PERFORMANCE CHARACTERISTICS

The **CII-ArboViroPlex rRT-PCR assay** has been developed using serum and urine samples spiked with culture derived target viruses as referenced below:

1. ZIKV culture supernatant (strain PRVABC59)
2. DENV type 1 virus culture supernatant (strain Hawaii)
3. DENV type 2 virus culture supernatant (strain New Guinea C)
4. 4. DENV type 3 virus culture supernatant (strain H87)
5. DENV type 4 virus culture supernatant (strain H241)
6. CHIKV culture supernatant (strain R80422)
7. WNV culture supernatant (strain HNY1999)

Using authentic clinical samples that were determined to be positive for ZIKV, DENV, CHIKV, or WNV by an independent and well-characterized method has also validated the assay.

Analytical Sensitivity Determination

Tentative Limit of Detection (LoD) for ZIKV, DENV, CHIKV, and WNV in Serum Samples

Serial dilutions in human serum of the virus stocks described above were prepared to provide an initial estimate of the limit of detection (LoD), i.e. the tentative LoD. For nucleic acid extraction, 225 µL of single donor serum aliquots were spiked individually with 25 µL of each serially diluted virus stock. The spiked serum mixes (250 µL in total) were extracted in triplicate by NucliSENS® easyMAG® (bioMérieux). Total nucleic acid was eluted in 50 µL elution buffer and immediately stored on ice for further processing. Each sample was tested with the **CII-ArboViroPlex rRT-PCR assay** on the CFX96TM touch Real-Time PCR Detection System (Bio-Rad). The lowest concentration at which all three replicates tested positive for virus signal was scored as the tentative LoD (bold and highlighted for each virus). All samples were found positive for RNase P. The serum LoD (virus copy number or genomic equivalent quantity/mL) results are described in Table 1.

Table 1. Determination of the tentative LoD for the **CII-ArboViroPlex rRT-PCR assay** with serum specimens.

Analytes	Virus Strain Tested	Stock Virus Titer (TCID50/ml)	Serial 10-Fold Dilution Factor	TCID50/mL Dilution Tested	Run 1 Ct	Run 2 Ct	Run 3 Ct	Average Ct	Call Rate	Virus copy number (GEQ/ml)
Zika Virus	PRVABC59	6.61 X 10*6	1	6.61 X 10*5	16.45	15.86	16.10	16.14	3/3	5.16E+07
			2	6.61 X 10*4	19.96	20.30	20.80	20.35	3/3	5.16E+06
			3	6.61 X 10*3	23.20	22.90	23.60	23.23	3/3	5.16E+05
			4	6.61 X 10*2	26.86	27.50	26.70	27.02	3/3	5.16E+04
			5	**6.61 X 10*1**	**30.87**	**31.00**	**31.30**	**31.06**	**3/3**	**5.16E+03**
			6	6.61 X 10*0	34.70	35.20	ND	34.95	2/3	5.16E+02
			7	6.61 X 10*-1	37.80	ND	ND	NA	1/3	5.16E+01
Dengue Virus Type 1	Hawaii	1.70 X 10*5	1	1.70 X 10*4	20.58	21.20	20.10	20.63	3/3	4.37E+06
			2	1.70 X 10*3	23.94	22.80	23.00	23.25	3/3	4.37E+05

			3	1.70 X 10*2	27.30	27.30	28.00	27.53	3/3	4.37E+04
			4	1.70 X 10*1	30.22	30.00	31.00	30.41	3/3	4.37E+03
			5	**1.70 X 10*0**	**34.01**	**34.50**	**32.00**	**33.50**	**3/3**	**4.37E+02**
			6	1.70 X 10*-1	ND	ND	ND	NA	0/3	4.37E+01
			7	1.70 X 10*-2	ND	ND	ND	NA	0/3	4.37E+00
Dengue Virus Type 2	New Guinea C	4.1 X 10*5	1	4.1 X 10*4	18.33	18.00	18.90	18.41	3/3	1.87E+06
			2	4.1 X 10*3	21.99	21.60	20.80	21.46	3/3	1.87E+05
			3	4.1 X 10*2	25.13	25.12	25.90	25.38	3/3	1.87E+04
			4	4.1 X 10*1	28.64	28.60	27.80	28.35	3/3	1.87E+03
			5	**4.1 X 10*0**	**31.54**	**32.00**	**31.90**	**31.81**	**3/3**	**1.87E+02**
			6	4.1 X 10*-1	33.75	34.10	ND	33.92	2/3	1.87E+01
			7	4.1 X 10*-2	ND	ND	ND	NA	0/3	1.87E+00
Dengue Virus Type 3	H87	1.70 X 10*5	1	1.70 X 10*4	20.58	19.50	19.80	19.96	3/3	4.63E+07
			2	1.70 X 10*3	23.94	23.00	23.50	23.48	3/3	4.63E+06
			3	1.70 X 10*2	27.30	27.10	26.50	26.97	3/3	4.63E+05
			4	1.70 X 10*1	30.22	30.90	31.20	30.77	3/3	4.63E+04
			5	**1.70 X 10*0**	**34.01**	**35.00**	**34.60**	**34.54**	**3/3**	**4.63E+03**
			6	1.70 X 10*-1	ND	ND	ND	ND	0/3	4.63E+02
			7	1.70 X 10*-2	ND	ND	ND	ND	0/3	4.63E+01
Dengue Virus Type 4	H241	1.26 X 10*6	1	1.26 X 10*5	20.43	20.20	19.70	20.11	3/3	2.87E+07
			2	1.26 X 10*4	24.03	23.90	24.50	24.14	3/3	2.87E+06
			3	1.26 X 10*3	27.42	27.00	27.40	27.27	3/3	2.87E+05
			4	1.26 X 10*2	31.26	32.00	32.40	31.89	3/3	2.87E+04
			5	**1.26 X 10*1**	**34.40**	**34.80**	**34.40**	**34.53**	**3/3**	**2.87E+03**
			6	1.26 X 10*0	ND	38.90	ND	NA	0/3	2.87E+02
			7	1.26 X 10*-1	ND	ND	ND	ND	0/3	2.87E+01
Chikungunya virus	R80422	3.56 X 10*6	1	3.56 X 10*5	17.69	17.60	17.00	17.43	3/3	5.39E+07
			2	3.56 X 10*4	21.37	21.20	22.00	21.52	3/3	5.39E+06
			3	3.56 X 10*3	24.87	23.50	23.80	24.06	3/3	5.39E+05
			4	3.56 X 10*2	27.99	27.60	28.00	27.86	3/3	5.39E+04
			5	**3.56 X 10*1**	**31.00**	**31.50**	**31.90**	**31.47**	**3/3**	**5.39E+03**
			6	3.56 X 10*0	34.48	35.00	ND	34.74	2/3	5.39E+02
			7	3.56 X 10*-1	ND	ND	38.50	NA	0/3	5.39E+01
West Nile Virus	HNY1999	1.61 X 10*7	1	6.61 X 10*6	15.14	14.90	14.60	14.88	3/3	2.10E+07
			2	6.61 X 10*5	18.55	17.50	17.60	17.88	3/3	2.10E+06
			3	6.61 X 10*4	22.15	22.10	22.50	22.25	3/3	2.10E+05
			4	6.61 X 10*3	26.53	26.00	26.50	26.34	3/3	2.10E+04
			5	6.61 X 10*2	30.06	30.90	30.00	30.32	3/3	2.10E+03
			6	**6.61 X 10*1**	**33.22**	**32.80**	**33.50**	**33.17**	**3/3**	**2.10E+02**
			7	6.61 X 10*-1	36.20	36.50	ND	36.35	2/3	2.10E+01

Confirmation of the LoD in Serum Samples

Based on the LoD estimates determined in range-finding studies, virus stocks were diluted into 20 serum sample aliquots to give a final virus concentration at the presumptive LoD. Nucleic acids were extracted by NucliSENS® easyMAG® (bioMérieux). All 20 extracted samples were tested with the **CII-ArboViroPlex rRT- PCR assay** on the CFX96TM Real-Time PCR Detection System (Bio-Rad). Results are shown in Table 2.

Table 2. LoD confirmation in serum specimens using the CFX96TM Real-Time PCR Detection System

No. Of replicate	ZIKV Ct	DENV 1 Ct	DENV 2 Ct	DENV 3 Ct	DENV 4 Ct	CHIKV Ct	WNV Ct
Virus concn (GEQ/ml)	**5.16E+03**	**4.37E+02**	**1.87E+02**	**4.63E+03**	**2.87E+03**	**5.39E+03**	**2.10E+02**
Replicate 1	31.86	32.87	31.05	33.68	31.81	31.65	33.81
Replicate 2	31.68	34.41	31.28	30.93	32.01	31.68	33.68
Replicate 3	31.79	33.52	31.16	31.95	31.68	31.59	34.00
Replicate 4	32.47	32.67	32.52	30.45	30.67	31.36	32.81
Replicate 5	32.69	33.49	32.51	30.28	30.77	31.39	32.62
Replicate 6	32.70	33.42	32.58	31.44	30.54	31.01	32.50
Replicate 7	30.42	31.93	32.29	30.18	31.85	31.26	34.19
Replicate 8	30.38	32.81	32.14	30.42	32.04	31.64	34.22
Replicate 9	30.48	32.41	32.49	31.42	31.56	31.63	33.60
Replicate 10	32.02	32.35	32.88	30.50	30.78	31.87	33.90
Replicate 11	32.70	32.89	32.27	31.85	30.72	31.72	33.92
Replicate 12	32.65	33.23	33.09	31.48	30.90	31.26	33.93
Replicate 13	31.58	32.90	32.40	30.70	31.23	31.34	33.39
Replicate 14	31.24	32.73	33.54	30.53	30.99	31.12	33.17
Replicate 15	31.23	32.82	33.26	30.73	31.16	31.44	33.31
Replicate 16	32.64	33.60	35.34	31.36	31.67	31.76	33.38
Replicate 17	32.24	31.66	32.14	31.09	31.34	31.51	33.95
Replicate 18	32.48	31.31	31.69	30.05	31.35	32.08	33.31
Replicate 19	31.48	31.96	31.55	30.49	30.46	31.94	33.30
Replicate 20	31.49	34.45	32.34	30.67	30.45	31.19	33.26
Average Ct	31.81	32.87	32.43	31.01	31.20	31.52	33.51
STD	0.76	0.80	0.93	0.81	0.52	0.28	0.48
%CV	2.39	2.43	2.88	2.63	1.66	0.88	1.44
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%

Tentative LoD for ZIKV in Urine Samples

Serial dilutions in urine of the ZIKV stock described previously was prepared. For nucleic acid extraction, 225 µL of single donor urine aliquots were spiked individually with 25 µL of each

serially diluted virus stock. The spiked urine mixes (250 µL in total) were extracted by NucliSENS® easyMAG® (bioMérieux). Total nucleic acid was eluted in 50 µL elution buffer and immediately stored on ice for further processing. Each sample was extracted in triplicate and tested with the **CII-ArboViroPlex rRT-PCR assay** on the CFX96TM Real-Time PCR Detection System (Bio-Rad). The lowest concentration at which all three replicates tested positive for viral signal was scored as the presumptive LoD (bold and highlighted for each virus). The urine LoD (virus copy number or genomic equivalent quantity/ mL) results are described in Table 3.

Table 3. Determination of the tentative LoD for ZIKV with the **CII-ArboViroPlex rRT-PCR assay** in urine

Analytes	Virus Strain Tested	Stock Virus Titer (TCID50/mL)	Serial 10-Fold Dilution Factor	TCID50/mL Dilution Tested	Run 1 Ct	Run 2 Ct	Run 3 Ct	Average Ct	Call Rate	Virus copy number (GEQ/ml)
Zika Virus	PRVABC59	6.61 X 10*6	1	6.61 X 10*5	18.59	18.1	19.2	18.63	3/3	5.16E+07
			2	6.61 X 10*4	22.2	22.1	21.6	21.97	3/3	5.16E+06
			3	6.61 X 10*3	25.75	24.6	25.5	25.28	3/3	5.16E+05
			4	6.61 X 10*2	29.23	29.1	28.9	29.08	3/3	5.16E+04
			5	**6.61 X 10*1**	**33.91**	**32.5**	**31.76**	**32.72**	**3/3**	**5.16E+03**
			6	6.61 X 10*0	ND	ND	37.6	NA	1/3	5.16E+02
			7	6.61 X 10*-1	ND	ND	ND	NA	0/3	5.16E+01

Confirmation of the LoD in Urine Samples

Based on the LoD estimates determined in range-finding studies, virus stocks were diluted into 20 urine sample aliquots to give a final virus concentration at the presumptive LoD. Nucleic acids were extracted by NucliSENS® easyMAG® (bioMérieux). All 20 extracted samples were tested with **CII-ArboViroPlex rRT-PCR assay** on the CFX96TM Real-Time PCR Detection System (Bio-Rad). Results are shown in Table 4.

Table 4. LoD confirmation of ZIKV in urine specimens using the CFX96TM Real-Time PCR Detection System

No. of replicate	ZIKV Ct
Virus concn (GEQ/ml)	**5.16E+03**
Replicate 1	31.86
Replicate 2	31.68
Replicate 3	31.79
Replicate 4	32.47
Replicate 5	32.69
Replicate 6	32.70
Replicate 7	30.42
Replicate 8	30.38
Replicate 9	30.48

Replicate 10	32.02
Replicate 11	32.70
Replicate 12	32.65
Replicate 13	31.58
Replicate 14	31.24
Replicate 15	31.23
Replicate 16	32.64
Replicate 17	32.24
Replicate 18	32.48
Replicate 19	31.48
Replicate 20	31.49
Average Ct	31.81
STD	0.76
%CV	2.39
Call rate	20/20
Call rate %	100%

FDA Sensitivity Study

The analytical sensitivity of the **CII-ArboViroPlex rRT-PCR assay** in serum and urine was also evaluated using reference materials (S1 and S2) and a standard protocol provided by the FDA, which included a LoD range finding study and a confirmatory LoD study. Results of the FDA Sensitivity Study using the **CII- ArboViroPlex rRT-PCR assay** are presented below in Table 5.

Table 5: Summary of LoD Confirmation Results Using FDA Reference Materials

Reference Materials	Specimen Type	Confirmed LoD* RNA NAAT Detectable Units/mL
S1	Serum	1000
S2	Serum	500
S1	Urine	1000
S2	Urine	500

*Study performed according to an FDA issued protocol

Laboratory Testing of Other ZIKV Isolates

Two additional isolates of ZIKV that represent the African genotype (MR776 and ArD158095) were also laboratory tested with **CII-ArboViroPlex rRT-PCR assay** at the concentrations shown below and Ct values were compared with the CDC Trioplex assay (Table 6).

Table 6: Detection of African ZIKV isolates by **CII-ArboViroPlex rRT-PCR assay**

Zika virus isolate	Source/Sample Type*	Concentration (GEQ/ml)	CDC Trioplex Assay (Ct)	CII-ArboViroPlex rRT-PCR Assay (Ct)
ZIKV MR776	Virus culture	1.70 X 10*6	16.52	15.85
ZIKV ArD158095	In vitro transcribed RNA	1.40 X 10*6	15.98	15.11

Laboratory Testing for Cross Reactivity

Cross-reactivity of the **CII-ArboViroPlex rRT-PCR assay** was evaluated by testing additional flaviviruses or purified nucleic acids from other viruses and bacteria causing febrile illness. To evaluate the analytical specificity of the **CII-ArboViroPlex rRT-PCR assay** we tested samples containing nucleic acids representing a wide range of human pathogens not targeted in the **CII-ArboViroPlex rRT-PCR assay**. Nucleic acids were extracted from cell culture supernatant or clinical specimens banked at the Center for Infection and Immunity, or provided by the NIH-Integrated Research Facility (Fort Detrick, MD). Samples were tested in triplicate with **CII-ArboViroPlex rRT-PCR assay** on the CFX96TM Real-Time PCR Detection System (Bio-Rad) and showed no evidence of cross-reactivity. Test results were only considered valid if samples were positive for the host transcript control RNase P. Results are shown in Table 7.

Table 7. Specificity of **CII-ArboViroPlex rRT-PCR assay**

Virus/Bacteria/Parasite	Strain	Source/Sample type	Concentration	Average Ct (3 replicates)
Yellow fever virus	17D-204	Extracted nucleic acid	2.1X 10*5 PFU /ml	ND
Japanese encephalitis virus	Nakayama	Extracted nucleic acid	~1.5X10*7 GEQ/ml	ND
Saint Louis encephalitis virus	Unknown	Extracted nucleic acid	~1.0X10*5 GEQ/ml	ND
Hepatitis C virus	Clinical isolate	Extracted nucleic acid	1.6X10*6 GEQ/ml	ND
Enterovirus D68	F02-3607 Corn	Virus culture	1.1X10*7 GEQ/ml	ND
Influenza A virus	H3N2-Panama	Virus culture	1X10*9 $TCID_{50}$/ml	ND
Human Adenovirus 6	HADV-6	Virus culture	1.64X 10*5 $TCID_{50}$/ml	ND
Human herpes virus 1	KOS	Virus culture	1X10*11 $TCID_{50}$/ml	ND
Ebola virus	Makona	Extracted nucleic acid	1.3X 10*5 PFU /ml	ND
Parvovirus B19	Clinical isolate	Extracted nucleic acid	~5.0X10*6 GEQ/ml	ND
Lassa virus	Josiah	Extracted nucleic acid	2.1X 10*5 PFU /ml	ND
Plasmodium falciparum	Clinical isolate	Extracted nucleic acid	1.5X10*7 GEQ/ml	ND
Salmonella Typhi	Clinical isolate	Extracted nucleic acid	2.9X10*5 GEQ/ml	ND
E.Coli	DH5a	Bacterial Culture	NA	ND

***In silico* evaluation of CII-ArboViroPlex rRT-PCR assay primers and probes**

Reactivity of **CII-ArboViroPlex rRT-PCR assay** primers and probes was also assessed *in silico* with other representative isolates of ZIKV, DENV, CHIKV, WNV. All primer and probe sequences showed 100% sequence identity with their expected target; this confirms the broad reactivity and specificity of primers and probes used in **CII-ArboViroPlex rRT-PCR assay** for their specified targets. *In silico* analysis was performed for other potentially cross reacting agents not available for laboratory testing. Sequences of the primers and probes included in the **CII-ArboViroPlex rRT-PCR assay** were evaluated for evidence of cross reactivity against the other target organisms (ZIKV, DENC, CHIKV and WNV) and potentially cross reactive organisms listed in Table 8. Analyses using the BLAST algorithm (NCBI) were performed using all possible combinations of primer and probes. Any correlations based on computational alignment were reviewed for potential formation of a PCR product through binding of the primers or probes in reasonable distance and orientation to target

nucleic acid. In only a few instances were more than one primer or probe of a set having >80% nucleotide homology with an organism sequence identified. Potential microbiological interference between DENV primer/probes and ZIKV strains was evaluated in a microbiological interference study described in the next section. *Salmonella typhi* vaccine strain and the CHIKV primer/probes, CHIK.NSP2.NQR and CHIK.NSP2.PROBE, where matched in the 80% range. However, wet testing of a *Salmonella typhi* clinical sample did not result in an amplification product with the CHIKV primer-probe set. Therefore, the **CII-ArboViroPlex rRT-PCR assay** is unlikely to create a spurious amplicon that would be detected in the assay.

Table 8. Potential Cross reactivity of **CII-ArboViroPlex rRT-PCR assay** primer and probes to other pathogens (*in silico* analysis)

Other flaviviruses	**Other viruses**
St. Louis encephalitis virus	Enterovirus, all serotypes
Japanese encephalitis virus	Adenovirus, all serotypes
Spondweni virus	Hepatitis B virus
Yellow fever virus	HIV
Yellow fever virus vaccine strain	Varicella Zoster virus (HHV-3)
Hepatitis C virus	Cytomegalovirus (HHV-5)
	Epstein Barr virus (HHV-4)
Other viruses	**Other bacterial and parasitic agents**
Eastern equine encephalitis virus	Rickettsia sp.
Western equine encephalitis virus	Borrelia burgdorferi
Ross River virus	Group A streptococcus
Barmah Forest virus	Leptospirosis
O'nyong-nyong virus	Plasmodium sp.
Sindbis virus, Tonate virus and Una virus	Plasmodium vivax
Measles virus	Trypanosoma cruzi (Chagas)
Rubella virus	Schistosomiasis
Hepatitis A virus vaccine	Salmonella typhi vaccine

Laboratory Testing of Microbiological Interference Between Primer/Probe Sets

In silico analysis revealed that in some instances certain primers or probes shared >80% identity to other agent's sequence, the potential for interference between ZIKV and DENV targets was evaluated with wet- testing. Two DENV primers had approx. 80% identity to ZIKV sequence and one ZIKV primer had approx. 70% identity to DENV sequence. Two different concentrations of ZIKV and DENV (1000xLoD and 3xLoD) were spiked in single donor serum and tested in triplicate with **CII-ArboViroPlex rRT-PCR assay** in individual wells or after mixing both targets in same sample well. Comparing Ct values in both situations, did not demonstrate a significant difference in Ct values despite the high sequence identity between individual primers and the alternative viral template (Table 9; no signals were detected, ND, for CHIKV or WNV). Thus, it was concluded that primer concentrations used in the **CII-ArboViroPlex rRT-PCR assay** provide primers at sufficient excess to tolerate the presence of templates that match in the 80% range a primer or probe without affecting

assay results. Likewise, this should also address concerns regarding potential interference between CHIKV and *Salmonella thyphi,* for which the *in silico* analysis indicated an 80% match with the CHIKV reverse primer and the CHIKV probe.

Table 9. Interference between ZIKV and DENV primer/probe sets of the **CII-ArboViroPlex rRT-PCR assay** (*in vitro* testing)

	ZIKV 1 Ct	ZIKV 2 Ct	ZIKV 3 Ct	Average ZIKV Ct	DENV 1 Ct	DENV 2 Ct	DENV 3 Ct	Average DENV Ct
ZIKV (1000 X LoD)	18.67	18.75	18.15	18.52	ND	ND	ND	ND
ZIKV (3 X LoD)	30.32	30.55	30.20	30.36	ND	ND	ND	ND
DENV (1000 X LoD)	ND	ND	ND	ND	18.17	18.56	17.65	18.13
DENV (3 X LoD)	ND	ND	ND	ND	29.50	30.10	30.50	30.03
ZIKV (1000 X LoD) +DENV (3 X LoD)	19.13	18.87	18.85	18.95	29.80	30.10	29.95	29.95
ZIKV (3 X LoD) + DENV (1000 X LoD)	30.15	30.66	31.44	30.75	18.20	18.65	18.50	18.45

Interference Substances Studies

Interfering substances studies were not performed for the **CII-ArboViroPlex rRT-PCR assay**. The **CII- ArboViroPlex rRT-PCR assay** uses conventional real-time RT-PCR and an established nucleic acid (EasyMAG) extraction protocol. In addition, the assay uses external (HSC and positive viral controls) and internal reaction controls (eHSC and RNase P).

Clinical Evaluation

The performance characteristics of the LightMix® Zika rRT-PCR Test have been established using a clinical study.

Negative specimens:

Samples used as negative controls were collected from patients with febrile illness (positive for Lyme disease or unspecified febrile illness). Serum samples were collected from geographic areas not known to be endemic for ZIKV; urine samples were collected from NYS and thus not suspect of being positive for ZIKV. All negative specimen samples were tested and did not show positive viral signals (negative for ZIKV, DENV, CHIKV, and WNV) with the **CII-ArboViroPlex rRT-PCR assay** and the comparator method (CM), but were positive for RNase P with both the assays.

Total Negative controls, n= 111 (61 negative serum and 50 negative urine samples)

Positive Zika virus Specimens:

The performance characteristics of the **CII-ArboViroPlex rRT-PCR assay** for detection of ZIKV in clinical samples were established using 67 serum (41 clinical serum samples and 26 contrived serum samples spiked at 1-3x LoD) and 52 urine samples (26 clinical and 26 contrived serum samples spiked at 1-3X LoD). Zika specimens were simultaneously also tested for DENV, CHIKV, and WNV by the 5-plex **CII-ArboViroPlex rRT-PCR assay** but were found negative for DENV, CHIKV, and WNV. Contrived samples were prepared by spiking 1X LoD and 3X LoD virus in 13 negative serum samples and 13 negative urine samples collected from individual patients with febrile illness but no indication of viral infection (resulting in a total of 26 ZIKV serum samples and 26 ZIKV urine samples). Positive specimens were obtained through NYCDOHMH, NYSDOH and Boca Biolistics, FL, USA and tested with various comparator methods as described in Table 10.

Total number	**n=119 (serum, n=67; urine, n=52)**
Total Serum Samples	**n= 67 positive samples (positive by the reference assay)**
Clinical	n= 41 (paired serum/urine, n=8; unpaired, n=33)
Contrived	n= 26 (n=13 at 1x LoD, this being 5.16E+03 GEQ/mL; and n=13 at 3x LoD, this being1.54 E+04 GEQ/mL)
Total Urine Samples	**n= 52 positive Samples**
Clinical	n= 26 (paired serum/urine, n=8; unpaired, n=18)
Contrived	n= 26 (n=13 at 1x LoD, this being 5.16E+03 GEQ/mL and n=13 at 3x LoD, this being1.54 E+04 GEQ/mL)

Positive Dengue Virus Specimens

The performance characteristics of the **CII-ArboViroPlex rRT-PCR assay** for detection of DENV in

clinical serum samples was established using 29 DENV positive clinical human serum samples (4 DENV-1, 4 DENV- 2, 6 DENV-3, 3 DENV-4 and 12 DENV positives for which serotype data was not available). In addition to clinical samples, 26 contrived serum samples were also used for validation. For preparing contrived samples 13 negative control serum samples collected from individual patients with febrile illness but no indication of viral infection were spiked with DENV3 at 1X LoD and 3X LoD. Positive DENV clinical serum specimens were collected at NYCDOHMH, NYSDOH, Fundação Oswaldo Cruz (FIOCRUZ, Brazil) and The Center for infection and Immunity (Columbia University) and tested with various comparator methods as described in Table 11.

Total Serum Samples	**n= 55 positive serum samples**
Clinical	n= 29
Contrived	n= 26 (n=13 at 1x LoD, this being 4.63 E+03 GEQ/mL and n=13 at 3x LoD, this being 1.39 E+04 GEQ/mL)

Positive Chikungunya Virus Specimens

The performance characteristics of CII-ArboPlex rRT-PCR assay with CHIKV was established using a clinical study with a **20 CHIKV positive serum samples**. Positive CHIKV specimens were collected at University of the West Indies, Trinidad and tested with an EUA comparator method as described in Table 12. In addition to clinical samples, 26 contrived serum samples (13 serum samples were spiked with CHIKV at 1X LoD and 3X LoD) were also used for validation.

Total Samples	**n= 46 positive serum samples**
Clinical	n= 20
Contrived	n= 26 (n=13 at 1x LoD, this being 3.65 E+02 GEQ/mL and n=13 at 3x LoD, this being 1.09 E+04 GEQ/mL)

West Nile Virus clinical evaluation

The performance characteristics of **CII-ArboPlex rRT-PCR assay** with WNV was also established using a clinical study with a **19 WNV positive serum samples** (Table 34). Positive WNV specimens were collected by the Blood System Research Institute and tested with a comparator method as described in Table 12. In addition to clinical samples, 26 contrived serum samples (13 serum samples were spiked with WNV at 1X LoD and 3X LoD) were also used for validation.

Total Samples	**n= 45 positive serum samples**
Clinical	n= 19
Contrived	n= 26 (n=13 at 1x LoD, this being 2.12 E+02 GEQ/mL and n=13 at 3x LoD, this being 6.36 E+02 GEQ/mL)

The results of the clinical performance evaluation are summarized in Table 10 for serum and Table 11 for urine. The comparator methods (CM) used in the clinical evaluation of the natural clinical specimens are described in Table 12. In the case of the contrived specimen's performance was evaluated against the expected result.

Table 10. Summary of Overall Clinical Performance for Serum

Serum Specimen Category*	Number Tested	CII-ArboViroPlex rRT-PCR assay result			
		ZIKV Positive	DENV Positive	CHIKV Positive	WNV Positive
ZIKV Positive					
Natural Clinical CM1	19	18/19[#]	0/19	0/19	0/19
Natural Clinical CM2	22	21/22[##]	0/22	0/22	0/22
Contrived					
1xLoD	13	13/13	0/13	0/13	0/13
3xLoD	13	13/13	0/13	0/13	0/13
DENV Positive^					
Natural Clinical CM3	20	0/20	20/20	0/20	0/20
Natural Clinical CM4	9	0/9	9/9	0/9	0/9
Contrived					
1xLoD	13	0/13	13/13	0/13	0/13
3xLoD	13	0/13	13/13	0/13	0/13
CHIKV Positive					
Natural Clinical CM5	20	0/20	0/20	20/20	0/20
Contrived					
1xLoD	13	0/13	0/13	13/13	0/13
3xLoD	13	0/13	0/13	13/13	0/13
WNV Positive					
Natural Clinical CM6	19	0/19	0/19	0/19	19/19
Contrived					
1xLoD	13	0/13	0/13	0/13	13/13
3xLoD	13	0/13	0/13	0/13	13/13
Negative					
CM9	61	0/60	1/61^	0/61	0/61
Overall Percent Agreement		**ZIKV**	**DENV**	**CHIKV**	**WNV**
Positive percent agreement		97.0% (65/67) 95% CI: 89.8-99.2%	100.0% (55/55) 95% CI: 93.5-100.0%	100.0% (46/46) 95% CI: 92.3-100.0%	100.0% (45/45) 95% CI: 92.1-100.0%
Negative percent agreement		100.0% (129/129)[$] 95% CI: 97.1-100.0%	99.5% (140/141)[$] 95% CI: 96.1-99.9%	100.0% (150/150)[$] 95% CI: 97.5-100.0%	100.0% (151/151)[$] 95% CI: 97.5-100.0%

*Comparator methods (CM) as described in the text and Table 10 below.

#CII-ArboViroPlex rRT-PCR assay CT of the specimen was 38.68 just above the 38.00 cut-off for a positive result interpretation.

##CII-ArboViroPlex rRT-PCR assay CT of the specimen was 38.4 just above the 38.00 cut-off for a positive result interpretation.

^One of the DENV specimens was negative by the comparator and positive by the **CII-ArboViroPlex rRT-PCR assay**.

$Includes clinical positive specimens for other viral targets as additional negatives, e.g. for Zika virus the CIKV, DENV and WNV positive specimens were included as negatives in the Zika virus performance.

Table 11. Summary of Overall Clinical Performance for Zika in Urine

Urine Specimen Category*	Number Tested	CII-ArboViroPlex rRT-PCR assay result			
		ZIKV Positive	DENV Positive	CHIKV Positive	WNV Positive
ZIKV Positive					
Natural Clinical CM7	15	14/15^	0/15	0/15	0/15
Natural Clinical CM8	11	11/11	0/11	0/11	0/11
Contrived					
1xLoD	13	13/13	0/13	0/13	0/13
3xLoD	13	13/13	0/13	0/13	0/13
Negative CM9	50	0/50	0/50	0/50	0/50
Positive percent agreement		98.1%(51/52) 95% CI: 89.9-99.7%			
Negative percent agreement		100%(50/50) 95% CI: 92.9-100.0%			

*Comparator methods (CM) as described in the text and Table 17 below.

^**CII-ArboViroPlex rRT-PCR assay** C_T of the specimen was 38.05 just above the 38.00 cut-off for a positive result interpretation.

Table 12. Description of Comparator Methods (CM) used in the clinical evaluation.

Virus	Assay	Description
ZIKV	CM1	Comparator method was an rRT-PCR IVD assay authorized by FDA for detection of ZIKV RNA in EDTA plasma with analytical sensitivity in the range 5,000-10,000 RNA Units/mL. Equivalency between serum and EDTA plasma was demonstrated.
ZIKV	CM2	Comparator method was a validated rRT-PCR IVD assay based on the Lanciotti et al., 2008. Detection of ZIKV RNA in serum with analytical sensitivity of 400 copies/mL
ZIKV	CM3	Comparator method was an TMA IVD assay authorized by FDA for detection of ZIKV RNA in urine with analytical sensitivity in the range 150-300 RNA Units/mL
ZIKV	CM4	Comparator method was a validated rRT-PCR IVD assay based on the Lanciotti et al., 2008. Detection of ZIKV RNA in urine with analytical sensitivity of 2000 copies/mL
DENV	CM5	Comparator method was an rRT-PCR IVD assay authorized by FDA for detection of ZIKV and DENV RNA in serum with analytical sensitivity in the range 2.68×10^4-8.25×10^4 copies/mL depending on the DENV serotype
DENV	CM6	Comparator method was an FDA-cleared rRT-PCR IVD assay for detection of DENV RNA in serum with analytical sensitivity in the range 1×10^3 pfu/mL for all DENV serotypes
CHIKV	CM7	Comparator method was an rRT-PCR IVD assay authorized by FDA for detection of ZIKV and CHIKV RNA in serum with analytical sensitivity of 1.28×10^5 copies/mL.
WNV	CM8	Comparator method was an rRT-PCR blood screening assay authorized by FDA for detection of WNVRNA in serum with analytical sensitivity in the range 8.2-9.8 copies/mL.
NEG	CM9	Comparator method was an rRT-PCR IVD assay authorized by FDA for detection of ZIKV RNA in serum and urine and DENV/CHIKV in serum.

Additional Contrived Multiplex Evaluation

In addition to the clinical evaluation described above, the **CII-ArboViroPlex rRT-PCR assay** was also evaluated in a limited contrived study designed to simulate potential co-infections. Single donor serum was spiked with a low concentration of ZIKV (2 X LoD) and higher concentration of CHIKV/DENV/WNV (100 X LoD). Total nucleic acid was extracted and tested in 5 replicates. Table 13 shows the data in comparison to samples spiked with a single viral target.

Table 13. CII-ArboViroPlex rRT-PCR assay specificity study spiking multiple viral targets

Sample X LoD	CHIKV (Ct)	DENV (Ct)	ZIKV (Ct)	WNV (Ct)	RNase P (Ct)
Only ZIKV-2X LoD	ND *	ND *	28.1 *	ND *	27.9 *
Only CHIKV- 100X LoD	25.2 *	ND *	ND *	ND *	27.8 *
Only DENV- 100X LoD	ND *	24.8 *	ND *	ND *	27.7 *
Only WNV- 100X LoD	ND *	ND *	ND *	27.3 *	27.5 *
ZIKV-2X LoD+ CHIKV- 100X LoD- Replicate 1	25.0	ND	28.3	ND	27.7
ZIKV-2X LoD+ CHIKV- 100X LoD- Replicate 2	25.0	ND	28.2	ND	27.7
ZIKV-2X LoD+ CHIKV- 100X LoD- Replicate 3	25.0	ND	28.3	ND	27.7
ZIKV-2X LoD+ CHIKV- 100X LoD- Replicate 4	25.1	ND	28.1	ND	27.8
ZIKV-2X LoD+ CHIKV- 100X LoD- Replicate 5	25.1	ND	28.2	ND	27.7
ZIKV-2X LoD+ DENV- 100X LoD- Replicate 1	ND	24.4	28.5	ND	27.3
ZIKV-2X LoD+ DENV - 100X LoD- Replicate 2	ND	24.5	28.8	ND	27.6
ZIKV-2X LoD+ DENV - 100X LoD- Replicate 3	ND	24.8	28.8	ND	27.7
ZIKV-2X LoD+ DENV - 100X LoD- Replicate 4	ND	24.5	28.7	ND	27.5
ZIKV-2X LoD+ DENV - 100X LoD- Replicate 5	ND	24.6	28.7	ND	27.5
ZIKV-2X LoD+ WNV- 100X LoD- Replicate 1	ND	ND	28.2	28.0	27.5
ZIKV-2X LoD+ WNV - 100X LoD- Replicate 2	ND	ND	28.6	28.3	27.5
ZIKV-2X LoD+ WNV - 100X LoD- Replicate 3	ND	ND	28.4	28.4	27.8
ZIKV-2X LoD+ WNV - 100X LoD- Replicate 4	ND	ND	28.3	27.9	27.4
ZIKV-2X LoD+ WNV - 100X LoD- Replicate 5	ND	ND	28.4	28.4	27.6
Serum control	ND	ND	ND	ND	27.5
Negative control	ND	ND	ND	ND	ND

* Mean Ct from 5 replicates

Information about any significant new findings observed during the course of the emergency use of the **CII-ArboViroPlex rRT-PCR assay** test will be made available at https://www.mailman.columbia.edu/research/center-infection-and-immunity.

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