

# **CII-ArboViroPlex rRT-PCR assay**

## **Instructions for Use**

- ❖ **For use under the Emergency Use Authorization (EUA) only**
  - ❖ For *in vitro* diagnostic use
  - ❖ For prescription use only
  - ❖ 11<sup>th</sup> August 2017

**The Center for Infection and Immunity,  
Columbia University, New York, 10032**

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## INTRODUCTION

Before using the **CII-ArboViroPlex rRT-PCR assay** this package insert must be read carefully. All the instructions must be followed strictly. Assay reliability and results are not guaranteed in case of deviations from the instructions provided in this package insert.

## NAME

CII-ArboViroPlex rRT-PCR assay

## INTENDED USE

The **CII-ArboViroPlex rRT-PCR assay** is a multiplex *in vitro* reverse transcription real time polymerase chain reaction (rRT-PCR) test intended for the qualitative detection and differentiation of RNA from Zika virus (ZIKV), dengue virus (DENV), chikungunya virus (CHIKV), and West Nile virus (WNV) in serum, and for the qualitative detection of Zika virus RNA in urine (collected alongside a patient-matched serum specimen). The assay is intended for use with specimens collected from individuals meeting CDC Zika virus clinical criteria (e.g., clinical signs and symptoms associated with Zika virus infection) and/or CDC Zika virus epidemiological criteria (e.g., history of residence in or travel to a geographic region with active Zika transmission at the time of travel). Use of the **CII-ArboViroPlex rRT-PCR assay** is limited in the United States to laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests, or similarly qualified laboratories outside the United States.

Assay results are for the identification of ZIKV, DENV, CHIKV, or WNV viral RNA. Zika virus RNA is generally detectable in serum and/or urine during the acute phase of infection and up to 14 days following onset of symptoms, if present. Positive results are indicative of current infection. Laboratories are required to report all positive results to the appropriate public health authorities.

Negative results do not preclude ZIKV, DENV, CHIKV, or WNV infections and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information. The **CII-ArboViroPlex rRT-PCR assay** is intended for use by trained clinical laboratory personnel, specifically instructed and trained in the techniques of nucleic acid amplification and *in vitro* diagnostic procedures. This assay is only for use under the Food and Drug Administration's Emergency Use Authorization.

## SUMMARY AND EXPLANATION OF THE TEST

The **CII-ArboViroPlex rRT-PCR assay** detects four agents (ZIKV, DENV, CHIKV, and WNV) that may be present in areas of endemic tropical disease. The **CII-ArboViroPlex rRT-PCR assay** detects the African and Asian ZIKV genotypes with similar sensitivity. Several Zika virus outbreaks have occurred in the past few years including the current outbreak in Brazil and a number of other Central and South American countries. In 2015, the number of suspected Zika virus cases in Brazil was estimated to be between 440,000 and 1.3 million. By September 2016, locally-transmitted cases had been reported from the U.S. and U.S. territories, 48 other countries and territories in the Americas, 8 countries and territories in Oceania/Pacific islands, Singapore and 1 country in Africa. Due to the

increasing evidence of a correlation between Zika virus infection and (1) the incidence of Guillain-Barré syndrome and (2) poor pregnancy outcomes, including microcephaly, the importance of diagnosing a Zika virus infection is rapidly increasing.

Zika virus is primarily transmitted by mosquitoes (including *Aedes aegypti*). In addition, human-to-fetus and sexually transmitted infections have been documented. Most cases of Zika virus infection are asymptomatic; however when symptoms occur they are generally mild and flu-like. Laboratories in the United States are in need of diagnostic tools for use in the acute phase for rapid diagnosis of Zika virus infection. A public health emergency has been declared by the Secretary of Health and Human Services (HHS) on February 26, 2016 justifying the authorization of emergency use of *in vitro* diagnostic tests for detection of Zika virus. The **CII-ArboViroPlex rRT-PCR assay** can augment clinically important laboratory evidence for individuals in the acute phase. Zika virus RNA is generally detectable in serum and/or urine during the acute phase of infection (approximately 14 days following onset of symptoms, if present).

Under emergency use authorization (EUA), the Center for Infection and Immunity, Columbia University, New York, distributes the **CII-ArboViroPlex rRT-PCR assay** for the detection and differentiation of RNA from ZIKV, DENV, CHIKV, and WNV in serum, and for the qualitative detection of Zika virus RNA in urine (collected alongside a patient-matched serum specimen) collected from individuals meeting CDC's Zika clinical and/or epidemiological criteria. FDA consulted with subject matter experts within HHS on the public health need for diagnostic devices to detect Zika virus nucleic acid. It is FDA's conclusion that there currently exists a public health need for devices like the **CII-ArboViroPlex rRT-PCR assay** for Zika virus detection during the public health emergency.

### **Assay Principle**

The **CII-ArboViroPlex rRT-PCR assay** is a multiplex one-step real-time reverse transcription polymerase chain reaction (rRT-PCR) test. The **CII-ArboViroPlex rRT-PCR assay** kit includes primers and dual-labeled BHQ® Probes to be used in the *in vitro* qualitative detection of isolated RNA from ZIKV, DENV, CHIKV, WNV and RNase P from clinical specimens. Dual-labeled BHQ probes are linear, dual labeled probes incorporating a fluorophore and quencher covalently attached to the 5' and 3' ends of the oligonucleotide, respectively. Fluorescence signal is generated through the 5' exo-nuclease activity of *Taq* polymerase, which cleaves off the fluorescent fluorophore-labeled nucleotide from the probe during digestion of the probe hybridized to its complementary sequence in the target strand and thus separating fluorophore from quencher. The five primer and probe sets are designed to detect ZIKV, DENV, CHIKV, WNV and RNase P RNA in serum and urine from subjects with signs and symptoms of virus infection and/or with epidemiological risk factors consistent with virus exposure. For the ZIKV specific probe, the signal from the fluorescent dye probe (Cal Fluor red 610) on the 5' end is quenched by BHQ-2 on its 3' end. For the DENV specific probe, the signal from the fluorescent dye probe (Cal Fluor red 560) on the 5' end is quenched by BHQ-1 plus on its 3' end. For the CHIKV specific probe, the signal from the fluorescent dye probe (FAM) on the 5' end is quenched by BHQ-1-plus on its 3' end. For the WNV specific probe, the signal from the fluorescent dye probe (Quasar 670) on the 5' end is quenched by BHQ-2 on its 3' end. For the RNase P specific probe, the signal from the fluorescent dye probe (Quasar 705) on the 5' end is quenched by BHQ-3 on its 3' end. After patient specimen collection and receipt by the laboratory, total nucleic acids are isolated and purified from serum or urine samples using the NucliSENS® easyMAG® automated extraction platform (bioMérieux). The purified nucleic acids are reverse transcribed and amplified by using the RNA UltraSense™ One-Step Quantitative

RT-PCR System (Thermo Fisher) with thermal cycling and detection on the CFX96 Real-Time PCR Detection System (Bio-Rad). In the process, the probe anneals to a specific target sequence located between the forward and reverse primers and generates a fluorescent signal upon its digestion by *Taq* polymerase, which is measured during the end of the PCR cycle. With each cycle of PCR, more probes are digested, resulting in an increase in fluorescence that is proportional to the amount of target nucleic acid. Fluorescence signal intensity is analyzed and data collected by the CFX Manager™ Software.

## KIT COMPONENTS

The **CII-ArboViroPlex rRT-PCR assay** kit includes four vials containing primers and probes for each of the viral targets (ZIKV-Mix, DENV-Mix, CHIKV-Mix, and WNV-Mix), one vial containing combined primers and probe for RNase P (RP-Mix), one vial each containing plasmid-derived *in vitro* transcribed RNA preparations for use as positive controls for ZIKV (ZPC), DENV (DPC), CHIKV (CPC), and WNV (WPC), respectively, and two vials of human specimen extraction control (HSC) for use as extraction control, one vial of extracted nucleic acid from human specimen control (eHSC) to be used as a positive control in rRT-PCR for detection of human RNase P mRNA and as a negative control for detection of viral RNA, two vials of sterile distilled H<sub>2</sub>O (NTC) to be used as non-template control, and one vial of nuclease-free water for reconstitution of primers and probes. Each **CII-ArboViroPlex rRT-PCR assay** kit provides the following reagents, which are sufficient for performing 96 reactions (Table 1).

**Table 1.** Kit components of **CII-ArboViroPlex rRT-PCR assay** (96 reactions)

S.No.	Vial Label	Vial component	Number of vials	Volume (µl/vial)
1	ZIKV-MIX	ZIKV forward primer	1	dried, reconstitute with 100 µl H <sub>2</sub> O
		ZIKV reverse primer		
		ZIKV probe		
2	DENV-MIX	DENV forward primer	1	dried, reconstitute with 100 µl H <sub>2</sub> O
		DENV reverse primer A		
		DENV reverse primer B		
		DENV probe		
3	CHIKV-MIX	CHIKV forward primer	1	dried, reconstitute with 100 µl H <sub>2</sub> O
		CHIKV reverse primer		
		CHIKV probe		
4	WNV-MIX	WNV forward primer	1	dried, reconstitute with 100 µl H <sub>2</sub> O
		WNV reverse primer		
		WNV probe		
5	RP-MIX	RNase P forward primer	1	dried, reconstitute with 100 µl H <sub>2</sub> O
		RNase P reverse primer		
		RNase P probe		
6	ZPC	ZIKV Positive Control	1	40 µl
7	DPC	DENV Positive Control	1	40 µl

8	CPC	CHIKV Positive Control	1	40 µl
9	WPC	WNV Positive Control	1	40 µl
10	HSC	Human specimen extraction control	2	1.5 ml
11	eHSC	Extracted nucleic acid from HSC	1	40 µl
12	NTC	Nuclease free water	2	750 µl
13	Diluent*	Nuclease free water	1	750 µl

\*Used to reconstitute assay reagents

## STORAGE CONDITIONS

- The **CII-ArboViroPlex rRT-PCR assay** kit is shipped on dry ice. The components of the kit should arrive frozen.
- If one or more components are not frozen upon receipt, or if tubes have been compromised during shipment, contact The Center for Infection and Immunity for assistance.
- Protect primer/probe mixes from light.
- All components should be stored at -20°C upon arrival. Do not store in a frost-free freezer.
- Always check the expiration date and do not use reagents beyond the expiration date.
- Repeated thawing and freezing of Master reagents (more than twice) should be avoided, as this might affect the performance of the assay.
- The reagents should be frozen in aliquots, if they are to be used intermittently.
- For making aliquots storage of original kit between +2°C and +8°C should not exceed a period of two hours.

## PREPARATION OF CII-ARBOVIROPLEX rRT-PCR ASSAY MASTER MIX

Instructions for the preparation of **CII-ArboViroPlex rRT-PCR assay** master mix:

1. Upon receipt, the **CII-ArboViroPlex rRT-PCR assay** kit should be stored at -20°C in the dark.
2. Lyophilized primers and probes (vial 1-5) must be reconstituted with 100 µl nuclease free distilled water, aliquoted and stored at -20°C or below. Thawed aliquots may be stored in the dark at 2-8°C for up to a week. Thawed aliquots should not be refrozen.
3. All reagents should be manipulated in a designated clean area for assay setup that is physically isolated from specimen extraction and handling areas.
4. Assay components should be kept cold when thawed using either ice or a cold block.
5. Tubes containing assay components should be gently flicked to mix and briefly spun to collect all liquid in the bottom of the tube.

## MATERIALS AND DEVICES REQUIRED BUT NOT PROVIDED

- ❖ **For Nucleic acid isolation**
  - NucliSENS® easyMAG® automated total nucleic acid extraction method:
    - NucliSENS® easyMAG® Magnetic Silica (bioMérieux catalog #280133)
    - NucliSENS® easyMAG® Disposables (bioMérieux catalog #280135)

- NucliSENS® easyMAG® Buffer 1 (bioMérieux catalog #280130)
- NucliSENS® easyMAG® Buffer 2 (bioMérieux catalog #280131)
- NucliSENS® easyMAG® Buffer 3 (bioMérieux catalog #280132)
- NucliSENS® easyMAG® Lysis Buffer (bioMérieux catalog #280134)
- Manual ([http://www.biomerieuxusa.com/sites/subsidiary\\_us/files/nuclisens\\_NucliSENS®easyMAG®\\_brochure-1.pdf](http://www.biomerieuxusa.com/sites/subsidiary_us/files/nuclisens_NucliSENS®easyMAG®_brochure-1.pdf))

NOTE: There is a product recall for certain lots of easyMAG extraction reagents. Though no shifts in performance associated with use of these reagents were observed when using them in combination with the Center for Infection and Immunity, Columbia University's **CII-ArboViroPlex rRT-PCR assay**, each lot of affected easyMAG extraction reagents should be evaluated at least weekly before use in extraction of diagnostic specimens. Laboratories should also closely monitor for any trend in Ct values of the External Positive Controls and the HSC controls during testing. Laboratories should refer to bioMérieux Product Safety Correction Notices for a list of impacted lots and advice for end users.

#### ❖ **PCR reagents**

- Molecular-grade water, nuclease-free
- RNA UltraSense™ One-Step Quantitative RT-PCR System (Thermo Fisher Scientific, catalog # 11732927); Manual ([https://tools.thermofisher.com/content/sfs/manuals/rnaulttrasense\\_man.pdf](https://tools.thermofisher.com/content/sfs/manuals/rnaulttrasense_man.pdf))

#### ❖ **Equipment required**

- BioMérieux NucliSENS® easyMAG® (bioMérieux; catalog #280140)
- CFX96 Real-Time PCR Detection System (Bio-Rad)
- Vortex mixer
- Microcentrifuge
- 96-well cold block (and/or ice bath)
- Micropipettes (2, 10, 20, 200 and 1000 µl)
- Multichannel micropipette (2-20 µl)
- Reaction tube racks

#### ❖ **Consumables**

- Hard-Shell® 96-Well PCR plates, low profile, thin wall, skirted, white/white (Bio-Rad, catalog # HSP9655)
- Microseal® 'B' PCR Plate Sealing Film, adhesive, optical (Bio-Rad, catalog # MSB1001)
- DNA AWAY™ (Thermo Scientific; catalog # 7010)
- RNase AWAY™ (Thermo Scientific; catalog # 7003)
- 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
- Disposable gloves
- Disposable gowns
- Permanent Alcohol/Waterproof Lab Markers
- Aerosol barrier sterile pipette tips for P2, P10, P20, P200, and P1000
- 0.5 mL and 1.5 mL reaction tubes

## QUALITY CONTROL

The **CII-ArboViroPlex rRT-PCR assay** should be conducted following strict quality control and quality assurance procedures. Quality control requirements should be followed in conformance with local, state, and federal regulations or accreditation requirements and the user laboratory's standard quality control procedures. Quality control procedures are intended to monitor reagent and assay performance. The following recommendations should be followed to minimize test failure and false negative/positive results.

1. Maintain good laboratory practices including the use of a positive extraction control in each nucleic acid isolation batch (provided in the kit as HSC).
2. Always include a non-template control (NTC), and the appropriate positive controls in each assay run.
3. Keep reagent tubes and reactions capped when not in use to prevent potential cross-contamination.
4. Use only aerosol barrier (filter) and DNase/RNase-free disposable pipette tips and calibrated pipettes.
5. Wear clean lab gown and protective laboratory gloves during assay reagent setup and handling of extracted nucleic acids. Change gloves whenever you suspect they may be contaminated.
6. Store primer/probe mixtures and enzyme master mix at appropriate temperatures (see product inserts).
7. Do not use any reagent past its expiration date.
8. Maintain separate areas, separate clean lab coats, and dedicated equipment for sample preparation, nucleic acid extraction, and assay setup.
9. Clean surfaces using an acceptable surface decontaminant (e.g. RNase AWAY™, DNA AWAY™, and/or 10% bleach) to avoid microbial and nuclease (DNase/RNase) contamination of the specimen and the components of the kit.
10. Do not bring extracted nucleic acid or PCR products into the assay setup area.

## WARNINGS AND PRECAUTIONS

1. The **CII-ArboViroPlex rRT-PCR assay** is for *in vitro* diagnostic use under the FDA Emergency Use Authorization only.
2. The **CII-ArboViroPlex rRT-PCR assay** is for prescription use only.
3. Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.
4. Use of this product is limited to specified laboratories and clinical laboratory personnel who have been trained on authorized instruments.
5. Do not use reagents from other manufacturers with this assay unless indicated in this manual.
6. Appropriate specimen collection, transport, storage and processing procedures are required for the optimal performance of this test. Improper collection, storage, or transport of specimens may lead to false negative results.



7. Specimens should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures. All specimens should be handled in accordance with the guidelines outlined in “Biosafety in Microbiological and Biomedical Laboratories” (BMBL; <https://www.cdc.gov/biosafety/publications/bmbl5/>) and “Healthcare Infection Control Practices Advisory Committee (HICPAC) Precautions to Prevent Transmission of Infectious Agents” ([http://www.cdc.gov/hicpac/2007IP/2007ip\\_part3.html](http://www.cdc.gov/hicpac/2007IP/2007ip_part3.html)). Use personal protective equipment (PPE) consistent with current guidelines. Additional information on safe handling of Zika virus specimens can be found at: <http://www.cdc.gov/zika/state-labs/index.htm>. A risk assessment should be performed for each laboratory for the specific procedures utilized. Congenital microcephaly and other neurodevelopmental defects have been documented in cases of ZIKV infection and should be considered as an additional factor in risk assessment if pregnant workers are involved in studies with ZIKV. For safety information on the instruments, reagents and chemicals, please refer to the respective user manuals or material safety data sheets (MSDS) available online on manufacturer’s websites. The waste materials produced during the assay are to be discarded in accordance with local safety regulations.

8. Always wear protective disposable powder-free gloves when handling kit components.

9. Good laboratory practice is essential for proper performance of this assay. Extreme care should be taken to preserve the purity of the components of the kit and reaction setups. All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.

10. Use separated and segregated working areas for (i) specimen preparation, (ii) reaction set-up and (iii) amplification/detection activities. Workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering different areas. Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.

11. Store positive and/or potentially positive material separated from all other components of the kit.

12. Do not open the reaction tubes/plates post amplification to avoid contamination with amplicons.

13. Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.

14. Do not use components of the kit that have passed their expiration date.

15. Discard sample and assay waste according to your local safety regulations.

16. Refer to the bioMérieux NucliSENS® easyMAG® and CFX96 Real-Time PCR Detection System (Bio-Rad) Manuals and Hazard sections for additional instructions on safety precautions.

## INSTRUCTIONS FOR USE

### ➤ Specimen collection, transportation and storage

With patient's consent specimens should be collected, transported and stored following CDC guidelines (<http://www.cdc.gov/zika/laboratories/test-specimens-bodyfluids.html>) in accordance with regulations for transport of potentially infectious biological specimens ([https://hazmatonline.phmsa.dot.gov/services/publication\\_documents/howtouse0507.pdf](https://hazmatonline.phmsa.dot.gov/services/publication_documents/howtouse0507.pdf)). To ensure nucleic acid integrity, serum/urine samples should be transported in dry ice or liquid nitrogen and, as feasible, aliquots (300-500 µl) should be generated before freezing and storage at ≤-70°C.

### ➤ Acceptable specimens for CII-ArboViroPlex rRT-PCR assay

- Human serum samples
- Human urine samples (collected alongside a patient-matched serum sample)

## Sample Preparation and Assay Setup

The multiplexed **CII-ArboViroPlex rRT-PCR assay** is performed on the CFX96 Real-Time PCR Detection platform (Bio-Rad), using nucleic acids that have been extracted from patient specimens using the NucliSENS® easyMAG® Instrument (bioMérieux); please note the disclaimer below regarding the use of reagents for this extraction procedure. After extraction, nucleic acids are stored frozen (≤-70°C) or for short-term handling are kept on ice or cold block until they are added to the rRT-PCR reaction mix. The fluorescence data generated during the assay are collected using the CFX Manager™ Software and interpreted to provide results of the diagnostic test. A detailed description of the steps involved in this process is provided below.

### Throughput:

Up to 88 sample tests can be performed on a single 96-well plate for the CFX96 Real-Time PCR Detection platform (see Table 2) for ZIKV, DENV, CHIKV, and WNV.

- Extraction of up to 96 samples: 4 easyMAG runs at 60 minutes each (4 x 24 samples) = 4 hours.
- Set-up a 96-well plate and perform **CII-ArboViroPlex rRT-PCR assay** = 2 hours.
- Total time required = approx. 6 hours to test one 96-well plate representing up to 88 samples/run on one CFX96 Real-Time PCR Detection instrument.

## Nucleic acid extraction and storage

- **Automated extraction with bioMérieux NucliSENS® easyMAG® instrument**

Total nucleic acids are isolated from patient samples as detailed in the manufacturer's manuals and instructions ([http://www.biomerieux-usa.com/sites/subsidiary\\_us/files/nuclisens\\_NucliSENS®\\_easyMAG®\\_brochure-1.pdf](http://www.biomerieux-usa.com/sites/subsidiary_us/files/nuclisens_NucliSENS®_easyMAG®_brochure-1.pdf)). Main steps are briefly summarized below:

Specimen input volume: 250 µl

Elution volume: 50-µl total nucleic acid

- Thaw the sample (serum or urine) on ice. Mix 250 µl sample with 750 µl NucliSENS® easyMAG® Lysis Buffer and incubate for 10-15 minutes at room temperature (standard ratio is 1:3 sample to lysis buffer).
- Add 50 µl NucliSENS® easyMAG® Magnetic Silica beads to the mix and ensure thorough mixing of the sample and magnetic silica by pipetting up and down 8-10 times.
- Transfer the mixture into a NucliSENS® easyMAG® sample cartridge.
- Process the sample according to manufacturer's instructions for extraction and instrument interface operation.
- When the run is completed, transfer 50 µl of eluted total nucleic acid into a clean reaction tube. Proceed with **CII-ArboViroPlex rRT-PCR assay** setup or store the nucleic acid at ≤ -70°C \*.

\* For short-term storage of extracted nucleic acid, place specimens in a cold block or on ice until testing. If testing is not being performed on the same day, the isolated nucleic acids should be frozen at ≤ -70°C immediately after making aliquots (2-5 aliquots). Do not freeze-thaw nucleic acid aliquots more than once before testing.

## Reagent Preparation and Reaction Plate Assembly

1. See Table 2 for preparation of Master Mix. For more than one reaction the recipe should be scaled accordingly.

**Table 2. CII-ArboViroPlex rRT-PCR assay mix components.**

<b>Assay Components</b>	<b>Volume per reaction (µl)</b>
RNA UltraSense™ 5X Reaction Mix	5
RNA UltraSense™ Enzyme Mix	1.25
ZIKV-MIX	1
DENV-MIX	1
CHIKV-MIX	1
WNV-MIX	1
RP-MIX	1
H <sub>2</sub> O	3.75
Template (extracted nucleic acid)	10
<b>Total reaction volume</b>	<b>25</b>

2. Reaction plate setup: The **CII-ArboViroPlex rRT-PCR assay** reagent mix (15 µl) should be aliquoted into each required well of Hard-Shell® 96-Well PCR plates (BioRad) and kept on a cold block until ready to be processed on the CFX96 Real-Time PCR Detection platform (Bio-Rad). Unused wells on the plate should be left empty.
3. Addition of clinical samples: Add 10 µl extracted nucleic acid template to each well and seal the plate properly with Microseal® 'B' PCR Plate Sealing Film (BioRad). Spin briefly (2-3 minutes) at 2000-4000 RPM on any PCR plate centrifuge to collect all liquid in the bottom.
4. Addition of controls: Each run must include positive controls for ZIKV, DENV, CHIKV, WNV, and human extraction control (HSC), an rRT-PCR control (extracted HSC control; eHSC) and 2 negative no template controls (NTC) (Table 3).

**Table 3.** Suggested CII-ArboViroPlex rRT-PCR assay sample plate set-up

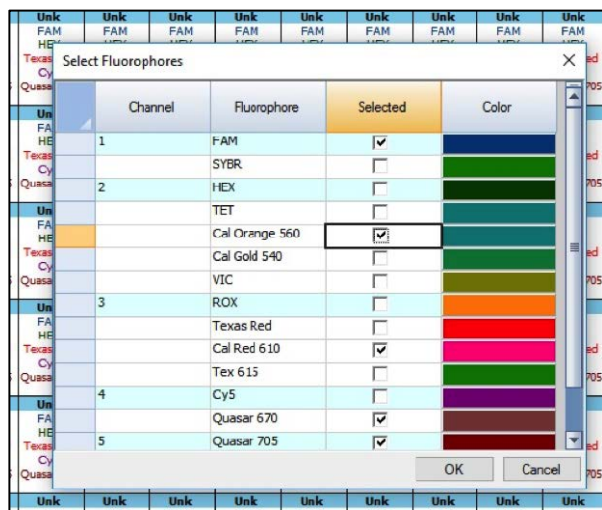
Plate Info	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
B	Sample											
C	Sample											
D	Sample											
E	Sample											
F	Sample											
G	Sample								NTC	ZIKV-Positive	WNV-Positive	DENV-Positive
H	Sample								NTC	CHIKV-Positive	HSC-Control	eHSC-Control

5. Setup of rRT-PCR on the CFX96 Real-Time PCR Detection platform: Program the CFX96 according manufacturer’s manual for multiplex detection selecting fluorophore channels CAL Fluor Red 610 for ZIKV, CAL Fluor Orange 560 for DENV, FAM for CHIKV, Quasar 670 for WNV and Quasar 705 for RNase P and the following cycling profile:

- 50.0°C for 30 min
- 95.0°C for 2 min
- 95.0°C for 15 sec
- 60.0°C for 1min
- +Plate Read
- Go to step 3 for 44 times
- End

➤ Fluorophore selections:

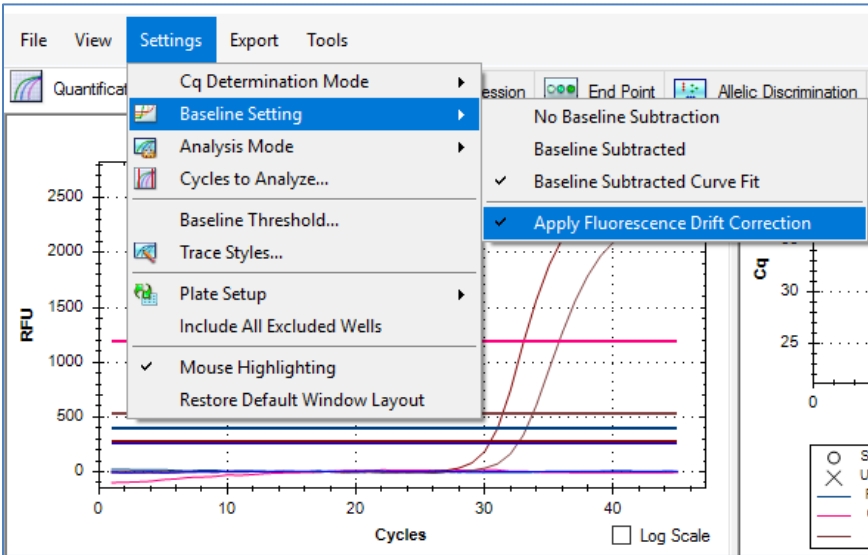
1. Open the “Plate Editor” window. Select wells containing samples or controls of the 96-well plate (Figure 1).
2. Select CAL Fluor Red 610 for ZIKV, CAL Fluor Orange 560 for DENV, FAM for CHIKV, Quasar 670 for WNV and Quasar 705 for RNase P.



**Figure 1.** Selection of fluorophores on CFX96 Real-Time PCR Detection platform (Bio-Rad)

3. Assign Sample Type (Standard/unknown sample/ NTC)

4. After the run is completed apply fluorescence drift correction prior to data analysis and reporting (Figure 2). Go to CFX Manager Software → Settings → Baseline Settings → Apply Fluorescence Drift Correction.



**Figure 2.** Instructions to apply fluorescence drift correction

### Amplification

➤ The **CII-ArboViroPlex rRT-PCR assay** includes primer and probe sets for the detection of ZIKV, DENV, CHIKV, WNV and RNase P control (Table 3). Primers and probes are manufactured by LGC BioSearch Technologies (CA, USA). Primers and probes were selected on the basis of global nucleotide alignments and optimized after testing for specificity, sensitivity and broad reactivity for the targeted viral agents. Primary testing for sensitivity and specificity was done by applying *in vitro* transcribed RNA targets for each virus and RNase P, followed by using virus culture isolates and finally clinical specimens. The primer and probe components for each target are provided as pre-assembled mixtures. Each vial contains reagents sufficient for 96 rRT-PCR reactions. A valid assay has to include all assay reagents outlined in Table 1.

**Table 3.** Primer and probe sets and linked reporter fluorophores and quenchers used for detection of ZIKV, DENV, CHIKV, WNV and RNase P in the **CII-ArboViroPlex rRT-PCR assay**.

Vial No.	Vial label	Vial Components (Primer/Probe. Gene name)	Probe modifications		Vial description
			5'-modification	3'-modification	
1	ZIKV-MIX	ZIKA.3P.QF			Zika virus universal primers and probe. Gene target- 3' UTR of ZIKV genomic RNA
		ZIKA.3P.QR			
		ZIKA.3P.Probe.R610	CAL Fluor Red 610	BHQ-2	
2	DENV-MIX	DENV.3P.QF			Dengue Virus universal primers and probe. Gene
		DENV.3P.QRA			
		DENV.3P.QRB			

		DENV.3P.Probe.O 560	CAL Fluor Orange 560	BHQ-1 plus	target- 3' UTR of DENV genomic RNA
3	CHIKV- MIX	CHIK.NSP2.NQF			Chikungunya virus universal primers and probe. Gene target- NSP2 of CHIKV genomic RNA
		CHIK.NSP2.NQR			
		CHIK.NSP2.Probe .FAM	FAM	BHQ-1 plus	
4	WNV- MIX	WNV.NS5.NQF			West Nile Virus universal primers and probe. Gene target- NS5 of WNV genomic RNA
		WNV.NS5.NQR			
		WNV.NS5.Probe. Q670	Quasar 670	BHQ-2	
5	RP- MIX	RNASE-P-QF			Human ribonuclease P virus universal primers and probe. Gene target- Homo sapiens ribonuclease P subunit p30 mRNA
		RNASE-P-QR			
		RNASE-P- Probe.Q705	Quasar 705	BHQ-3	

## Assay controls

All the assay controls must be included in each plate and tested concurrently with clinical samples.

### Extraction control

Human Specimen Control (HSC)

- A human cell culture preparation known to contain RNase P template but negative for the investigated viral targets. The HSC is included with each batch of test specimens to be extracted. The extracted HSC nucleic acid is included with the concurrently extracted test samples on each PCR plate and analyzed by rRT-PCR.
- The HSC should generate negative results for viral targets (no fluorescent signal from the respective probes/fluorophores), but a positive result should be obtained for RNase P (fluorescence signal for Quasar 705 fluorophore). The HSC is a component of the **CII-ArboViroPlex rRT-PCR assay** kit (vial 'HSC').

### Assay performance control

Extracted Human Specimen Control (eHSC)

- RNase P control/rRT-PCR Control (eHSC). Extracted total nucleic acid from a human cell culture preparation known to contain RNase P (eHSC), but negative for viral targets, is used as a control for performance of RNase P primer/probe set and PCR reagent function. The eHSC control is a component of the **CII-ArboViroPlex rRT-PCR assay** kit (vial 'eHSC').

### Positive controls for viruses

Positive controls for viruses should be positive for only one virus. The following positive controls should be tested on each PCR plate:

- **ZIKV Positive Control.** Synthetic RNA transcribed *in vitro* from a plasmid construct containing the amplified ZIKV sequence and mixed into a background of yeast t-RNA is used as a control for performance of ZIKV primer/probe set and PCR reagent function. The ZIKV positive control is a component of the **CII-ArboViroPlex rRT-PCR assay** kit (vial 'ZPC').

- **DENV Positive Control.** Synthetic RNA transcribed *in vitro* from a plasmid construct containing the amplified DENV sequence and mixed into a background of yeast t-RNA is used as a control for performance of DENV primer/probe set and PCR reagent function. The DENV positive control is a component of the **CII-ArboViroPlex rRT-PCR assay** kit (vial 'DPC').
- **CHIKV Positive Control.** Synthetic RNA transcribed *in vitro* from a plasmid construct containing the amplified CHIKV sequence and mixed into a background of yeast t-RNA is used as a control for performance of CHIKV primer/probe set and PCR reagent function. The CHIKV positive control is a component of the **CII-ArboViroPlex rRT-PCR assay** kit (vial 'CPC').
- **WNV Positive Control.** Synthetic RNA transcribed *in vitro* from a plasmid construct containing the amplified WNV sequence and mixed into a background of yeast t-RNA is used as a control for performance of WNV primer/probe set and PCR reagent function. The WNV positive control is a component of the **CII-ArboViroPlex rRT-PCR assay** kit (vial 'WPC').
- **RNase P control in clinical samples.** All clinical samples are tested for human RNase P, using the RP primer and probe set, to control for specimen quality and as an indicator that nucleic acid resulted from the extraction process.

#### **No template control**

- No Template Control (NTC) – Two NTC (sterile, nuclease-free water) should be run on each PCR plate. The NTC is a component of the **CII-ArboViroPlex rRT-PCR assay** kit (vial 'NTC').

## **INTERPRETATION OF RESULTS**

All data from test controls must be examined prior to interpretation of patient specimen's results. If the controls are not valid, the patient results cannot be interpreted. Each assay must be performed with proper positive and negative controls. The **CII-ArboViroPlex rRT-PCR assay** provides positive, negative, extraction and rRT-PCR controls. All controls must yield the expected results for sample results to be interpretable (Table 8).

**NTCs** – Negative controls. The NTCs must be negative; a positive result for ZIKV, DENV, CHIKV, or WNV, or RNase P indicates contamination and all sample results must be disregarded.

Clean bench areas, tools and pipettes in all work areas with DNA AWAY, RNase AWAY, and bleach. Repeat assay using fresh reagent aliquots and/or a fresh NTC aliquots. If repeat testing fails again, contact the manufacturer for support.

**Positive viral controls** – Positive controls for ZIKV, DENV, CHIKV, and WNV must be positive yielding results within the expected Ct value range (Ct between 25 and 35). Negative results with one or more positive viral controls most likely indicate missing components in the reaction mix.

Repeat the assay. If repeat testing continues to generate negative result from one or more positive control(s), contact the manufacturer for support.

**Extracted human specimen control (eHSC)** – Assay performance control. The eHSC must give positive result for RNase P within the expected Ct value range (Ct between 25 and 35). A

negative result most likely indicates missing components in the reaction mix. In addition, the eHSC must generate negative results for ZIKV, DENV, CHIKV, and WNV.

If RNase P is negative: Repeat the assay. If repeat testing continues to generate negative result for RNase P, contact the manufacturer for support.

If eHSC is positive for viral signal: Clean bench areas, tools and pipettes in all work areas with DNA AWAY, RNase AWAY, and bleach. Repeat assay using fresh reagent aliquots. If repeat testing fails again, contact the manufacturer for support.

**Human specimen extraction control (HSC)** – Extraction control, Must be extracted concurrently with test samples and is included as a sample during rRT-PCR. The HSC should generate a positive result for RNase P. In addition, the HSC must generate negative results for ZIKV, DENV, CHIKV, and WNV.

If RNase P in HSC is negative: The nucleic acid extraction might have failed only for the HSC sample but other results on that plate may reportable; if the eHSC on the plate is positive and no contamination by positive virus controls is indicated, then results from individual patient sample wells can be reported provided they yield a positive RNase P signal confirming that the extraction for that well worked (see also ‘RNase P signal in samples’ below).

If HSC is positive for viral signal: Clean bench areas, tools and pipettes in all work areas with DNA AWAY, RNase AWAY, and bleach. Repeat extraction and assay using fresh reagent aliquots. If repeat testing continues to generate positive viral signal, contact the manufacturer for support.

**RNase P in sample wells** – RNase P should be positive for each sample to confirm successful extraction. If RNase P is negative in some sample wells, either these individual extractions failed (e.g. machine failure for the respective sample slots) or the samples may not contain enough RNase P for detection. If RNase P is negative for a sample but a positive viral signal is recorded and the eHSC on the plate is positive for RNase P, the result for that sample can be reported. For samples without viral signal and RNase P signal sample extraction should be repeated from a new specimen aliquot. If the samples remain negative for RNase P, the result for these samples is inconclusive. If RNase P is negative in all sample wells and for eHSC, repeat assay using fresh reagent aliquots. If repeat testing continues to generate negative results for RNase P, contact the manufacturer for support.

**Table 4.** Description of positive and negative controls used in **CII-ArboViroPlex rRT-PCR assay.**

Control name		Purpose	Anticipated Result					Expected Ct Values
			ZIKV	DENV	CHIKV	WNV	RNase P	
NTC		Detect reagent or environmental contamination during assay setup	-	-	-	-	-	No Detection
Positive	ZPC	Detect failure of viral primers,	+	-	-	-	-	25-35 Ct



viral controls	DPC	probes and other assay components, and/or pipetting errors during assay setup	-	+	-	-		
	CPC		-	-	+	-		
	WPC		-	-	-	+		
eHSC	1. Detect failure of RNase P primers and probe (compare to HSC)							
	2. Detect failure of other assay components, and/or pipetting errors during assay setup (compare to positive viral controls)	-	-	-	-	+	Only RNase P Ct 25-35	
	3. Detect reagent or environmental contamination during assay setup (compare to NTC and HSC)							
HSC	1. Detect failure in nucleic acid extraction protocol							
	2. Detect failure of RNase P primers and probe (compare to eHSC)							
	3. Detect failure of other assay components, and/or pipetting errors during assay setup (compare to positive viral controls and eHSC)	-	-	-	-	+	Only RNase P Ct 25-35	
	4. Detect reagent or environmental contamination during assay setup (compare to NTC and eHSC)							

**Interpretation of Patient Specimen Results:**

The **CII-ArboViroPlex rRT-PCR assay** uses the testing algorithm described in table 4 and table 5. Nucleic acid is extracted from serum or urine and subjected to rRT-PCR analysis using the kit components described above (Table 1). Signal indicative of the presence of viral nucleic acid in a sample extract is considered to be evidence of viral infection, see Table 5, if the negative control does not show viral signal and all other controls work appropriately (Table 4). The absence of signal for viral nucleic acid does not exclude the possibility of infection.

**Table 5. CII-ArboViroPlex rRT-PCR assay results interpretation and reporting instructions**

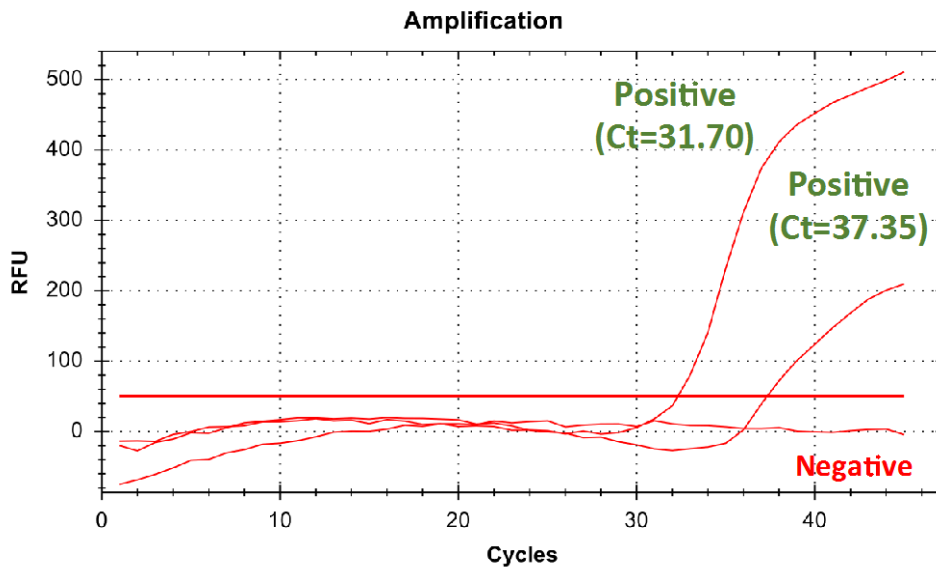
ZIKV	DENV	CHIKV	WNV	RNase P *	Interpretation	Reporting
-	-	-	-	+	No ZIKV, CHIKV, DENV or WNV RNA detected	Report test result
						Note: If date of onset of symptoms is unknown or if patient is asymptomatic, confirmatory testing may be warranted**
-	-	-	-	-	Inconclusive results for ZIKV, CHIKV, DENV and WNV RNA detection since extraction of nucleic acid is questionable (no RNase P signal)	Inconclusive test; no result can be reported.
						Repeat extraction and rRT-PCR with fresh aliquot of specimen and reagents. If repeat testing is again inconclusive, report inconclusive results.
+	-	-	-	+/-	ZIKV RNA detected but no CHIKV, DENV or WNV RNA detected	ZIKV positive
-	+	-	-	+/-	DENV RNA detected but no CHIKV, DENV or WNV RNA detected	DENV positive
-	-	+	-	+/-	CHIKV RNA detected but no CHIKV, DENV or WNV RNA detected	CHIKV positive
-	-	-	+	+/-	WNV RNA detected but no CHIKV, DENV or WNV RNA detected	WNV positive
+	+	-	-	+/-	ZIKV and DENV RNA detected but no CHIKV or WNV RNA detected	ZIKV and DENV positive
+	-	+	-	+/-	ZIKV and CHIKV RNA detected but no DENV or WNV RNA detected	ZIKV and CHIKV positive
+	-	-	+	+/-	ZIKV and WNV RNA detected but no CHIKV or DENV RNA detected	ZIKV and WNV positive
-	+	+	-	+/-	DENV and CHIKV RNA detected but no ZIKV or WNV RNA detected	DENV and CHIKV positive
-	+	-	+	+/-	DENV and WNV RNA detected but no ZIKV or CHIKV RNA detected	DENV and WNV positive
-	-	+	+	+/-	CHIKV and WNV RNA detected but no ZIKV or DENV RNA detected	CHIKV and WNV positive

+	+	+	-	+/-	ZIKV, DENV and CHIKV RNA detected but no WNV RNA detected	ZIKV, DENV and CHIKV positive
+	+	-	+	+/-	ZIKV, DENV and WNV RNA detected but no CHIKV RNA detected	ZIKV, DENV and WNV positive
+	-	+	+	+/-	ZIKV, CHIKV and WNV RNA detected but no DENV RNA detected	ZIKV, CHIKV and WNV positive
-	+	+	+	+/-	DENV, CHIKV and WNV RNA detected but no ZIKV RNA detected	DENV, CHIKV and WNV positive
+	+	+	+	+/-	ZIKV, DENV, CHIKV and WNV RNA detected	ZIKV, DENV, CHIKV and WNV positive

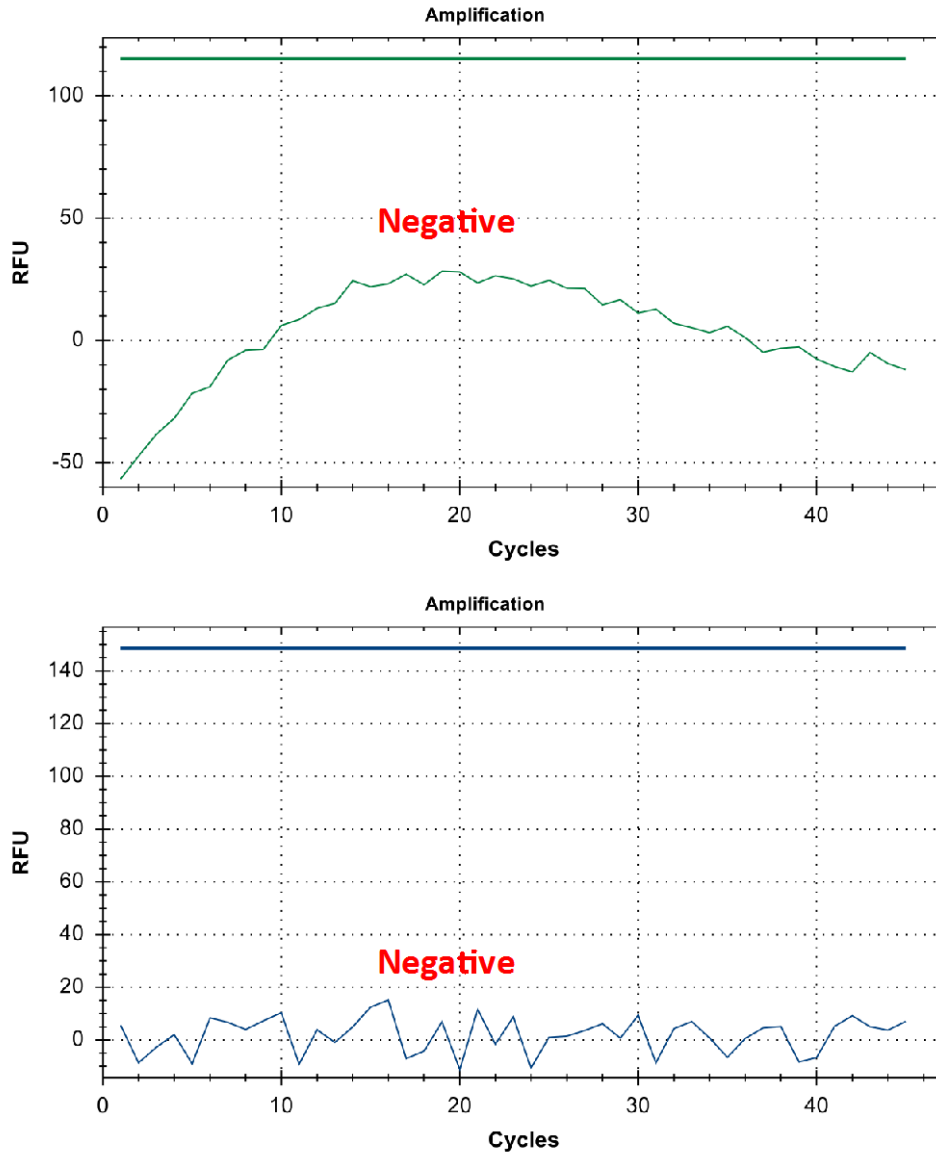
\* RNase P should be positive, but negative results may be observed for samples with low RNase P; if RNase P is negative but a viral signal is obtained, the test is valid and data can be reported (specimen likely contained insufficient levels of RNase P for detection, but viral RNA was extracted).

\*\* A patient-matched serum specimen is currently required for serological follow up testing of negative RT-PCR results per the CDC testing algorithm (found at <http://www.cdc.gov/zika/index.html>).

The user should follow **CII-ArboViroPlex rRT-PCR assay** results interpretation and reporting instructions (Table 5). All controls should yield the expected results in order to allow analysis of specimen data (Table 4). True positives should produce exponential curves with Ct values  $\leq 38.00$  (Figure 3). Samples that do not show exponential amplification or do not cross the threshold within 38 cycles are considered negative (Figure 4a, 4b).



**Figure 3.** Determination of positive results in **CII-ArboViroPlex rRT-PCR assay**



**Figure 4a, 4b.** Determination of negative results in **CII-ArboViroPlex rRT-PCR assay**

**Examination of Patient Specimen Results:**

Assessment of clinical specimen test results should be performed after the results of positive and negative controls have been examined and determined to be valid. If the PCR amplification curve based on increasing fluorescence for a primer/probe set crosses the threshold within ( $\leq$ ) 38.00 cycles, the result is positive. If the amplification curve for a primer probe set crosses the threshold above ( $>$ ) 38 cycles, the result is negative.

## ASSAY LIMITATIONS

- **A patient matched serum specimen is required for serological follow up testing of negative RT-PCR results, per the CDC testing algorithm. (Found at <http://www.cdc.gov/zika/index.html>).**
- Laboratories are required to report all positive results to the appropriate public health authorities.
- Negative results do not preclude infection with Zika virus and should not be used as the sole basis of a patient treatment/management decision. All results should be interpreted by a trained professional in conjunction with review of the patient's history and clinical signs and symptoms.
- Interpretation of RT-PCR test results must account for the possibility of false negative and false positive results.
- False negative results can arise from:
  - Poor sample collection or
  - Degradation of the viral RNA during shipping/transport or storage or
  - Specimen collection conducted prior to symptom onset
  - Specimen collection after nucleic acid can no longer be found in the patient (approximately 14 days post-onset of symptoms for sera)
  - Failure to follow the authorized assay procedures
  - Failure to use authorized extraction kit and platform
  - Presence of RT-PCR inhibitors
- False positive results can arise from:
  - Cross-contamination by target organisms, their nucleic acids or amplified product
- Performance of the **CII-ArboViroPlex rRT-PCR assay** has only been established with the specimen types listed in the Intended Use. Other specimen types have not been evaluated and should not be used with this assay.
- Performance of the **CII-ArboViroPlex rRT-PCR assay** has only been evaluated with the extraction methods/instruments and real-time PCR instruments listed in these instructions for use. Other extraction methods/instruments and real-time PCR instruments have not been evaluated and should not be used with this assay.
- The impact of the administration of Zika virus vaccines and/or therapeutics on the ability to detect Zika Virus RNA in patient specimens has not been evaluated.
- This assay must not be used on the specimen directly. Appropriate nucleic acid extraction must be conducted prior to using this assay.
- Potential mutations within the target regions of the virus genome covered by the primer and/or probes of the test may result in failure to detect the presence of the pathogen.

## CONDITIONS OF AUTHORIZATION FOR THE LABORATORIES

The **CII-ArboViroPlex rRT-PCR assay** Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients and authorized labeling are available on the FDA website:

<https://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm>. Use of the **CII-ArboViroPlex rRT-PCR assay** must follow the procedures outlined in these manufacturer's Instructions for Use and the conditions of authorization outlined in the Letter of Authorization. Deviations from the procedures outlined are not permitted under the Emergency Use Authorization. To assist clinical laboratories running the **CII-ArboViroPlex rRT-PCR assay**, the relevant Conditions of Authorization are listed below.

- Authorized laboratories will include with reports of the results of the **CII-ArboViroPlex rRT-PCR assay** the authorized Fact Sheet for Healthcare Providers and the authorized Fact Sheet for Patients. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.
- Authorized laboratories will perform the **CII-ArboViroPlex rRT-PCR assay** using the NucliSENS easyMAG automated extraction platform (bioMérieux) or with other authorized extraction methods.
- Authorized laboratories will perform the **CII-ArboViroPlex rRT-PCR assay** on the CFX96 Real-Time PCR Detection System (Bio-Rad), or other authorized instruments.
- Authorized laboratories will perform the **CII-ArboViroPlex rRT-PCR assay** for Zika virus, dengue virus, chikungunya virus, and West Nile virus on human serum or other authorized specimen types.
- Authorized laboratories will perform the **CII-ArboViroPlex rRT-PCR assay** for Zika virus on human urine when collected alongside a patient-matched serum specimen and other authorized whole blood derived specimen types.
- Authorized laboratories will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.
- Authorized laboratories will collect information on the performance of the test and report to DMD/OIR/CDRH (via email [CDRH-EUA-Reporting@fda.hhs.gov](mailto:CDRH-EUA-Reporting@fda.hhs.gov)) and Columbia University any suspected occurrence of false positive or false negative results of which they become aware.
- All laboratory personnel using the test should be appropriately trained in RT-PCR techniques and use appropriate laboratory and personal protective equipment when handling this kit, and use the test in accordance with the authorized labeling.
- Columbia University, its authorized distributor(s), and authorized laboratories, will ensure that any records associated with this EUA are maintained until notified by FDA. Such records will be made available to FDA for inspection upon request.

## 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. 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  $\mu$ L of single donor serum 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 by NucliSENS<sup>®</sup> easyMAG<sup>®</sup> (bioMérieux). Total nucleic acid was eluted in 50  $\mu$ L elution buffer and immediately stored on ice for further processing. Each sample was tested with the **CII-ArboViroPlex rRT-PCR assay** on the CFX96<sup>™</sup> 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 6:

**Table 6.** 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 <sup>6</sup>	1	6.61 X 10 <sup>5</sup>	16.45	15.86	16.10	16.14	3/3	5.16E+07
			2	6.61 X 10 <sup>4</sup>	19.96	20.30	20.80	20.35	3/3	5.16E+06
			3	6.61 X 10 <sup>3</sup>	23.20	22.90	23.60	23.23	3/3	5.16E+05
			4	6.61 X 10 <sup>2</sup>	26.86	27.50	26.70	27.02	3/3	5.16E+04
			<b>5</b>	<b>6.61 X 10<sup>1</sup></b>	<b>30.87</b>	<b>31.00</b>	<b>31.30</b>	<b>31.06</b>	<b>3/3</b>	<b>5.16E+03</b>
			6	6.61 X 10 <sup>0</sup>	34.70	35.20	ND	34.95	2/3	5.16E+02
			7	6.61 X 10 <sup>-1</sup>	37.80	ND	ND	NA	1/3	5.16E+01
Dengue Virus Type 1	Hawaii	1.70 X 10 <sup>5</sup>	1	1.70 X 10 <sup>4</sup>	20.58	21.20	20.10	20.63	3/3	4.37E+06
			2	1.70 X 10 <sup>3</sup>	23.94	22.80	23.00	23.25	3/3	4.37E+05

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



			3	6.61 X 10 <sup>4</sup>	22.15	22.10	22.50	22.25	3/3	2.10E+05
			4	6.61 X 10 <sup>3</sup>	26.53	26.00	26.50	26.34	3/3	2.10E+04
			5	6.61 X 10 <sup>2</sup>	30.06	30.90	30.00	30.32	3/3	2.10E+03
			<b>6</b>	<b>6.61 X 10<sup>1</sup></b>	<b>33.22</b>	<b>32.80</b>	<b>33.50</b>	<b>33.17</b>	<b>3/3</b>	<b>2.10E+02</b>
			7	6.61 X 10 <sup>-1</sup>	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 CFX96™ Real-Time PCR Detection System (Bio-Rad). Results are shown in Table 7.

**Table 7.** LoD confirmation in serum specimens using the CFX96™ 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)	<b>5.16E+03</b>	<b>4.37E+02</b>	<b>1.87E+02</b>	<b>4.63E+03</b>	<b>2.87E+03</b>	<b>5.39E+03</b>	<b>2.10E+02</b>
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 CFX96™ 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 8.

**Table 8.** 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 <sup>6</sup>	1	6.61 X 10 <sup>5</sup>	18.59	18.1	19.2	18.63	3/3	5.16E+07
			2	6.61 X 10 <sup>4</sup>	22.2	22.1	21.6	21.97	3/3	5.16E+06
			3	6.61 X 10 <sup>3</sup>	25.75	24.6	25.5	25.28	3/3	5.16E+05
			4	6.61 X 10 <sup>2</sup>	29.23	29.1	28.9	29.08	3/3	5.16E+04
			<b>5</b>	<b>6.61 X 10<sup>1</sup></b>	<b>33.91</b>	<b>32.5</b>	<b>31.76</b>	<b>32.72</b>	<b>3/3</b>	<b>5.16E+03</b>
			6	6.61 X 10 <sup>0</sup>	ND	ND	37.6	NA	1/3	5.16E+02
			7	6.61 X 10 <sup>-1</sup>	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 CFX96™ Real-Time PCR Detection System (Bio-Rad). Results are shown in Table 9.

**Table 9.** LoD confirmation of ZIKV in urine specimens using the CFX96™ Real-Time PCR Detection System

No. of replicate	ZIKV Ct
Virus concn (GEQ/ml)	<b>5.16E+03</b>
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 10.

**Table 10:** 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 Triplex assay (Table 11).

**Table 11:** 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 <sup>6</sup>	16.52	15.85
ZIKV ArD158095	In vitro transcribed RNA	1.40 X 10 <sup>6</sup>	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 CFX96™ 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 12.

**Table 12.** 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 <sup>5</sup> PFU /ml	ND
Japanese encephalitis virus	Nakayama	Extracted nucleic acid	~1.5X10 <sup>7</sup> GEQ/ml	ND
Saint Louis encephalitis virus	Unknown	Extracted nucleic acid	~1.0X10 <sup>5</sup> GEQ/ml	ND
Hepatitis C virus	Clinical isolate	Extracted nucleic acid	1.6X10 <sup>6</sup> GEQ/ml	ND
Enterovirus D68	F02-3607 Corn	Virus culture	1.1X10 <sup>7</sup> GEQ/ml	ND
Influenza A virus	H3N2-Panama	Virus culture	1X10 <sup>9</sup> TCID <sub>50</sub> /ml	ND
Human Adenovirus 6	HADV-6	Virus culture	1.64X 10 <sup>5</sup> TCID <sub>50</sub> /ml	ND
Human herpes virus 1	KOS	Virus culture	1X10 <sup>11</sup> TCID <sub>50</sub> /ml	ND
Ebola virus	Makona	Extracted nucleic acid	1.3X 10 <sup>5</sup> PFU /ml	ND
Parvovirus B19	Clinical isolate	Extracted nucleic acid	~5.0X10 <sup>6</sup> GEQ/ml	ND
Lassa virus	Josiah	Extracted nucleic acid	2.1X 10 <sup>5</sup> PFU /ml	ND
Plasmodium falciparum	Clinical isolate	Extracted nucleic acid	1.5X10 <sup>7</sup> GEQ/ml	ND
Salmonella Typhi	Clinical isolate	Extracted nucleic acid	2.9X10 <sup>5</sup> 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 13. 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, were 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 13.** Potential Cross reactivity of **CII-ArboViroPlex rRT-PCR assay** primer and probes to other pathogens (*in silico* analysis)

<b>Other flaviviruses</b>	<b>Other viruses</b>
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)
<b>Other viruses</b>	<b>Other bacterial and parasitic agents</b>
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 14; 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 14.** 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<sup>®</sup> 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 17.

<b>Total number</b>	<b>n=119 (serum, n=67; urine, n=52)</b>
<b>Total Serum Samples</b>	<b>n= 67 positive samples</b> (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 being 1.54 E+04 GEQ/ml)
<b>Total Urine Samples</b>	<b>n= 52 positive Samples</b>
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 being 1.54 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 17.

<b>Total Serum Samples</b>	<b>n= 55 positive serum samples</b>
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 17. 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.

<b>Total Samples</b>	<b>n= 46 positive serum samples</b>
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 17. 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.

<b>Total Samples</b>	<b>n= 45 positive serum samples</b>
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 15 for serum and Table 16 for urine. The comparator methods (CM) used in the clinical evaluation of the natural clinical specimens are described in Table 17. In the case of the contrived specimens performance was evaluated against the expected result.

**Table 15. 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
<b>ZIKV Positive</b>					
Natural Clinical CM1	19	18/19 <sup>#</sup>	0/19	0/19	0/19
Natural Clinical CM2	22	21/22 <sup>##</sup>	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
<b>DENV Positive<sup>^</sup></b>					
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
<b>CHIKV Positive</b>					
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
<b>WNV Positive</b>					
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
<b>Negative</b>					
CM9	61	0/60	1/61 <sup>^</sup>	0/61	0/61
<b>Overall Percent Agreement</b>		<b>ZIKV</b>	<b>DENV</b>	<b>CHIKV</b>	<b>WNV</b>
<b>Positive percent agreement</b>		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%
<b>Negative percent agreement</b>		100.0% (129/129) <sup>§</sup> 95% CI: 97.1-100.0%	99.5% (140/141) <sup>§</sup> 95% CI: 96.1-99.9%	100.0% (150/150) <sup>§</sup> 95% CI: 97.5-100.0%	100.0% (151/151) <sup>§</sup> 95% CI: 97.5-100.0%

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

<sup>#</sup>CII-ArboViroPlex rRT-PCR assay C<sub>T</sub> of the specimen was 38.68 just above the 38.00 cut-off for a positive result interpretation.

<sup>##</sup>CII-ArboViroPlex rRT-PCR assay C<sub>T</sub> of the specimen was 38.4 just above the 38.00 cut-off for a positive result interpretation.

<sup>^</sup>One of the DENV specimens was negative by the comparator and positive by the CII-ArboViroPlex rRT-PCR assay.

<sup>§</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 16. 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
<b><u>ZIKV Positive</u></b>					
Natural Clinical CM7	15	14/15 <sup>^</sup>	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
<b><u>Negative</u></b>					
CM9	50	0/50	0/50	0/50	0/50
<b>Positive percent agreement</b>		98.1%(51/52) 95% CI: 89.9-99.7%			
<b>Negative percent agreement</b>		100%(50/50) 95% CI: 92.9-100.0%			

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

<sup>^</sup>CII-ArboViroPlex rRT-PCR assay C<sub>T</sub> of the specimen was 38.05 just above the 38.00 cut-off for a positive result interpretation.

**Table 17. 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 $2.68 \times 10^4$ - $8.25 \times 10^4$ 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 $1 \times 10^3$ 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 $1.28 \times 10^5$ copies/mL.
WNV	CM8	Comparator method was an rRT-PCR blood screening assay authorized by FDA for detection of WNV RNA 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 18 shows the data in comparison to samples spiked with a single viral target.

**Table 18. 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

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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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