGCTCCGCTGTCCCTGTGAA	24		
WNV.NS5.NQR	NS5	CCACTCTCCTCCTGCATGGATGGAC	25		
WNV.NS5.Probe.Q670	NS5	TGGGTCCTACCGAAGAACCACGT	25	Quasar 670	BHQ-2
RNASE-P-QF	RPP30	AGATTTGGACCTGCGAGCG	19		
RNASE-P-QR	RPP30	GAGCGGCTGTCTCCACAAGT	20		
RNASE-P.Probe.Q705	RPP30	TTCTGACCTGAAGGCTCTGCGCG	23	Quasar 705	BHQ-3

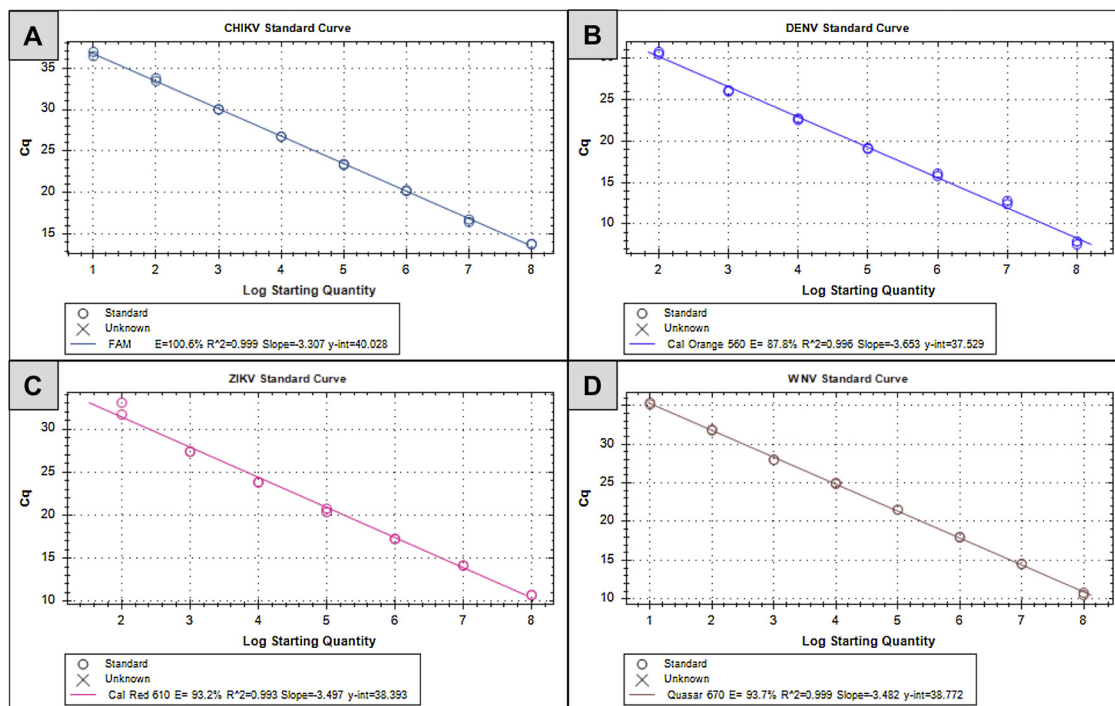
Primer and probe sequences from the targeted viruses were mapped amongst each other to test homology and avoid non-specific detection (Supplementary Table 1). All primer and probe sequences showed 100% sequence identity with their expected targets. Furthermore, specificity for the cognate templates was also assessed *in silico* comparison to other agents that may cause febrile illness but were not available for laboratory testing (Supplementary Table 2). All primers and probes were evaluated using the BLASTn algorithm (NCBI). We did not identify > 80% nucleotide homology with any of our primer or probe sequences. Empirical testing of cross-reactivity of the primers and probes was performed by testing additional viruses and bacteria (Supplementary Table 3) which may cause febrile illness. No amplification was observed with any of the templates, providing no evidence of potential false positive results due to closely related organisms.

### 3.2. Plasmid synthesis and generation of *in vitro* transcribed RNA standard curves

The 249 bp long 3' UTR region of ZIKV (ArD165531), the 284 bp long 3' UTR region of DENV-3 (449335\_Boyaca\_CO\_2015), the 245 bp long NSP2 region of CHIKV (S27-African prototype), and the 174 bp long NS5 region of WNV (HNY1999) were commercially synthesized and cloned into pUC57 plasmid DNA Vector (GenScript, USA). Plasmids were digested using BamHI and XbaI restriction enzymes to release target segments (New England Biolabs) which included a T7 promoter site on 5' end after digestion (Supplementary Fig. 1). Gel purified target inserts were quantified by NanoDrop (ThermoFisher) and used as templates for *in vitro* transcription with RiboMAX Large Scale RNA Production Systems (Promega, USA). *In vitro* transcribed RNA was DNase-treated, purified with Trizol (ThermoFisher), and quantitated by NanoDrop. Based on optical density and insert size, RNA copy numbers (RNA copies) were calculated and serially diluted ( $10^9$ – $10^0$  RNA copies/μL) in distilled water with a background of yeast tRNA (50 ng/μL).

### 3.3. Assay performance controls

*In-vitro* transcribed synthetic RNA standards for ZIKV, DENV, CHIKV and WNV (serial dilutions of  $10^5$ – $10^0$  RNA copies/Rn) were used as virus positive controls. Sterile, nuclease-free water was used as a no template control (NTC). Extracted total nucleic acid from a human cell culture preparation known to contain RNase P was used as RNase P control/rRT-PCR control. Each of the rRT-PCR plate (experiment) contained positive control, negative control and rRT-PCR control.



**Fig. 1.** Standard curves for CHIKV (A), DENV (B), ZIKV (C) and WNV (D) were generated from Ct values obtained for serial 10-fold dilutions plotted against the amounts of standard RNA copy number. All the assays were performed in duplicate.

### 3.4. Clinical specimens

Viral RNA positive clinical specimens (serum or urine) were obtained from patients who were ZIKV, DENV, CHIKV or WNV RNA positive using established rRT-PCRs [14–17]. These samples included sera from 41 patients with ZIKV, 24 patients with DENV (4 DENV-1, 4 DENV-2, 6 DENV-3, 3 DENV-4 and 7 DENV of unknown serotype), 20 patients with CHIKV, and 19 patients with WNV infection. We also obtained urine from 24 patients with ZIKV infection. Sixty one sera and 50 urine samples from patients with a febrile illness not attributed to infection with ZIKV, DENV, CHIKV or WNV were used as negative clinical controls.

### 3.5. Nucleic acid extraction

Total nucleic acid (TNA) was extracted from 250  $\mu$ L of tissue culture supernatant, serum or urine by using the NucliSENS easyMAG automated extraction platform (bioMérieux). TNA was eluted in 50  $\mu$ L of elution buffer and stored at  $-80^{\circ}\text{C}$  until further use.

### 3.6. Multiplex one-step real-time RT-PCR assay

The CII-ArboViroPlex rRT-PCR assay includes primers and dual-labeled BHQ probes (Biosearch Technologies, Novato, CA) to detect RNA from ZIKV, DENV, CHIKV, WNV and RNase P. Dual-labeled BHQ probes are linear, incorporating a fluorophore and quencher covalently attached to the 5' and 3' ends of the oligonucleotide, respectively. One-step rRT-PCR reactions were performed in 25  $\mu$ L volumes containing all primers and probes as described in the Supplementary Table 4. Ten microliters of purified *in-vitro* transcribed RNA or extracted TNA from specimens were reverse transcribed and amplified using RNA UltraSense One-Step Quantitative RT-PCR System (ThermoFisher) with thermal cycling and detection on the CFX96 Real-Time PCR Detection System (Bio-Rad). The thermal cycling conditions were used as follows; a) reverse transcription step at  $50^{\circ}\text{C}$  for 30 min, b) reverse transcription inactivation and Taq polymerase enzyme activation at  $95^{\circ}\text{C}$  for 2 min, c) followed by 45 cycles of qPCR at  $95^{\circ}\text{C}$  for 15 s (denaturation) and, d)

$60^{\circ}\text{C}$  for 1 min (annealing and extension). Fluorescence signal intensity was detected after completion of each qPCR cycle and data was collected using CFX Manager Software.

### 3.7. Virus strains, cell culture, virus amplification, and concentration

ZIKV (strain PRVABC59), DENV type 1 (strain Hawaii), DENV type 2 (strain New Guinea C), DENV type 3 (strain H87), DENV type 4 (Strain H241), CHIKV (strain R80422) and WNV (strain HNY1999) were cultured for this study (Supplementary methods, Supplementary table 5). Genomic equivalent quantities (GEQ) were estimated as viral copy number/ Rn of each viral supernatant and quantified using serial dilutions of quantitated *in vitro* T7-transcribed RNA standards diluted in yeast t-RNA (50 ng/ $\mu$ L) as background.

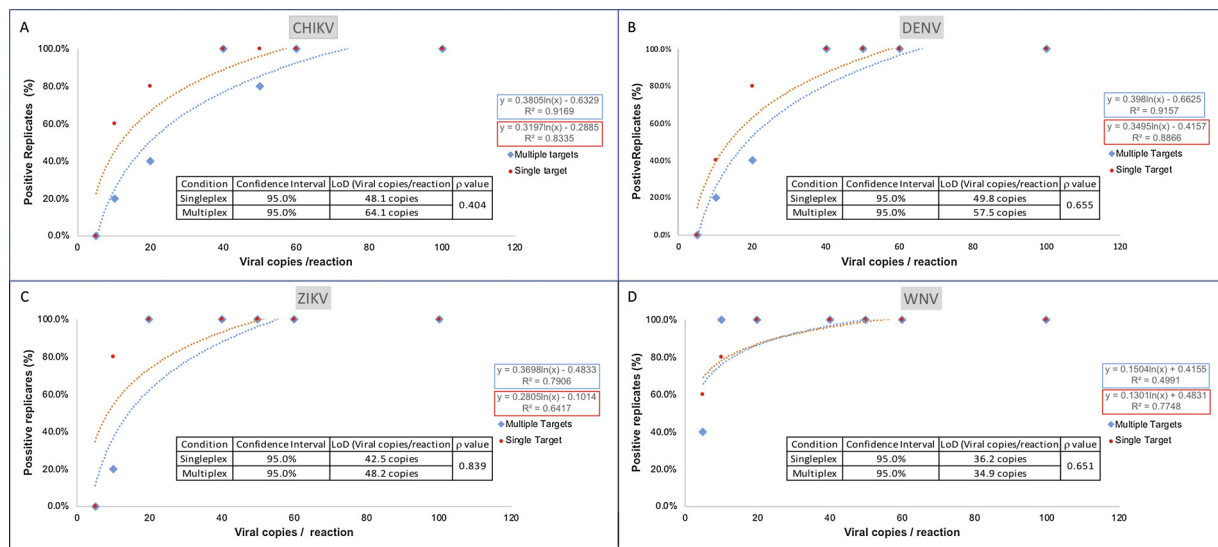
## 4. Results

### 4.1. Assay establishment

The assay comprises positive, negative, and rRT-PCR controls. The assay runs were considered valid only when amplification products were obtained for the relevant positive controls and not for irrelevant viral template targets. The sample was considered positive if the rRT-PCR amplification curve, based on increasing fluorescence for a primer/probe set, crossed the threshold within ( $\leq$ ) 38.00 cycles. If the amplification curve for a primer probe set crossed the threshold above ( $>$ ) 38 cycles, the sample result was considered negative. The Ct value '38' cutoff was selected because lowest dilution of *in-vitro* transcribed RNA standards for ZIKV, DENV, CHIKV and WNV were consistently positive in duplicate runs with similar Ct values and proper sigmoid amplification curve.

### 4.2. Estimation of the limit of detection (LOD)

Analytical sensitivities were determined by testing serial dilutions (ten-folds) of RNA standards for all four viruses. Ten microliters of serially diluted ( $10^7$ – $10^0$  copies) RNA standards for each virus were tested



**Fig. 2.** Probit regression analysis of singleplex versus multiplex CII-ArboViroPlex rRT-PCR. Ten replicates of 10,000, 5000, 1,000, 500, 250, 100, 60, 50, 40, 20, 10 and 5 of *in vitro* transcribed viral RNA copies were run in CII-ArboViroPlex assays in singleplex format (one template per assay; red dot symbols) or in multiplex format (all four viral templates per assay; blue diamond symbols). Probit analysis was performed by MATLAB v12 and trendline curves were calculated in Microsoft Excel v16.16.12 for CHIKV (A), DENV (B), ZIKV (C) and WNV (D). LoDs at 95% CI and p-values (MATLAB) were calculated comparing singleplex and multiplex results. Curves are shown for the range of 5–100 copies/Rn; for 250 to 10,000 copies/Rn all replicates (100%) were positive with comparable Ct values in all cases (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

in duplicate with the rRT-PCR reaction mix (Supplementary Table 4). The highest RNA dilution positive in duplicates was considered as limit of detection (LOD) for that virus. The LOD for CHIKV and WNV RNA standards was 10 copies/Rn. The LOD for DENV and ZIKV RNA standards was 100 copies/Rn. The coefficient of determination ( $R^2$  values) for each assay was greater than 0.99 in all cases (0.999 for CHIKV, 0.996 for DENV, 0.993 for ZIKV, and 0.999 for WNV; Fig. 1).

To estimate assay performance in the window between 10–100 copies/Rn and in the presence of competing templates, assays with one template (singleplex) and all four viral templates present (multiplex) were performed at 5, 10, 20, 40, 60, 100, 250, 500, 1,000, 5000 and 10,000 copies/Rn with 10 replicates each. Probit regression analysis [18, 19] indicated comparable performance for all four viruses (ZIKV, DENV, CHIKV and WNV) in singleplex and multiplex as indicated by closely matched LoD values at 95% confidence interval (CI) detection (Fig. 2). Since cases of infection with two or more viruses have been reported [20–23], these results suggest sensitive detection of simultaneous infection even with multiple viruses.

Serially diluted ( $10^5$ – $10^2$  copies/Rn) synthetic RNAs were tested in triplicate and compared with extracted TNA from virus culture supernatants using similar viral concentrations (GEQ/Rn). Synthetic *in vitro* transcribed RNA and TNA extracts from cultured virus generated comparable Ct values for the matching dilutions (Supplementary Fig. 2).

LODs of viral RNA in clinical samples were estimated by serial dilutions of virus stocks (Supplementary table 5) spiked in single donor human serum (SDHS). For TNA extraction, 225  $\mu$ L of SDHS aliquots were spiked individually with 25  $\mu$ L of each serially diluted virus stock. The spiked serum mixes (250  $\mu$ L in total) were extracted in triplicate and eluted in 50- $\mu$ L of elution buffer. 10  $\mu$ L of TNA was tested in triplicate and the lowest concentration at which all replicates were positive was determined as the tentative LOD (GEQ/Rn) for that virus. Only results with a positive RNase P signal were included. The LODs were  $2.6 \times 10^2$  GEQ/Rn for ZIKV,  $2.2 \times 10^1$  GEQ/Rn for DENV-1,  $9.4 \times 10^0$  GEQ/Rn for DENV-2,  $2.3 \times 10^2$  GEQ/Rn for DENV-3,  $1.4 \times 10^3$  GEQ/Rn for DENV-4,  $2.7 \times 10^2$  GEQ/Rn for CHIKV, and  $1.05 \times 10^1$  GEQ/Rn for WNV (Table 2). Based on the tentative LODs, 20 replicates of viral stock spiked into SDHS at the LOD were processed. The LOD from urine samples spiked with quantitated ZIKV viral stock was  $5.6 \times 10^2$  GEQ/

Rn. Based on the tentative LOD 20 replicates of viral stock spiked into single donor urine sample at LOD were processed. All samples were positive (Ct 31.01–33.51), yielding a > 95% confidence for detection (Table 2).

### 4.3. Assay assessment with clinical samples

#### 4.3.1. Negative specimens

All negative specimens were tested with the CII-ArboViroPlex rRT-PCR assay, the comparator CDC-Triplex (ThermoFisher) assay, and a published WNV qPCR [16] assay. None of these assays showed signal for ZIKV, DENV, CHIKV or WNV (Table 3). RNase P was positive for all the negative specimens by CII-ArboViroPlex rRT-PCR and CDC-Triplex assays.

#### 4.3.2. Positive Specimens

Thirty-nine of 41 sera and 25/26 ZIKV positive urine samples were positive for ZIKV in the CII-ArboViroPlex rRT-PCR assay. All 67 were negative for DENV, CHIKV and WNV. All 24 DENV positive sera were positive for DENV in the CII-ArboViroPlex rRT-PCR assay. All 24 were negative for ZIKV, CHIKV and WNV. All 20 sera positive for CHIKV were found positive for CHIKV in the CII-ArboViroPlex rRT-PCR assay. All 20 were negative for ZIKV, DENV and WNV. All 19 sera that were WNV positive were positive for WNV in the CII-ArboViroPlex rRT-PCR assay. All 19 were negative for ZIKV, DENV and CHIKV (Table 3).

Potential cross binding of individual primers or probes that share nucleotide identity to other agent's sequences may occur in some instances and affect the sensitivity of multiplex PCR assays [20]. To address this concern, we performed an interference experiment for ZIKV and DENV targets. DENV-3 P.F primer, DENV.3 P.PROBE had ~80% identity to ZIKV sequence and ZIKA.3 P.PROBE had ~70% identity to DENV sequence (Supplementary Table 1). Two different concentrations of ZIKV and DENV (high concn =  $1000 \times$  LoD and low concn =  $3 \times$  LoD) were spiked in SDHS and tested in triplicate after extractions with CII-ArboViroPlex rRT-PCR assay as single agent and also as co-infection in same sample well. No noticeable difference in Ct values were recorded despite the high sequence identity with individual primers/probes and in the presence of the low and high backgrounds of non-cognate viral templates (Table 4). We did not detect any signal for



**Table 2**

Determination of the presumptive and final limit of detection (LOD) of the ZIKV, DENV 1, DENV 2, DENV 3, DENV 4, CHIKV and WNV for the CII-ArboViroPlex rRT-PCR assay with serum specimens and ZIKV with urine specimens.

	ZIKV	DENV 1	DENV 2	DENV 3	DENV 4	CHIKV	WNV
<b>LoD</b>							
Virus concn (Copies/reaction)	2.58E + 02	2.19E + 01	9.35E + 00	2.32E + 02	1.44E + 03	2.70E + 02	1.05E + 01
Dilution factor from stock	1.00E – 05	1.00E – 05	1.00E – 05	1.00E – 05	1.00E – 05	1.00E – 05	1.00E – 06
<b>Confirmation of the LoD</b>							
Average Ct (20 replicates)	31.81	32.87	32.43	31.01	31.2	31.52	33.51
standard deviation	0.76	0.8	0.93	0.81	0.52	0.28	0.48
Call rate	20/20	20/20	20/20	20/20	20/20	20/20	20/20
Call rate %	100%	100%	100%	100%	100%	100%	100%

CHIKV or WNV. Based on our experiment we do not anticipate sensitivity of the assay will be compromised in presence of more than one agent in the same sample.

The concordance for results obtained using the CII-ArboViroPlex rRT-PCR assay and other established assays including the LightMix Zika rRT-PCR test (Roche), Trioplex Assay (Thermofisher), NYS-approved LDT assays [14], CDC-Dengue virus real-time RT-PCR assay [15], and WNV-NS3 RT-PCR Assay [16] is shown in Supplementary Table 6. All of the samples were also tested with the CDC-Trioplex-Assay (Thermo-fisher) and a WNV-NS3 RT-PCR Assay [16]. The positive percentage agreement was 95.1% for ZIKV serum assays, 96.2% for ZIKV urine assays, and 100% for DENV, CHIKV and WNV serum assays.

**5. Discussion**

Differentially diagnosing ZIKV, DENV, CHIKV, and WNV infection is important for epidemiology and risk assessment. As additional antiviral interventions are developed, we anticipate that differential diagnosis may also become important for clinical management. Molecular methods are rapidly replacing culture in viral diagnostics due to advantages in cost, sensitivity, ease of use and time to result. Virus isolation from a clinical sample is the gold standard for detection, but is a laborious and time-consuming method. Virus culture at reference labs is also not clinically feasible because it requires extensive laboratory setup and these methods are time consuming and exhaustive. There are several singleplex and multiplex RT-PCR assays for detection of arboviruses [24]; however, the CII-ArboViroPlex rRT-PCR assay has the

**Table 3**

Clinical Performance of CII-ArboViroPlex rRT-PCR assay for ZIKV, DENV, CHIKV and WNV in Serum and for ZIKV in urine.

Category	ZIKV Positive	DENV Positive	CHIKV Positive	WNV Positive
<b>Positive Controls</b>				
ZIKV Positive (Serum) n = 41				
CII ArboViroPlex	39/41 <sup>a</sup>	0/41	0/41	0/41
Comparator assays#	41/41	0/41	0/41	0/41
ZIKV Positive (Urine) n = 26				
CII ArboViroPlex	25/26 <sup>a</sup>	0/26	0/26	0/26
Comparator assays#	26/26	0/26	0/26	0/26
DENV Positive (n = 24 serum)				
CII ArboViroPlex	0/24	24/24	0/24	0/24
Comparator assays#	0/24	24/24	0/24	0/24
CHIKV Positive (n = 20 serum)				
CII ArboViroPlex	0/20	0/20	20/20	0/20
Comparator assays#	0/20	0/20	20/20	0/20
WNV Positive (n = 19 serum)				
CII ArboViroPlex	0/19	0/19	0/19	19/19
Comparator assays#	0/19	0/19	0/19	19/19
<b>Negative Controls</b>				
Negative Controls(n = 61 serum)				
CII ArboViroPlex	0/61	0/61	0/61	0/61
Comparator assays#	0/61	0/61	0/61	0/61
Negative (n = 50 urine)				
CII ArboViroPlex	0/50	0/50	0/50	0/50
Comparator assays#	0/50	0/50	0/50	0/50
<b>Positive agreement (Serum)</b>				
Positive % agreement	(39/41)	(24/24)	(20/20)	(19/19)
95% CI	95.10%	100.00%	100.00%	100.00%
Negative agreement (Serum)	89.8–99.2%	93.5–100.0%	92.3–00.0%	92.1–100.0%
Negative % agreement	(124/124) <sup>b</sup>	(141/141) <sup>b</sup>	(145/145) <sup>b</sup>	(146/146) <sup>b</sup>
95% CI	100.00%	100.00%	100.00%	100.00%
Positive agreement (Urine)	97.1–100.0%	96.1–99.9%	97.5–100.0%	97.5–100.0%
Positive percent agreement	(25/26)	NA	NA	NA
95% CI	96.20%			
Negative agreement (Urine)	89.9–99.7%	NA	NA	NA
Negative % agreement	100%			
95% CI	(50/50)			
	92.9–100.0%			

<sup>a</sup> Individual Ct value for each sample is provided in Supplementary Table 6.

<sup>b</sup> Includes clinical positive specimens for other viral targets as additional negatives, e.g. for Zika virus the CHIKV, DENV and WNV positive specimens were included as negatives in the Zika virus performance.

**Table 4**Interference experiment between ZIKV and DENV primer/probe sets of the CII-ArboViroPlex rRT-PCR assay (*in vitro* testing).

	Ct values in ZIKV (Cal fluor red 610) channel				Ct values in DENV (Cal fluor orange 560) channel			
	ZIKV 1 Ct	ZIKV 2 Ct	ZIKV 3 Ct	Average ZIKV Ct	DENV 1 Ct	DENV 2 Ct	DENV 3 Ct	Average DENV Ct
High ZIKV concn (1000 × LoD)	18.67	18.75	18.15	18.52	ND	ND	ND	ND
Low ZIKV concn (3 × LoD)	30.32	30.55	30.20	30.36	ND	ND	ND	ND
High DENV concn (1000 × LoD)	ND	ND	ND	ND	18.17	18.56	17.65	18.13
Low DENV concn (3 × LoD)	ND	ND	ND	ND	29.50	30.10	30.50	30.03
High ZIKV (1000 × LoD) + Low DENV (3 × LoD)	19.13	18.87	18.85	18.95	29.80	30.10	29.95	29.95
Low ZIKV (3 × LoD) + High DENV (1000 × LoD)	30.15	30.66	31.44	30.75	18.20	18.65	18.50	18.45

advantage of allowing simultaneous detection and quantitation of ZIKV, DENV (all 4 serotypes), CHIKV, and WNV. In addition, it detects a human housekeeping gene (RNase P) as an internal control for ensuring the integrity of RNA template and overall assay performance. Out of 67 ZIKV positive clinical samples, 2 sera and 1 urine sample that were negative at 38 cycles in the CII-ArboViroPlex rRT-PCR assay were found to be positive with comparator assays (1 sample each in LightMix Zika rRT-PCR test, Wadsworth Center New York State-approved ZIKV assay, and the Triplex assay). These samples were positive at 38.63, 38.40 and 38.05 cycles respectively in the CII-ArboViroPlex rRT-PCR assay. We speculate that freeze-thaw cycles may have caused degradation of template. Based on its performance characteristics the CII-ArboViroPlex rRT-PCR assay received emergency use authorization by the FDA for detection of ZIKV, DENV, WNV and CHIKV in serum and for ZIKV in urine [25].

## 6. Conclusions

We developed a quantitative one-step pentaplex rRT-PCR assay “CII-ArboViroPlex rRT-PCR” for the molecular detection and differential diagnosis of ZIKV, DENV, CHIKV and WNV and a human housekeeping gene (RNase P) which ensures assay validity in single-PCR reaction.

## CRedit authorship contribution statement

**Nischay Mishra:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **James Ng:** Methodology, Software, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Jennifer L. Rakeman:** Resources. **Michael J. Perry:** Resources. **Dominick A. Centurioni:** Resources. **Amy B. Dean:** Resources. **Adam Price:** Data curation. **Riddhi Thakkar:** Data curation. **Andreina Garcia Angus:** Validation. **Phillip Williamson:** Resources. **Eric Delwart:** Resources. **Christine Carrington:** Resources. **Nikita Sahadeo:** Resources. **Xiaoyu Che:** Formal analysis. **Thomas Briese:** Writing - original draft. **Rafal Tokarz:** Writing - original draft. **W. Ian Lipkin:** Conceptualization, Writing - original draft, Funding acquisition.

## Declaration of Competing Interest

NM, WIL, RT and TB are listed as inventors in patent application No. PCT/US2017/023012 that covers the CII-ArboViroPlex rRT-PCR assay. The remaining authors declare no competing interests.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jcv.2019.08.011>.

## References

- [1] F. Krauer, M. Riesen, L. Reveiz, O.T. Oladapo, R. Martinez-Vega, T.V. Porgo, A. Haefliger, N.J. Broutet, N. Low, Group WHOZCW, Zika virus infection as a cause of congenital brain abnormalities and Guillain-Barre syndrome: systematic review, *PLoS Med.* 14 (2017) e1002203.
- [2] S.A. Rasmussen, D.J. Jamieson, M.A. Honein, L.R. Petersen, Zika virus and birth defects—reviewing the evidence for causality, *N. Engl. J. Med.* 374 (2016) 1981–1987.
- [3] J.L. Slon Campos, J. Mongkolsapaya, G.R. Screaton, The immune response against flaviviruses, *Nat. Immunol.* 19 (2018) 1189–1198.
- [4] T. Briese, K.A. Bernard, West Nile virus—an old virus learning new tricks? *J. Neurovirol.* 11 (2005) 469–475.
- [5] J.J. Sejvar, West Nile virus infection, *Microbiol. Spectr.* 4 (2016).
- [6] T.J. Pinheiro, L.F. Guimaraes, M.T. Silva, C.N. Soares, Neurological manifestations of chikungunya and zika infections, *Arq. Neuropsiquiatr.* 74 (2016) 937–943.
- [7] G. Pialoux, B.A. Gauzere, S. Jaureguiberry, M. Strobel, Chikungunya, an epidemic arbovirolosis, *Lancet Infect. Dis.* 7 (2007) 319–327.
- [8] PAHO-WHO, Zika Suspected and Confirmed Cases Reported by Countries and Territories in the Americas Cumulative Cases 2015–2018 (2018) PAHO Website <http://www.paho.org/zikavirus>.
- [9] E.B. Kauffman, L.D. Kramer, Zika virus mosquito vectors: competence, biology, and vector control, *J. Infect. Dis.* 216 (2017) S976–S990.
- [10] J.J. Waggoner, L. Gresh, M.J. Vargas, G. Ballesteros, Y. Tellez, K.J. Soda, M.K. Sahoo, A. Nunez, A. Balmaseda, E. Harris, B.A. Pinsky, Viremia and clinical presentation in Nicaraguan patients infected with Zika virus, chikungunya virus, and dengue virus, *Clin. Infect. Dis.* 63 (2016) 1584–1590.
- [11] W. Wu, J. Wang, N. Yu, J. Yan, Z. Zhuo, M. Chen, X. Su, M. Fang, S. He, S. Zhang, Y. Zhang, S. Ge, N. Xia, Development of multiplex real-time reverse-transcriptase polymerase chain reaction assay for simultaneous detection of Zika, dengue, yellow fever, and chikungunya viruses in a single tube, *J. Med. Virol.* 90 (2018) 1681–1686.
- [12] D.C. Nyan, K.L. Swinson, A novel multiplex isothermal amplification method for rapid detection and identification of viruses, *Sci. Rep.* 5 (2015) 17925.
- [13] E.P. Calvo, F. Sanchez-Quete, S. Duran, I. Sandoval, J.E. Castellanos, Easy and inexpensive molecular detection of dengue, chikungunya and Zika viruses in febrile patients, *Acta Trop.* 163 (2016) 32–37.
- [14] R.S. Lanciotti, O.L. Kosoy, J.J. Laven, J.O. Velez, A.J. Lambert, A.J. Johnson, S.M. Stanfield, M.R. Duffy, Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007, *Emerg Infect Dis* 14 (2008) 1232–1239.
- [15] CDC, CDC DENV-1-4 Real-Time RT-PCR Assay, Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases, Division of Vector-Borne Diseases, Dengue Branch, 1324 Canada Street, San Juan, PR 00920, 2013.
- [16] T. Briese, W.G. Glass, W.I. Lipkin, Detection of West Nile virus sequences in cerebrospinal fluid, *Lancet* 355 (2000) 1614–1615.
- [17] G.A. Santiago, J. Vazquez, S. Courtney, K.Y. Matias, L.E. Andersen, C. Colon, A.E. Butler, R. Roulo, J. Bowzard, J.M. Villanueva, J.L. Munoz-Jordan, Performance of the Triplex real-time RT-PCR assay for detection of Zika, dengue, and chikungunya viruses, *Nat. Commun.* 9 (2018) 1391.
- [18] H. Jalal, H. Stephen, M.D. Curran, J. Burton, M. Bradley, C. Carne, Development and validation of a rotor-gene real-time PCR assay for detection, identification, and quantification of Chlamydia trachomatis in a single reaction, *J. Clin. Microbiol.* 44 (2006) 206–213.

- [19] G. Lutfalla, G. Uze, Performing quantitative reverse-transcribed polymerase chain reaction experiments, *Methods Enzymol.* 410 (2006) 386–400.
- [20] C.B.F. Vogels, C. Ruckert, S.M. Cavany, T.A. Perkins, G.D. Ebel, N.D. Grubaugh, Arbovirus coinfection and co-transmission: a neglected public health concern? *PLoS Biol.* 17 (2019) e3000130.
- [21] M.Y. Carrillo-Hernandez, J. Ruiz-Saenz, L.J. Villamizar, S.Y. Gomez-Rangel, M. Martinez-Gutierrez, Co-circulation and simultaneous co-infection of dengue, chikungunya, and zika viruses in patients with febrile syndrome at the Colombian-Venezuelan border, *BMC Infect. Dis.* 18 (61) (2018).
- [22] N. Acevedo, J. Waggoner, M. Rodriguez, L. Rivera, J. Landivar, B. Pinsky, H. Zambrano, Zika virus, chikungunya virus, and dengue virus in cerebrospinal fluid from adults with neurological manifestations, guayaquil, Ecuador. *Front. Microbiol.* 8 (42) (2017).
- [23] W.E. Villamil-Gomez, A.J. Rodriguez-Morales, A.M. Uribe-Garcia, E. Gonzalez-Arismendy, J.E. Castellanos, E.P. Calvo, M. Alvarez-Mon, D. Musso, Zika, dengue, and chikungunya co-infection in a pregnant woman from Colombia, *Int. J. Infect. Dis.* 51 (2016) 135–138.
- [24] K. Pabbaraju, S. Wong, K. Gill, K. Fonseca, G.A. Tipples, R. Tellier, Simultaneous detection of zika, chikungunya and dengue viruses by a multiplex real-time RT-PCR assay, *J. Clin. Virol.* 83 (2016) 66–71.
- [25] Center for Infection and Immunity CU, CII-ArboViroPlex rRT-PCR assay: authorization of the emergency use of in vitro diagnostics for detection of zika virus and/or diagnosis of zika virus infection, U.S. Food Drug Administration. (2017).