Zika Virus Real-time RT-PCR

Viracor-IBT Laboratories, Inc.

For use under an Emergency Use

Authorization only

Instructions For Use

Table of Contents

Introduction

This document describes the use of the Viracor-IBT Zika Virus Real-time RT-PCR assay for the *in- vitro* qualitative detection of RNA from Zika Virus in human plasma and serum, or urine (collected alongside a patient matched serum or plasma specimen).

Intended Use

The Viracor-IBT Zika Virus Real-time RT-PCR test is a real-time RT-PCR test intended for the qualitative detection of RNA from the Zika virus in plasma and serum, or urine (collected alongside a patient matched serum or plasma specimen) from individuals meeting Centers for Disease Control and Prevention (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, or other epidemiologic criteria for which Zika virus testing may be indicated). Testing is limited to Viracor-IBT Laboratories, Inc. in Lee's Summit, MO, which is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests.

Test results are for the identification of Zika viral RNA. Zika virus RNA is generally detectable in serum during the acute phase of infection (approximately 7 days following onset of symptoms, if present). Positive results are indicative of current infection. Viracor-IBT Laboratories, Inc. in Lee's Summit, MO is required to report all positive results to the appropriate public health authorities.

Negative results do not preclude Zika virus infection 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 Viracor-IBT Zika Virus Real-time RT-PCR test is intended for use by trained clinical laboratory personnel qualified by state and federal regulations who have received specific training on the use of the Viracor-IBT Zika Virus Real-time RT-PCR test. The test is only for use under the Food and Drug Administration's Emergency Use Authorization.

Protocol Use Limitations

The Viracor-IBT Zika Virus Real-time RT-PCR test has not been extensively tested with clinical specimens. Modifications of this test (i.e., use of PCR instruments or chemistries other than those described) are not permitted.

Assay Principle

The Viracor-IBT Zika Virus Real-time RT-PCR assay is a real-time reverse transcription polymerase chain reaction (RT -PCR) test. The Zika virus primer and probe set is designed to detect RNA from the Zika virus in plasma, serum or urine from patients presenting signs and symptoms of the Zika virus infection

in conjunction with epidemiological risk factors.

Following patient specimen collection and receipt by the laboratory, nucleic acids are isolated and purified from plasma, serum or urine using the bioMerieux NucliSENS easyMag extraction platform using a protocol for total nucleic acid extraction. The purified nucleic acid is reverse transcribed and amplified using Life Technologies TaqPath™ 1-step RT-qPCR master mix reagent with thermocycling and detection in an ABI 7500 real-time PCR instrument. In the process, the probe anneals to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by the ABI 7500 real-time PCR instrument.

The following controls will be used with the Viracor-IBT Zika Virus Real-time RT-PCR test:

1. A "no template" (negative) control (NTC) is included in each RT-PCR run to ensure that all amplification reagents are free of target RNA or amplicon and is used to demonstrate that detection of target Zika genomic RNA is not due to false positive results.

2. Two Zika virus positive amplification curve controls (S2 and S4) are included in each RT-PCR run to ensure that the target Zika genomic RNA can be detected by the RT-PCR assays and are used to demonstrate that the anticipated level of sensitivity has been achieved.

3. A Zika virus negative extraction control (NEC), which is a known-negative sample, is included in each run to ensure that all extraction and amplification reagents are free of target RNA or amplicon and is used to demonstrate that detection of target Zika genomic RNA is not due to false positive results.

4. A Zika virus positive extraction control (PEC), which is a known-negative sample spiked with live Zika whole virus (Zeptometrix #0810092CF), is included in each extraction and RT-PCR run to ensure that extraction and amplification procedure was carried out accurately. The PEC demonstrates that detection of target Zika genomic RNA achieved expected results and the assay performance is within the performance specifications.

5. An internal control (IC), bacteriophage MS2, is added to each sample prior to extraction. Detection of the MS2 RNA in the final extracted nucleic acid demonstrates that lysis and extraction steps were correctly performed for each sample. A lack of fluorescence signal, or a delay in the occurrence of a detectable fluorescence signal, for the internal control indicates that either nucleic acid extraction was not effective or that PCR inhibitors are present.

Specimens

Acceptable Specimens

- Plasma, Serum and Urine (collected alongside a patient matched serum or plasma specimen)

Specimen Handling And Storage

- For plasma, collect 4-5 mL whole blood in EDTA or ACD tube, centrifuge and transfer 2 mL plasma to sterile, screw top tube (minimum volume 1 mL). Specimens can be shipped at ambient or refrigerated temperature Monday through Friday. Specimens shipped at ambient temperature must be received within 96 hrs of collection.
- For serum, collect 4-5 mL whole blood in red top tube, centrifuge and transfer 2 mL serum to sterile, screw top tube (minimum volume 1 mL). Specimens can be shipped at ambient or refrigerated temperature Monday through Friday. Specimens shipped at ambient temperature must be received within 96 hrs of collection.
- For urine, 2 mL sample collected in a sterile urinalysis container then transferred to sterile, screw top tube for shipment (minimum volume 1 mL). Specimens can be shipped at ambient or refrigerated temperature Monday through Friday. Specimens shipped at ambient temperature must be received within 96 hrs of collection.
- Please refer to www.viracoribt.com/zika for additional specimen collection and handling requirements.

Safety Precautions

Strict universal safety precautions must be taken and good laboratory practice is compulsory for all activities that require handling of clinical specimens that may be biohazardous and infectious.

- The Positive Control contains live Zika virus, and all specimens should be treated as potentially infectious. All controls, samples and equipment coming into contact with these specimens should be considered potentially infectious and decontaminated or disposed of with proper biohazard precautions. CDC and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2. The Biosafety in Microbiological and Biomedical Laboratories states that Zika virus is considered a BSL-2 pathogen.
- Lab coat and gloves must be worn by all personnel while handling clinical specimens.
- Generation of aerosols, splashes, and spills of potentially infectious material should be avoided.
- All laboratory glassware, equipment, disposable materials and wastes suspected or known to be biohazardous must be decontaminated, preferably in an autoclave.
- Sample preparation must be performed in biological safety cabinet.
- Work surface should be decontaminated using a fresh 10% aqueous solution of bleach at the end of each use.
- Specially cleaned equipment, racks, etc. are designated specifically for nucleic acid extraction, reagent preparation or PCR use. Such materials are not to be removed from their respective locations.

- Real-time PCR reactions are performed in sealed 96-well plates. Real-time PCR reactions are to be disposed immediately after amplification.

Equipment and Consumables

MATERIALS PROVIDED BY VIRACOR-IBT LABORATORIES, INC

- **Zika Virus Quantitative Real-time RT-PCR test**
    - Oligonucleotide primers - Biosearch Technologies, 81 Digital Drive, Novato, CA 94949-5728
    - Dual-labeled hydrolysis probes (TaqMan)
        - FAM-BHQ-1 labeled and Quasar670-BHQ-2 labeled oligonucleotide probes: Biosearch Technologies, 81 Digital Drive, Novato, CA 94949-5728
    - Bacteriophage MS2 - American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20108
    - Zika virus stock - 0810092CF with a concentrated titer of 1.15x107 TCID50/mL, Zeptometrix, 878 Main Street, Buffalo, NY 14202
    - pIDTBlue modified plasmid with inserted nucleotide regions from the Zika strain KU497555 (Brazil, 2015) - Reference ID 143425698 ZIKV_519-723_918-1049_1168-1330, Integrated DNA Technologies, Inc. 1710 Commercial Park Coralville, Iowa 52241

MATERIALS REQUIRED BUT NOT PROVIDED

- Biomerieux easyMAG reagents, cat # 2801403 (lysis buffer, cat. # 280134; buffer 1, cat. # 280130; buffer 2, cat. # 280131; buffer 3, cat. # 280132; magnetic silica, cat. # 280133)
- TaqPath™ 1-step RT-qPCR master mix, CG; (Life Technologies, catalog # A15299 or A15300)
- Zika virus negative plasma, serum, urine
- Zika virus positive control
- Zika virus positive amplification curve controls (S2 and S4)
- RNase-, DNase-free water, Fisher Scientific, cat # BP561-1

EQUIPMENT

- Biomerieux easyMAG instrument with disposables
- ABI 7500 standard Instrument with disposables
- Freezer (manual defrost) at -10 to -30°C (for kit component frozen storage)
- Freezer (manual defrost) at -10 to -30°C (for specimen frozen storage)
- Refrigerator at 2 to 8°C
- Class II Biosafety cabinet (BSC) for specimen handling prior to extraction
- Bench top centrifuge for low speed centrifugation of 96-well plates or other reaction vessels
- Microcentrifuge
- Vortex mixer

CONSUMABLES

- RNase/DNase-free 5 mL tubes
- RNase/DNase-free 1.5 mL polypropylene microcentrifuge tubes and racks
- RNase/DNase-free 96 well thermocycler plate rack or appropriate tube rack
- 96-well optical reaction plate or other reaction vessels
- Optical adhesive cover (for use with 96-well optical reaction plate)
- Appropriate personal protective equipment (PPE) including disposable, powder-free gloves
- Cooler racks for 1.5 mL microcentrifuge tubes and 96-well 0.2 mL PCR reaction tubes or plates
- Single or multi-channel micropipette(s) with an accuracy range between 1-10 µL, 10-100 µL and 100-1000 µL. **NOTE:** A separate set of micropipettes is required for extraction, sample addition and amplification areas.
- Pipette tips with aerosol barrier: 10 µL, 200 µL, and 1000 µL sizes

Quality Control

Real-Time RT-PCR is a sensitive method and should be conducted following strict quality control and quality assurance procedures. Following these guidelines will help minimize the chance of false-negative and false-positive results

ASSAY CONTROLS

- Zika Virus Positive Extraction Control: A Zika virus positive extraction control (PEC), which is a known-negative sample spiked with live Zika whole virus, is included in each extraction and RT-PCR run to ensure that extraction and amplification procedure was carried out accurately. The PEC demonstrates that detection of target Zika genomic RNA achieved expected results and the assay performance is within the performance specifications. The PEC should have C_T values $\geq$ 29 but $\leq$ 32.
- Zika Virus Positive Amplification Curve Controls (low and high concentrations): Two Zika virus positive amplification curve controls (S2 and S4) are included in each RT-PCR run to ensure that the target Zika genomic RNA can be detected by the RT-PCR assays and are used to demonstrate that the anticipated level of sensitivity has been achieved. The low concentration positive amplification curve control (S2) should have C_T values $\geq$ 28 but $\leq$ 30. The high concentration positive amplification curve control (S4) should have C_T values $\geq$ 21 but $\leq$ 23.
- Zika Virus Negative Extraction Control: A Zika virus negative extraction control (NEC), which is a known-negative sample, is included in each run to ensure that all extraction and amplification reagents are free of target RNA or amplicon and is used to demonstrate that detection of target Zika genomic RNA is not due to false positive results. The NEC should have no detectable signal after 45 cycles of amplification in each reaction mix.
- Internal Control: An internal control (IC), bacteriophage MS2, is added to each sample prior to extraction. Detection of the MS2 RNA in the final extracted nucleic acid demonstrates that lysis and extraction steps were correctly performed for each sample. A lack of fluorescence signal, or

a delay in the occurrence of a detectable fluorescence signal, for the internal control indicates that either nucleic acid extraction was not effective or that PCR inhibitors are present. The expected C_T value for MS2 is ≤ 35. In samples with no Zika target detected, a C_T value less than or equal to this value for MS2 RNA demonstrates that effective nucleic acid extraction has been achieved and the absence of RT-PCR inhibition.

Nucleic Acid Extraction

Notes on Extraction

- Only acceptable specimens extracted using the prescribed extraction method may be tested with this assay.
- One positive and one negative control will be included in each run.
- Internal control RNA is added to the sample after lysis buffer addition and is co-purified and co-detected in each specimen and control.
- Retain specimen RNA extracts in cold block or on ice until testing.

Automated Extraction

- bioMerieux NucliSENS easyMAG extraction
- Clinical specimens are extracted using the protocol for total nucleic acid extraction. Follow the manufacturer's instructions using reagents specified by cat # 2801403 (lysis buffer, cat. # 280134; buffer 1, cat. # 280130; buffer 2, cat. # 280131; buffer 3, cat. # 280132; magnetic silica, cat. # 280133).
- Extraction uses the following volumes:
    - Specimen input volume: 500 µL
    - Elution volume: 100 µL

Storage of Nucleic Acid Specimens

Store the RNA for up to 15 minutes refrigerated prior to use. RNA should be frozen at -60ºC or colder if longer storage is required before use and may be stored at least 2 weeks at -60ºC or colder.

Real Time RT-PCR Assay

Quality Control

- **Internal Amplification Control**
    - To ensure the absence of non-specific PCR inhibition of a sample, an internal control RNA (IC) is added to the easyMAG Lysis Buffer prior to nucleic acid extraction. A sample can be interpreted as negative only if the IC C_T. value is within the acceptable range but no signal from target reporter dye has been detected. The acceptable range for each new lot/preparation of the internal control will be set at ±3 C_T from the mean (29) of the internal control C_T values obtained from 20 replicates (data generated from negative and positive controls over multiple runs). The acceptance ranges will be generated for each lot of Internal Control.
- **Controls Use Frequency**
    - A Zika virus positive extraction control (PEC), which is a known-negative sample spiked with live Zika whole virus, is included in each extraction and RT-PCR run to ensure that extraction and amplification procedure was carried out accurately.
- **Controls Tolerance Limits**
    - Negative Control should be listed as "Not detected" for the RT-PCR reaction. If the Negative Control is positive (C_T value < 45), then this control is invalid. This indicates possible contamination of prepared samples. Positive patient results cannot be reported. Positive specimens on this run must be repeated. Negative specimens may be reported given that all other assay run criteria are met.
    - If the Zika Virus Positive Control C_T values are within the acceptable range, the Positive Control is considered valid and acceptable. If the Zika Virus Positive Control C_T values are above or below the acceptable range for one or both mixes, the Positive Control is considered invalid and unacceptable. All patient specimens must be re-assayed.
- **Review Patient Data**
    - Review patient results for unusual patterns, trends or distributions in patient results, such as an unusually high percentage of abnormal results, or unusually high percentage of Not Detected, or positive results.

Procedure

For all procedures involving specimens, buttoned lab coats, gloves, and face protection are required minimum personal protective equipment.

Nucleic Acid extraction (to be performed in the Extraction Room)

Startup

easyMAG Startup

1. Verify each reagent loaded on the easyMAG is within expiration date.
2. Login to the instrument software.
3. Document daily startup on maintenance log.

Reagent Preparation

Lysis Buffer Preparation for Off-Board Extraction

For RNA qPCR assays:

1. Add 2 mL lysis buffer to each sample vessel well. Add 5 µL of the working stock of MS2 dilution 4 (1:10,000 dilution) per 2 mL lysis buffer.
2. Alternatively, for RNA qPCR assays, add 2 mL of lysis buffer containing the appropriate internal control to an easyMAG cartridge (as shown in example below).
3. Visually verify every cartridge well has the correct volume of Lysis buffer.

Preparation of Silica Bead Mix

Each EMP (Biohitt Electronic Multipipettor) program is set to prepare and dispense silica for eight specimens. Repeat steps as needed for each sample strip of eight.

EMP Program 1 is set to aspirate and dispense 550 µL of buffer (molecular grade water) and silica solution. For this program, load EMP with one tip.

1. Place the tip in molecular grade water and hit start button once to aspirate 550 µL.
2. Place tip in 2 mL aliquot tube and hit start button once to dispense 550 µL of molecular grade water. Discard tip by hitting eject button twice.
3. Vortex silica tube. Load EMP with new tip and place in silica tube. Hit start button once to aspirate 550 µL of silica solution.
4. Place tip in 2 mL tube from step 2 above and hit start button once to dispense 550 µL of silica solution.
5. Discard tip by hitting eject button twice.

EMP Program 2 is set to transfer 8 volumes of silica mix to 8-well ELISA strip. For this program, load the EMP with one tip.

1. Place the tip in the silica mix prepared above in Preparation of Silica Bead Mix. Hit start button once to aspirate 1050 µL of the mix.
2. Leave the tip in the tube and hit start once to dispense 25 µL back into the reservoir to reset the pipette.
3. Place the tip sequentially into the wells of the ELISA strip and hit start button once for every 125 µL dispense step.
4. Place the tip back in the container holding the silica mix and hit start button once to discard the remaining 25 µL solution.
5. Discard tip by hitting eject button twice.

easyMAG Extraction Procedure

All extraction runs require a Positive extraction control and Negative extraction control (NEC).

1. Refer to easyMAG Extraction Chart on next page for appropriate controls, volumes, and protocols for each type of sample/test.
2. Determine number of extraction wells required per sample and extraction run according to the chart below:

Tests per Sample or per extraction run	easyMAG extraction wells for sample and controls
≤ 6 tests	1 well (single extract)
7 - 12 tests	2 wells (double extract)
≥ 13 tests	3 wells (triple extract)

3. Vortex capped sample aliquot.
4. Pipette appropriate volume of sample into cartridge. Visually verify correct volumes in cartridges.
5. Record lot numbers, Lysis time and CLS performing extraction, loading of instrument and elution transfer on the easyMAG run map.

easyMAG EXTRACTION CHART

	NEC	POS CTRL (as applicable)	Internal Control (volume/well)	Control and Sample volume	Easy-MAG protocol	Onboard or Offboard (2mL lysis/well + internal control)	Elution volume
Zika virus**	Plasma NEC**	ZIKA PEC	MS2(-4) (5 µL)	500 µL*	Plasma CSF Other or Urine	Onboard or Offboard	100 µL

***500 µL for full volume extraction**. Notate any low sample volume samples on map.

**** Zika virus** extraction requires the use of plasma NEC for both plasma and urine extractions.

Additional instructions for specific specimen types or tests

Urine samples:

Urine specimens must be segregated and extracted on a separate easyMAG run from other specimen types due to potential for contamination.

Positive Identification checks for manual pipetting of samples into easyMAG Cartridges

Maintain positive identification when pouring off specimens into aliquot tubes and when pipetting samples into cartridges according to identifiers shown on the easyMAG run map.

a. Ensure the master tube and aliquot tubes match by patient name, date of collection, time of collection.
b. Do not combine multiple samples into the same aliquot tube unless the sample type, patient name, date of collection, and time of collection all match.
c. Refer to posted *Clinical Processing Guide*, *Pre-processing Procedures for ID tests and Pre-processing Temperature Requirements for ID and AI* and Hemolysis chart when determining acceptability of samples and handling and testing requirements for different sample types.

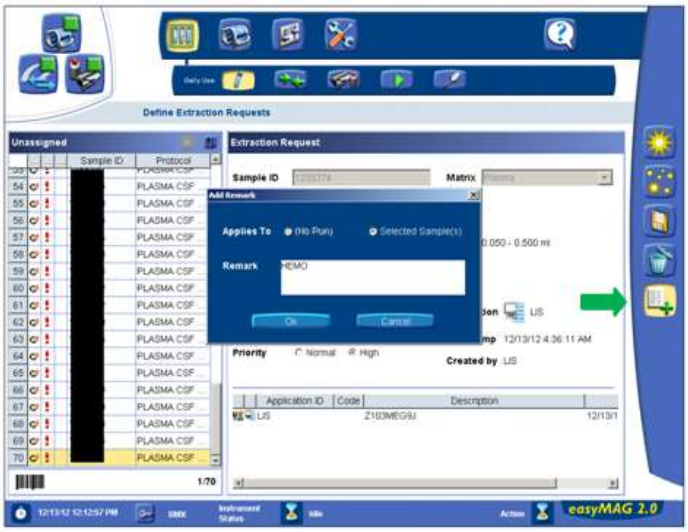
Programming easyMAG for Off Board Lysis

1. Insert the sample strips and aspirator disposables into the instrument in the order indicated on the easyMAG Run Map, ensuring tips are seated properly.
2. In easyMAG Software, check to ensure adequate volume is in each reagent.
3. Document easyMAG instrument number and the next sequential run number on the map.

On the touch screen, touch the ***Settings icon***, software defaults to ***Application Settings***. The screen will prompt for entries in the following parameters*;* ***Default Request, Run Name Prefix, Sample ID Prefix, Sample Type, and Sample Addition Guidance.*** **Options**	**Enter the following:**
Default Protocol:	Chose appropriate extraction protocol per easyMAG extraction Chart
Run Name Prefix Type:	Literal
Run Name Prefix :	Date E<instrument number>-<run number>. 021214 E9-1 021214 EOF9-1
Sample ID Prefix:	Leave blank
Sample Type:	Lysed selected
Workflow Defaults:	On Board silica incubation selected
Reagent Tracking:	none selected

Starting the easyMAG extraction run

1. Hold the Load easyMAG sample strip by the indent and slide the edges of the strip into the sample strip carriage arms, beneath the tabs in either arm as shown above. Slide the strip back until the lock tab at the back clicks into the slot. Ensure the lock tabs are aligned correctly.
2. Load the Aspirator disposables and ensure they are clipped into the instrument in order for the instrument to aspirate fluid from the sample strips. There must be an aspirator disposable in the corresponding position for each sample easyMAG sample strip.
3. Verify samples entered in the software screen are in the same order as easyMAG run map.
4. For specimens with input volumes less than the default input volume, select accession number in easyMAG and enter the adjusted input volume.
5. For specimens that require comments, select accession number and enter the appropriate comment using the **Add Remark** icon in **Define Extraction Request** menu in the easyMAG software. Once entered, verify the comment was added by viewing the window below for each example.



6. Comments must be entered exactly as written below or the comments will not appear on the ABI map. Do not add additional spaces before or after the comments indicated as doing so may cause the easyMAG run to fail.

Comments	***Indicates***
HEMO	*Hemolysis*
BLDY	*Bloody*
SUPV	*Supervisor*
RXT	*Re-extract*
PALS	*Patient Advocates for Laboratory Services*
Free text	*Comment by coordinator/supervisor*

7. To assign specimens to a run touch **Organize Run** icon. Touch **Create Run** icon and name the run in the following format: <Date> <Instrument number>-<run number>.
    a. Ex. 010112 E1-2
8. Move accession numbers from **Unassign** to **Layout**.
9. Touch **Load Run** icon. Select desired run. Print worklist by touching **Print Worklist** icon. Verify that protocol, accession numbers, and order of samples match run map. Verify that low volumes have been entered. Correct any errors before scanning cartridges.
10. Scan cartridge locations (i.e. A, B, C), then scan the cartridge barcode for each location; verify barcodes scanned correctly.
11. Verify Lysis incubation is >10 minutes per time noted on the extraction map.
12. Obtain silica mix contained in 8-well ELISA strip plate. EMP Program 3 on Biohit electronic multipipettor is set to transfer and mix 8 volumes of silica mix to 8 wells of the easyMAG sample strip.
13. Verify that 10 minutes of Lysis incubation has completed, then perform the following steps:
    a. Load the EMP with 1-8 tips, depending on the number of sample vessel wells in use.
    b. Place the tips in the ELISA wells containing the silica mix and hit start button once more to mix and aspirate 100 µL of the silica mixture.
    c. Place the tips in the wells of the sample vessel strip and hit start button once to aspirate an additional 800 µL out of the sample vessel and perform 3 mix cycles.
    d. Place pipette above biohazard waste container and press eject button twice to discard tips.
14. Start Run. Complete run map documentation.
15. As appropriate make copy of extraction map for mapmaker or for lab assistant to create labeled nucleic acid microcentrifuge tubes.
16. Enter adjusted volumes and disclaimer comments in LIMS Software. Refer to *Correction Factors for Specimen Input and Elution Volume Changes* for acceptable input volumes.

Elution Transfer

Positive Identification prior to Removing Final Product

1. Ensure elution is transferred within 30 minutes of run completion so the bead pellet does not drop into elution volume.

2. Magnets may be used to pull the beads out of the elution volume.
3. Verify elution volumes are as expected and beads are uniform for each well of cartridges. If elution volumes and/or bead size are not as expected, inform a coordinator or supervisor.
4. Print easyMAG map and select “yes” to assess run.
5. Review and verify the content on the extraction map, load map and the easyMAG run report :
    a. The physical instrument number.
    b. Run number on the map.
    c. Accession numbers scanned correctly into the software.
    d. Correct input volumes are printed.
    e. Correct comments are printed.
    f. Protocol setting is correct.
    g. Cartridge barcodes scanned correctly into the software, and match the order loaded on easyMAG.

Setting Up Real-time RT-PCR Reactions

1. Create ABI Plate Map
    a. Obtain a printed copy of a Run-Result Map and write the accession numbers of patient samples and controls to be tested on the run
    b. Include a minimum of one well for a NEC, NTC, and positive control for each analyte run. Include a minimum of one well each for positive amplification curve controls (S2 and S4). On the plate map, record the run-map number and date. Add accession numbers of patient samples tested for each analyte.
    c. Patient samples are to be run in single well per analyte.
    d. Include any extraction or elution volume differences requiring additional calculation on the run map.

2. Prepare Master Mix for ABI PCR plate - Master Mix room
    a. Prepare master mix only in designated laboratory space (master mix room) with designated pipettes and disposables. Do not bring in disposables or pipettes into the master mix room from other areas of the lab.
    b. Complete appropriate master mix form. Record the number of wells for each assay requiring master mix. Calculate the amount of master mix and primers/probe preparation required for each assay by multiplying the number of wells by the volume of master mix reagents and primers/probe (P/P) per well.
    c. Record the run date and lot numbers and expiration dates for primer/probe mix and master mix on master mix Reagent Log. If a new lot of reagent is placed in use, record the map run numbers containing new lot of reagent at the end of the Master Mix Reagent Log form.
    d. Label epMotion trough or 2 mL screwtop tube for master mix with the map number and the assay name.
    e. Prepare master mix for each assay according to calculations and add each assay’s master mix to appropriate wells as designated on the Run-Result Map. Use master mix volume per well specified on the master mix calculation sheet.

Note: TaqPath 1-step RT-qPCR Master Mix is stored at -25°C to -15°C. The master mix will not freeze but gelling may occur. 30 minutes before assembling the plate, allow master mix to thaw in the refrigerator, protected from light. It is normal for this master mix to have a faint yellow hue. TaqPath 1-step RT-qPCR Master Mix is a 4X formulation and it is more viscous than most master mixes. Pipette slowly and be sure that master mix, primers and probes, and water are thoroughly mixed before transfer into PCR plate.

f. Record master mix completion time and initial for preparation of master mix on the form. Circle the dead air box used during master mix preparation.
g. Obtain appropriate ABI PCR plate per form
    i. Place ABI Fast PCR plates in cold plate holder (silver 96 well cold plate stored in refrigerator). Ensure prepared master mix is labeled with the assay name and map number.
    ii. Place ABI Standard PCR plate in frozen cold block for reaction assembly. Ensure prepared master mix is labeled with the assay name and map number. Prepared plate must be loaded with nucleic acid templates immediately following preparation for Superscript and TaqPath reactions.
h. Vortex master mix prior to use.
i. Pipette master mix volume per well as specified on the master mix calculation sheet. Add each assay's master mix to appropriate wells as designated on the Run-Result Map.
    i. For Post EVO maps with assays greater than 2 columns in the ABI plate map, each assay master mix must be placed in a epMotion reagent reservoir(s) labeled with assay and map number. Master mix is then added to the ABI plate by epMotion instrument in the plating room. Circle the epMotion used to deliver master mix.
    ii. For Post EVO maps with assays less than or equal to 2 columns in the ABI plate map, each assay master mix must be placed in a 2 ml screwtop skirted tube labeled with assay and map number. Master mix is then plated into the ABI plate in the plating room DAB. Circle the DAB used to pipette master mix.

Add Nucleic Acid – Plating room

IMPORTANT: This step requires a high level of accuracy and precision. Great care must be taken to ensure that the precise amount of template is added to wells designated on the plate map. Potential for cross contamination is significant and care must be taken not to create aerosols during addition of patient nucleic acid or standards. A new pipette tip is required for each transfer of nucleic acid or master mix in the plating room.

Prior to the start and at the end of any of the following pipetting steps, visually verify the proper volume of master mix or nucleic acid is added to the ABI plate according to the run map.

<u>Addition of Nucleic Acid</u>

1. Obtain plate prepared with master mix placed in cold plate holder, Plate Map, Calculation Sheet, and samples to be analyzed according to the Plate Map.

2. In laminar flow hood or dead air box add patient nucleic acid or controls to the wells indicated on the Run-Result Map. On the Master Mix Calculation Sheet, circle all dead air boxes used during run preparation.

Addition of Master Mix

1. Obtain the ABI plate with nucleic acid placed in cold plate holder, Plate Map, Calculation Sheet.
2. On the Master Mix Calculation Sheet, circle all dead air boxes used during run preparation.

Add Standards or Curve Controls and Negative Template Control – Amplification Room

1. Place ABI plate into a cold plate holder.
2. Verify all Standards or Curve Controls are in date. Expiration date is 1 year from open date unless the manufacturer expiration date is earlier.
3. Document Standards or Curve Controls lot numbers, expiration dates, ABI map number on Standards Log.
4. Vortex standards.
5. Use minicentrifuge for a few seconds to spin droplets to bottom of standards vial.
6. Pipette volume of Standards or Curve Controls to the ABI plate per map (refer to master mix calculation sheet for appropriate volume of Standard to use).
7. Pipette volume of Negative Template Control (RNAse and DNAse free water).
   Note: RNAse and DNAse free water has an expiration date of 24 hours upon open.
8. Circle the dead air box used to pipette the Standards or Curve Controls, initial STDs plated by on ABI map.
9. Visually verify all wells have appropriate volume per map.
10. Seal ABI plate with ABI Optical Sealer ensuring all wells are sealed
11. Vortex ABI plate at 2 opposing corners. Do not place wells directly on vortex platform.
12. Centrifuge ABI plate at 3500 rpm for 30 seconds using program 5.

Manually enter map into ABI software, Load plate and Start Run

1. Power on Laptop, ABI instrument, Open ABI software.
2. Click Create New Document.
3. Click Browse.
4. Select Desktop Shortcut to Assay Template. Select the appropriate Assay Template
5. Click Finish.

Manual Entry of Maps into ABI software

1. If New Document Wizard is used to enter the map and a stored standard curve is not available, the standard values for quantitative tests should be entered according to Standard Values for Quantitative Assays. The concentrations listed in the benchtop include a calculation to compensate for specimen to elution concentration as well as converting µL to mL so that results will be provided as copies/mL.

2. In the Set Up> Plate window use the Well Inspector (View>Well Inspector) to modify settings for each well to include: 'Analyte code-accession number' and appropriate detector(s) for target analyte and UIC or MS2 if multiplexing. See Analyte Targets for appropriate Analyte Codes and detectors.
   Note: Multiplexed reactions require selection of all applicable detectors for standards as well as test patients.

Load ABI plate

1. Verify ABI Software map matches content of ABI printed map and verify that ABI wells are filled appropriately according to ABI printed map.
2. Verify plate seal is applied and load ABI plate with well A1 matching the A1 indicator on the instrument. Load Plate. Close door.

Start ABI run

1. Click Instrument tab on ABI software.
2. Verify map name on ABI computer matches ABI map.
3. Click Start.
4. The ABI run is confirmed to have started appropriately if the countdown of elapsed run time begins.
5. Document ABI instrument ID, start time and initial on the ABI map.
6. Save the plate document.

Single Well/Stored Standard Curve

1. When run is complete, press **OK** and **Disconnect**.
2. From Results>Amplification Plot Tab, Analysis Settings, set threshold and baseline.
3. Verify correct analysis settings.
4. Set Line Color: Detector Color.
5. Select **Analyze**. Initial map to document settings are correct.

Standards or Curve Controls:

1. Verify Curve Control values are within established ranges listed in Acceptable Assay Standard Control Values. Document whether the values are within criteria with either "Accept" or "Alert" in STD Accept or Alert section on the ABI Map for each assay.
2. Review the Negative Template Control (NTC) for each assay. Document the absence of assay target signal in each of the NTC wells on the ABI plate map.
3. Document the absence of assay target signal in the Negative Extraction Control (NEC) as Not Detected with the C_T value.

Patient Wells:

1. Review each patient well for valid amplification curves. Visually examine the amplification plot of each patient reaction.

a. Curves cross the threshold at its exponential phase.
b. If pathogen target amplifies, verify that the exponential phase is parallel to the exponential phase of the standard curve for that range.
c. For patient wells with no target amplification and an internal control C_T value >35:

2. If there is a questionable curve, check Component view.
3. Review each patient well for any background amplification that crosses the threshold, if Component view confirms background amplification, enter "0" in the Report column and enter "background" under Result Override column. After reviewing each well, select each column and/or the whole plate and review for any background amplification. This additional review is required to ensure all background curves are correctly identified.
4. Notate the well for background on the map, date and initial.
5. Omit any well which contain plating errors or invalid curves. Document the omission on the ABI plate map, date and initial. Notate the well for plating error on the map, date and initial.
6. After review of each of the wells on the map, review for background amplification again by selecting each column or the entire map.

Interpreting Test Results

REPORTING RESULTS AND REPEAT CRITERIA

- Clinical laboratory personnel qualified by applicable state and/or federal regulations must review all individual assay results and quality control data prior to releasing results.
- All assay controls must be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted.
- A C_T value will be assigned for each amplification reaction occurring in a reaction well. The C_T value indicates the cycle at which fluorescence in the well exceeds the set threshold. A C_T value can be the result of amplification of the desired target or can be the result of non- specific fluorescence ("NSF").
- Amplification plots and component data should be examined for all samples below the LOD of the assay. If the amplification plot shows an exponential increase of fluorescence crossing the threshold and the component plot shows an increase in fluorescence of the detector, the target has been amplified. If the amplification plot does not exhibit an exponential increase crossing the threshold or the component plot does not show an increase in fluorescence, amplification of the target has not occurred.
- If a C_T value has been assigned to a well but the amplification plot does not exhibit an exponential increase crossing the threshold or the component plot does not show increase in fluorescence, the C_T value is the result of non-specific fluorescence the C_T value as "NSF" on the worksheet). "NSF" results are considered not to be interpretable and sample analysis must be repeated.
- An internal control (IC) is included with every sample. The IC C_T value of all negative samples must have a C_T value $\leq$ 35. This criterion must be met in order for the result to be valid. A C_T value greater than 35 or non-amplification of the IC indicates possible inhibition of PCR reaction (due to presence of inhibitors co-purified with the nucleic acids).

Examine the Zika Virus Positive and Negative Controls

- If the Negative Control is:
    - Positive (C_T value < 45 and has a valid amplification curve), then this control is invalid. This indicates possible contamination of prepared samples. Positive patient results cannot be reported. Positive specimens on this run must be repeated. Negative specimens may be reported given that all other assay run criteria are met.
    - Negative (C_T values listed as "Not detected"), then this control is valid and acceptable.
- Zika Virus Positive Control
    - If the Zika Virus Positive Control C_T values are above or below the acceptable range, the Positive Control is considered invalid and unacceptable. All patient specimens must be re-assayed.
    - If the Zika Virus Positive Control C_T values are within the acceptable range, the Positive Control is considered valid and acceptable.
- Zika Amplification Curve Positive Controls
    - If the Zika Amplification Curve Positive Control C_T values are above or below the acceptable range, the control are considered invalid and unacceptable. All patient specimens must be re-assayed.
    - If the Zika Amplification Curve Positive Control C_T values are within the acceptable range, the controls are considered valid and acceptable.

Examination of Patient Specimen Results

- Zika Virus RNA positive results must be reported to the appropriate Public Health agency as well as to the CDC.
- Examination of clinical specimen results should be performed after the Negative and Positive Controls have been examined and determined to be valid and acceptable. Zika virus results must be examined for each patient specimen.
- If the Zika virus results of a patient specimen are listed as "Not detected" and the IC C_T values for the specimen are $\leq$ 35, the result is reported as "Not Detected."
- If the Zika virus results of a patient specimen are listed as "Not detected" but the IC C_T values of the specimen are > 35, possible RT-PCR inhibition has occurred for the specimen. The specimen should be rerun. If upon repeat testing the same situation occurs the patient result is reported as "Indeterminate due to inhibition" with the additional comment: "After repeat analysis, non-amplification of the internal control suggests the presence of PCR inhibitors in the patient sample. An additional sample should be submitted for testing if clinically warranted."
- If the C_T values for Zika virus results of a patient specimen are $\leq$ 41, the result is reported as "Detected."

Assay Limitations

1. Interpretation of test results must account for the possibility of false-negative and false-positive results. False-negative results can arise from:
    a. poor sample collection or
    b. degradation of the viral RNA during shipping or storage or
    c. specimen collection conducted prior to symptom onset
    d. specimen collection after nucleic acid can no longer be found in the patient specimen
    e. failure to follow the authorized assay procedures
    f. failure to use authorized extraction kit and platform
2. 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 from this and other tests must be used in conjunction with the clinical history, epidemiological data and other data available to the clinician evaluating the patient.
3. This test is for in vitro diagnostic use under FDA Emergency Use Authorization only and is limited to the qualified laboratories designated by Viracor-IBT Laboratories, Inc., and, in the United States, certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests.
4. Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
5. Extraction of nucleic acid from clinical specimens must be performed with the specified extraction methods listed in this procedure. Other extraction methods have not been evaluated for use with this assay.
6. False-negative results may occur when the infecting organism has genomic mutations, insertions, deletions, or rearrangements or when performed very early in the course of illness.
7. False-negative results may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.
8. False-positive results may occur. Repeat testing or testing with a different device may be indicated in some settings.
9. The performance of this test has not been established for patients without symptoms of Zika virus infection.
10. The performance of this test has not been established for monitoring treatment of Zika virus infection.
11. This test cannot rule out diseases caused by other bacterial or viral pathogens.
12. This assay has not been validated with specimens except plasma, serum and urine.

Performance Characteristics

Analytical Sensitivity

Analytical sensitivity – Limit of detection (LOD) studies were performed to determine the lowest detectable concentration of Zika virus at which approximately 95% of all (true positive) replicates test positive. The LOD was initially determined by limiting dilution studies using a Zika viral stock 0810092CF (Zeptometrix lot# 307586) with a concentrated titer of $1.15x10^7$ $TCID_{50}$/mL, processed in an identical manner to clinical specimens, and Probit analysis. The concentration of Zika virus sub-stock of approx. 2,000 copies/mL or $2.3x10^2$ $TCID_{50}$/mL used for LOD was determined in four independent experiments, with *in vitro* transcribed RNA (independently quantified by a Ribogreen dye binding method) used as standard curve material. The replicate experiments for Zika virus stock concentration determination were performed at a concentration of approx. 2,000 copies/mL and exhibited a %CV of 6.96% for plasma samples and 2.67% for urine samples based on log_{10} copies/mL values.

Following determination of the Zika virus stock concentration, 20 replicates of a two-fold dilutions series across eight dilutions were analyzed (using the entire test system over multiple extraction and amplification runs), beginning at approximately 390 copies/mL in Zika virus negative human plasma and urine. The results for individual dilutions for plasma and urine are shown in **Tables 1 and 2**, respectively. Probit analysis for plasma demonstrated 95% detection (i.e. dose for 95th centile) at 68 copies/mL (95% confidence interval 44.1 - 137.6 copies/mL, *P*=0.0004 for slope). Probit analysis for urine demonstrated 95% detection (i.e. dose for 95th centile) at 211 copies/mL (95% confidence interval 113 - 597 copies/mL, *P*=0.0005 for slope). The plasma LOD based on these results is 97 copies/mL and the urine LOD is 98 copies/mL.

Table 1. Replicate results for Zika virus LOD determination in plasma using a Zika viral stock 0810092CF

Expected $TCID_{50}$/mL	Expected copies/mL	No. Replicates	No. Positive	% Positive
27.56	387	20	20	100%
13.78	194	20	20	100%
6.89	**97**	**20**	**20**	**100%**
3.44	48	20	18	90%
1.72	24	20	14	70%
0.86	12.10	20	10	50%
0.43	6.05	20	4	20%
0.22	2.98	20	3	15%

Table 2. Replicate results for Zika virus LOD determination in urine using a Zika viral stock 0810092CF

Expected $TCID_{50}$/mL	Expected copies/mL	No. Replicates	No. Positive	% Positive
27.56	392	19	19	100%
13.78	196	20	20	100%
6.89	**98**	**20**	**19**	**95%**
3.44	49	20	13	65%
1.72	25	20	12	60%
0.86	12.26	20	8	40%
0.43	6.13	20	4	20%
0.22	2.52	20	7	35%

Additional experiments for LOD demonstration were performed using a characterized Zika Asian lineage strain (Zika Virus strain PRVABC59, ATCC VR-1843 Lot 64104231). Initially a range finding study was performed in which the Zika virus stock was spiked into negative human plasma using 0.3x $\log_{10}$-fold dilutions starting at 2.6 $\log_{10}$ copies/mL (398 copies/mL) down to 1.1 $\log_{10}$ copies/mL (13 copies/mL). Three replicates were prepared for each dilution and tested throughout the entire assay system. The lowest concentration at which all three replicates were positive was treated as the tentative LOD. In plasma, the lowest concentration at which the Viracor-IBT Zika Virus Real-time RT-PCR detected 3/3 replicates was 25 copies/mL, as shown in **Table 3**. After the range finding study was completed the LOD confirmation study was performed using the same Zika virus stock (strain PRVABC59). The LOD was then confirmed by spiking 20 replicates in negative human plasma using five 0.15x$\log_{10}$-fold dilutions above the tentative LOD (25 copies/mL), and one dilution below the tentative LOD. All replicates were tested throughout the entire assay system. The lowest concentration at which $\geq$ 95% of replicates were positive was considered the confirmed LOD for plasma. As shown in **Table 4**, the concentration at which $\geq$95% of replicates were positive was 50 copies/mL.

Table 3. Summary of LOD range finding results in plasma using Zika Virus strain PRVABC59

Sample Name	Nominal copies/mL	Mean C_T Zika RNA	No. Tested	No. Detected	% Detection	Mean Int. control C_T
ZIKV PL 398	398	35.76	3	3	100%	23.04
ZIKV PL 200	200	36.84	3	3	100%	22.99
ZIKV PL 100	100	37.28	3	3	100%	23.44
ZIKV PL 50	50	38.53	3	3	100%	24.49
ZIKV PL 25	25	39.01	3	3	100%	24.20
ZIKV PL 13	13	38.59	2	3	66.7%	23.45

Table 4. Summary of LOD confirmation results in plasma using Zika Virus strain PRVABC59

Sample Name	Nominal copies/mL	Mean C_T Zika RNA	No. Tested	No. Detected	% Detection	Mean Int. control C_T
ZIKV PL 141	141	36.48	20	20	100%	27.40
ZIKV PL 100	100	36.15	20	20	100%	27.09
ZIKV PL 71	**71**	**37.59**	**20**	**19**	**95%**	**28.11**
ZIKV PL 50	50	37.92	20	19	95%	27.29
ZIKV PL 35	35	38.16	20	15	75%	26.88
ZIKV PL 25	25	38.34	20	10	50%	26.84
ZIKV PL 18	18	38.35	20	11	55%	26.50

For urine, a range finding study was performed in which the Zika virus stock (strain PRVABC59) was spiked into negative human urine using 0.3x$\log_{10}$-fold dilutions starting at 2.6 $\log_{10}$ copies/mL (398 copies/mL) down to 1.1 $\log_{10}$ copies/mL (13 copies/mL). Three replicates were prepared for each dilution and tested throughout the entire assay system. The lowest concentration at which all three replicates were positive was treated as the tentative LOD. The lowest concentration at which the Viracor-IBT Zika Virus Real-time RT-PCR detected 3/3 replicates was 13 copies/mL, as shown in **Table 5**. After the range finding study was completed the LOD confirmation study was performed using the same Zika virus stock (strain PRVABC59). The LOD was confirmed by spiking 20 replicates in negative human urine using three 0.15x$\log_{10}$-fold dilutions above the tentative LOD (13 copies/mL). All replicates were tested throughout the entire assay system. The lowest concentration at which $\geq$ 95% of replicates were positive was considered the confirmed LOD. As shown in **Table 6**, the concentration at which $\geq$95% of replicates were positive was 35 copies/mL.

Table 5. Summary of LOD range finding results in urine using Zika Virus strain PRVABC59

Sample Name	Nominal copies/mL	Mean C_T Zika RNA	No. Tested	No. Detected	% Detection	Mean Int. control C_T
ZIKV UR 398	398	33.11	3	3	100%	22.38
ZIKV UR 200	200	34.12	3	3	100%	22.35
ZIKV UR 100	100	34.71	3	3	100%	22.43
ZIKV UR 50	50	36.25	3	3	100%	22.34
ZIKV UR 25	25	37.51	3	3	100%	22.34
ZIKV UR 13	13	35.73	3	3	100%	22.51

Table 6. Summary of LOD confirmation results in urine using Zika Virus strain PRVABC59

Sample Name	Nominal copies/mL	Mean C_T Zika RNA	No. Tested	No. Detected	% Detection	Mean Int. control C_T
ZIKV UR 35	**35**	**36.67**	**20**	**20**	**100%**	**25.73**
ZIKV UR 25	25	37.71	20	18	90%	26.09
ZIKV UR 18	18	37.68	20	17	85%	26.02

Analytical Specificity:

Reactivity/Inclusivity:

To initially assess inclusivity, in-silico analysis was performed for the Zika Virus Quantitative Real-time RT-PCR assay using all available Zika virus sequences in the genomic region amplified and detected by the assay. Results of this analysis are shown in **Table 7**.

Table 7. Zika virus assay, in-silico assessment

Oligo Name	Tm	GC%	Length	Complete Coverage	%Complete Coverage	Coverage w/ 1 max mismatch	%Coverage w/ 1 max mismatch
ZIKV 1186 F	58.3	64.7	17	42/60	70%	43/60	72%
ZIKV 1183 F	58.8	55	20	9/60	15%	10/60	15%
ZIKV 1184 F	58.5	57.9	19	6/60	10%	7/60	12%
Total Coverage				**57/60**	**95%**	**59/60**	**98%**
ZIKV 1207 P	68.2	45.2	31	50/60	83%	51/60	85%
ZIKV 1207 P2	68.5	48.4	31	5/60	8%	6/60	10%
Total Coverage				**55/60**	**92%**	**57/60**	**95%**
ZIKV 1262 R	58.4	41.7	24	29/48	60%	30/48	63%
ZIKV 1262 R2	61	56.4	24	0/48	0%	8/48	17%
ZIKV 1264 R	58.7	38.5	26	9/48	19%	9/48	19%
Total Coverage				**38/48**	**79%**	**47/48**	**98%**

Reactivity/inclusivity was assessed by testing Zika virus positive samples of commercially available Zika virus strains rocessed through the entire test system. Additionally, analysis with synthetic DNA ultramers by RT-PCR was performed in order to evaluate the inclusivity of the assay with various strains containing mismatches within the primer and probe binding sites. All spiked Zika virus samples and ultramer samples were positive for Zika virus, demonstrating acceptable inclusivity of the assay. Results of inclusivity testing of viral samples and ultramers are shown in **Table 8**.

Table 8. Analytical specificity – inclusivity

ID	Source	Organism / Strain	Zika Virus Real-time RT-PCR C_T
0810092CF	Zeptometrix	ZIKV (strain ID unavailable; not Asian)	29.08
VR-1838	ATCC	ZIKV MR 766 (Uganda 1947)	16.09
VR-1839	ATCC	ZIKV IB H 30656 (Nigeria 1968)	16.35
VR-1843	ATCC	ZIKV PRVABC59 (Puerto Rico 2015)	17.44
143450369	IDT	ZIKV HQ234500 Ultramer (Nigeria 1968)	28.36
143450370	IDT	ZIKV HQ234501 Ultramer (Senegal 1984)	30.36
143450371	IDT	ZIKV KF268948 Ultramer (CAR 1976)	35.13
143450367	IDT	ZIKV KU497555 Ultramer (Brazil 2015)	28.38
143450368	IDT	ZIKV NC_012532 Ultramer (Uganda 1947)	29.09

Cross Reactivity:

Potential cross-reactivity was evaluated with various pathogens that could cause similar symptoms, pathogens closely related to Zika virus due to sequence identity, and pathogens transmitted by the same mosquito vector. No signal was detected for the non-target organisms tested with the Zika Virus Real-time RT-PCR assay as shown in **Table 9**. Additional in silico analysis of key pathogens demonstrated a lack of cross-reactivity as shown in **Table 10**.

Table 9. Analytical specificity – cross-reactivity

ID	Source	Organism	Concentration	Zika Virus Real-time RT-PCR C_T
NATWNV-0005	Zeptometrix	West Nile Virus	5,000 copies/mL	ND[1]
0810088CF	Zeptometrix	Dengue Virus serotype 1	5.01X103 $TCID_{50}$/mL	ND
0810089CF	Zeptometrix	Dengue Virus serotype 2	5.01X103 $TCID_{50}$/mL	ND
0810090CF	Zeptometrix	Dengue Virus serotype 3	4.17X103 $TCID_{50}$/mL	ND
0810091CF	Zeptometrix	Dengue Virus serotype 4	9.55X104 $TCID_{50}$/mL	ND
NATCHIKV-ST	Zeptometrix	Chikungunya virus	1.25X106 $TCID_{50}$/mL	ND
0810095CF	Zeptometrix	Yellow Fever Virus strain 17D	2.19X104 $TCID_{50}$/mL	ND
VR-73	ATCC	Ilheus virus	1.26X106 LD_{50}/mL	ND
VR-1277	ATCC	Mayaro virus	1.58X106 LD_{50}/mL	ND
0810093CF	Zeptometrix	Banzi Virus	2.45X103 $TCID_{50}$/mL	ND
VR-1265CAF	ATCC	St. Louis Encephalitis Virus ascetic fluid V-524-401-562	N.A.[3]	ND
VR-837	ATCC	BK virus	1.4x104 $TCID_{50}$/mL	ND
53592	ATCC	Chlamydophila pneumoniae	7.9x104 $TCID_{50}$/mL	ND
VR-807	ATCC	Cytomegalovirus	1.4x103 $TCID_{50}$/mL	ND
0810047CF	Zeptometrix	Enterovirus	9x104 $TCID_{50}$/mL	ND
081008CF	Zeptometrix	Epstein-Barr virus	3x107 copies/ mL	ND
VR-15	ATCC	Adenovirus	3.16x106 $TCID_{50}$/mL	ND
950150	Acrometrix	Hepatitis B virus	>1.0X105 $TCID_{50}$/mL	ND
950350	Acrometrix	Hepatitis C virus	>1.0X105 $TCID_{50}$/mL	ND
25 000411	VIBT	Hepatitis D virus	1x104 copies/ mL	ND
6329/10	WHO	Hepatitis E virus	>1.0X104 $TCID_{50}$/mL	ND
081006CF	Zeptometrix	Herpes simplex virus-1	6.6X104 $TCID_{50}$/mL	ND
081005CF	Zeptometrix	Herpes simplex virus-2	1.0X107 $TCID_{50}$/mL	ND
960406	Acrometrix	HIV	8.10x108 copies/mL	ND
VR-1480	Zeptometrix	Human herpesvirus-6	N.A.	ND
0810071CF	Zeptometrix	Human herpesvirus-7	1.1X105 $TCID_{50}$/mL	ND
0810104CF	Zeptometrix	Human herpesvirus-8	1.0X104 $TCID_{50}$/mL	ND
VR-1583	ATCC	JC virus	7.9x104 $TCID_{50}$/mL	ND
1498735	Clinical specimen	Parvovirus B19	1.0x108 copies/mL	ND
0801512	Zeptometrix	Group A strep	>1.0X104 CFU/mL	ND
30932	ATCC	Plasmodium falciparum	N.A.	ND
35210	ATCC	Borrelia burdorferi	N.A.	ND
VR-1796	ATCC	Influenza H1N1 2009 Pandemic	>1.0X103 $TCID_{50}$/mL	ND
VR-95	ATCC	Influenza A H1N1 Seasonal	7.9X106 $TCID_{50}$/mL	ND
VR-822	ATCC	Influenza H3N2 Seasonal	7.9X105 $TCID_{50}$/mL	ND
0810037CF	Zeptometrix	Influenza B	>1.0X103 $TCID_{50}$/mL	ND
VR-26	ATCC	RSV A	>1.0X103 $TCID_{50}$/mL	ND
VR-24	Zeptometrix	Measles virus	5.8X105 $TCID_{50}$/mL	ND
0810048CF	Zeptometrix	Rubella virus	>1.0X103 $TCID_{50}$/mL	ND

VR-1367	ATCC	Varicella-Zoster virus	4.5x104 $TCID_{50}$/mL	ND

[1] ND, Not Detected

[3] N.A., Not Available

Table 10. Organisms analyzed in silico for cross-reactivity with Zika Virus Quantitative Real-time RT-PCR primers and probes

	query name	ZIKV 1183 F	ZIKV 1184 F	ZIKV 1186 F	ZIKV 1262 R	ZIKV 1262 R2	ZIKV 1264 R	ZIKV 1207 P	ZIKV 1207 P2
	query function	Forward primer-1	Forward primer-2	Forward primer-3	Reverse primer-1	Reverse primer-2	Reverse primer-3	Probe-1	Probe-2
	query length	20	19	17	24	24	26	31	31
	strand match	plus/plus	plus/plus	plus/plus	plus/minus	plus/minus	plus/minus	plus/plus	plus/plus
Organism	**tax ID**	**Percent homology for alignment with lowest E value for each analysis (vector sequences excluded)**							
Dengue virus 1	11053	65%	89%	94%	50%	50%	46%	N.A.[a]	N.A.
Dengue virus 2	11060	90%	89%	100%	46%	46%	42%	N.A.	N.A.
Dengue virus 3	11069	65%	53%	59%	54%	50%	46%	N.A.	N.A.
Dengue virus 4	11070	55%	58%	65%	46%	46%	42%	N.A.	N.A.
Yellow Fever	40005	50%	58%	65%	54%	54%	46%	55%	N.A.
West Nile Virus	11082	65%	53%	76%	50%	58%	42%	N.A.	N.A.
Chikungunya Virus	37124	50%	65%	59%	38%	42%	42%	N.A.	N.A.
Mayaro Virus	59301	50%	53%	59%	46%	46%	42%	N.A.	35%
St Louis encephalitis virus	11080	45%	53%	59%	54%	54%	50%	45%	45%
Japanese encephalitis virus	11071	55%	58%	65%	54%	58%	50%	N.A.	N.A.
Spondweni Virus	64318	40%	58%	65%	33%	33%	31%	42%	42%
Eastern equine encep. virus	11021	50%	53%	59%	54%	54%	50%	N.A.	N.A.
Western equine encep. virus	11039	45%	42%	47%	42%	42%	38%	35%	35%
Ross River Virus	11029	45%	47%	53%	38%	38%	35%	45%	61%
Barmah Forest Virus	11020	40%	42%	47%	33%	33%	31%	39%	42%
O'nyong-nyong Virus	11027	40%	42%	47%	38%	38%	35%	32%	58%

[a]No significant similarity found

Interference Studies

Interference studies were not performed since conventional, well-established methods were used for both nucleic acid extraction and RT-PCR amplification.

Clinical Evaluation

Contrived Clinical Specimens Testing:

Accuracy of the Zika Virus Real-time RT-PCR assay was assessed for both plasma and urine using contrived and negative samples. Testing was completed using spiked (contrived) specimens from unique individual donor samples for both plasma and urine specimens. A total of 145 samples, 75 plasma and 60 urine, were tested through the entire assay system (**Tables 11** and **12**). For plasma specimens, 10 un-spiked negative plasma samples were tested along with 10 samples spiked at 0.3xLOD, 20 samples at 0.5xLOD, 10 samples at 1.0xLOD, 10 samples at 1.6xLOD, 15 samples at 2.0xLOD, and 10 at 5.0xLOD. For urine samples, 5 samples were spiked at 1.5xLOD, 3 samples at 5.0xLOD, and 2 samples at 10.0xLOD. All spiked samples were contrived using the Zika Virus Culture Fluid ATCC VR-1843 Lot 64104231 (strain PRVABC59). All samples were blinded and randomized for testing. As shown in **Table 11,** Zika virus was detected in 90.1% of all reactions, and 100% of reactions spiked at ≥1.6xLOD. For spiked urine samples, Zika virus was detected in 100% of all reactions.

Table 11. Summary of accuracy results using contrived plasma and urine samples using Zika virus strain PRVABC59

Fold of LOD	Specimen	No. Tested	No. Detected	% Detected
0.3	Plasma	10	7	70%
0.5	Plasma	20	17	85%
1.0	Plasma	10	9	90%
1.6	Plasma	10	10	100%
2.0	Plasma	15	15	100%
5.0	Plasma	10	10	100%
	TOTAL	**75**	**68**	**90.1%**
1.5	Urine	5	5	100%
5.0	Urine	3	3	100%
10.0	Urine	2	2	100%
	TOTAL	**10**	**10**	**100%**
0 (unspiked)	Plasma	10	0	0%

Additionally, 25 urine samples were spiked at 1.5xLOD, 10 samples at 5.0xLOD, and 5 samples at 10.0xLOD, with 10 negative (unspiked) urine controls; all urine samples were collected from unique individual donors. For this second set of spiked urine samples, testing was also performed with a comparator RT-PCR assay (Altona RealStar Zika Virus RT-PCR Kit). The results are summarized in **Table 12**. For the urine samples tested by both the Viracor-IBT Zika Virus Real-time RT-PCR assay and the comparator RT-PCR (Altona) assay, 100% Zika virus detection (100% agreement) was observed for all contrived samples at 1.5x, 5.0x and 10.0xLOD. All 10 un-spiked known negative urine samples returned a result of "Not Detected" for both assays.

Table 12. Summary of accuracy results using contrived urine samples using Zika virus strain PRVABC59 and testing by Viracor-IBT Zika Virus Real-time RT-PCR assay and comparator Altona RealStar Zika Virus RT-PCR Kit assay

Fold of LOD	Specimen	No. Tested	No. Detected		% Agreement
			Viracor-IBT Zika assay	Comparator assay	
1.5	Urine	25	25	25	100%
5.0	Urine	10	10	10	100%
10.0	Urine	5	5	5	100%
TOTAL		**40**	**40**	**40**	**100%**
0 (unspiked)	Urine	10	0	0	100%

Natural Clinical Specimens Testing:

The performance characteristics of the Zika Virus Real-time RT-PCR were further evaluated using retrospectively collected natural clinical samples. Multiple serum and plasma samples were collected in a surveillance study from Colombia, South America. Eleven (11) plasma samples and 32 serum samples were from Zika presumptive-positive patients (as determined by clinical presentation), 30 plasma samples were from asymptomatic individuals considered to be at risk since they were geographically located in mosquito infested areas where Zika is currently endemic (Colombia, South America). A total of 80 expected Zika RNA negative specimens were from asymptomatic normal donors in the United States. These expected Zika RNA negative samples were composed of 30 serum, 20 plasma, and 30 urine specimens collected from individual healthy donors from multiple ages, gender, and race from different geographic areas within the United States. A summary of the number of samples tested in each group and the positivity rate as determined by the Viracor-IBT Zika Virus Real-time RT-PCR assay is shown in **Table 13**.

Table 13. Samples distribution and positivity rate as determined by the Viracor-IBT Zika Virus Real-time RT-PCR assay for retrospectively collected individual patient/subject samples from Colombia and the United States[a]

Sample Distribution	Plasma - No. positive/No. tested (% positive)	Serum - No. positive/No. tested (% positive)	Urine - No. positive/No. tested (% positive)
Symptomatic - Colombian origin (clinical signs compatible with Zika infection)	3/11 (27.3%)	3/32 (9.4%)	N/A
Asymptomatic - Colombian origin (high risk surveillance area)	17/30 (56.6%)	N/A	N/A
Symptomatic - United States origin	N/A	2/2 (100%)	1/1 (100%)
Asymptomatic - United States origin	0/20 (0%)	0/21[a] (0%)	0/30 (0%)

[a] Results shown do not include 9 samples with an Invalid results (i.e., internal control C_T values >35 and no Zika virus detected).

Basic information such as specimen collection date, date of onset of symptoms (if any), clinical diagnosis, demographics, and gestational time (if applicable) were collected at the time of donation. All natural clinical samples, presumptive positives, asymptomatic samples, and negative samples were blinded and randomized at the time of testing at VIBT and samples tested for confirmatory and comparator testing purposes was handled in the same manner. All clinical samples were processed as previously described utilizing the entire workflow. Results of testing by the Viracor-IBT Zika Virus Real-time RT-PCR assay, as well as a comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA), are shown in **Tables 14, 15, 16** and **17**.

Table 14. Summary of Zika virus natural clinical samples collected from symptomatic individuals in Colombia, South America with compatible clinical signs of Zika virus infection

			Viracor-IBT Zika Virus Real-time RT-PCR Result			Comparator Assay[a] Result		
Blinded ID	**Sample**	**Specimen**	**ZIKV C_T**	**Result**	**Internal control C_T**	**ZIKV C_T**	**Result**	**Internal control C_T**
3	ZIKV Acc 3	Plasma	ND	Not detected	26.99	ND	Negative	29.97
8	ZIKV Acc 8	Plasma	29.17	Detected	27.5	27.10	Positive	29.67
67	ZIKV Acc 67	Plasma	ND	Not detected	25.69	ND	Negative	29.13
79	ZIKV Acc 79	Plasma	ND	Not detected	26.08	ND	Negative	29.44
109	ZIKV Acc 109	Plasma	ND	Not detected	27.86	ND	Negative	30.04
61	ZIKV Acc 61	Plasma	ND	Not detected	30.04	ND	Negative	28.91
90	ZIKV Acc 90	Plasma	39.08	Detected	30.27	36.83	Positive	29.44
66	ZIKV Acc 66	Plasma	39.97	Detected	25.96	ND	Negative	31.02
126	ZIKV Acc 126	Plasma	ND	Not detected	28.54	ND	Negative	29.60
27	ZIKV Acc 27	Plasma	ND	Not detected	25.85	ND	Negative	30.00
5	ZIKV Acc 5	Plasma	ND	Not detected	29.47	ND	Negative	29.80
88	ZIKV Acc 88	Serum	ND	Not detected	26.02	ND	Negative	29.09
89	ZIKV Acc 89	Serum	ND	Not detected	26.74	ND	Negative	29.74
114	ZIKV Acc 114	Serum	ND	Not detected	27.08	ND	Negative	30.06
10	ZIKV Acc 10	Serum	ND	Not detected	26.44	ND	Negative	29.38
37	ZIKV Acc 37	Serum	ND	Not detected	25.45	ND	Negative	29.65
30	ZIKV Acc 30	Serum	ND	Not detected	26.94	ND	Negative	30.02
65	ZIKV Acc 65	Serum	ND	Not detected	26.05	ND	Negative	29.88
34	ZIKV Acc 34	Serum	ND	Not detected	26.52	ND	Negative	30.68
93	ZIKV Acc 93	Serum	ND	Not detected	26.11	ND	Negative	29.48
23	ZIKV Acc 23	Serum	ND	Not detected	26.53	ND	Negative	29.69
150	ZIKV Acc 150	Serum	ND	Not detected	28.35	ND	Negative	29.51
19	ZIKV Acc 19	Serum	ND	Not detected	26.99	ND	Negative	29.94
58	ZIKV Acc 58	Serum	ND	Not detected	26.83	ND	Negative	29.24
40	ZIKV Acc 40	Serum	ND	Not detected	26.07	ND	Negative	30.06
83	ZIKV Acc 83	Serum	ND	Not detected	26.29	ND	Negative	29.78
142	ZIKV Acc 142	Serum	ND	Not detected	26.26	ND	Negative	29.52
35	ZIKV Acc 35	Serum	ND	Not detected	26.1	ND	Negative	30.00
7	ZIKV Acc 7	Serum	37.38	Detected	25.76	35.44	Positive	28.84
51	ZIKV Acc 51	Serum	ND	Not detected	27.27	ND	Negative	29.42
100	ZIKV Acc 100	Serum	ND	Not detected	26.42	ND	Negative	29.44
80	ZIKV Acc 80	Serum	ND	Not detected	25.68	ND	Negative	29.71
111	ZIKV Acc 111	Serum	ND	Not detected	27.13	ND	Negative	30.52

71	ZIKV Acc 71	Serum	ND	Not detected	26.24	ND	Negative	30.10
146	ZIKV Acc 146	Serum	ND	Not detected	26.94	ND	Negative	29.45
52	ZIKV Acc 52	Serum	ND	Not detected	28.84	ND	Negative	29.92
22	ZIKV Acc 22	Serum	27.90	Detected	25.35	27.61	Positive	29.41
38	ZIKV Acc 38	Serum	31.22	Detected	27.01	ND	Negative	29.71
145	ZIKV Acc 145	Serum	ND	Not detected	27.54	ND	Negative	29.85
36	ZIKV Acc 36	Serum	ND	Not detected	26.41	ND	Negative	29.86
14	ZIKV Acc 14	Serum	ND	Not detected	27.12	ND	Negative	30.04
129	ZIKV Acc 129	Serum	ND	Not detected	27.92	NA	NA	NA
18	ZIKV Acc 18	Serum	ND	Not detected	26.82	ND	Negative	29.59

[a]Altona RealStar Zika Virus RT-PCR under an EUA

Table 15. Summary of Zika virus natural clinical samples collected from asymptomatic individuals in Colombia, South American residing in high-risk Zika virus surveillance areas

			Viracor-IBT Zika Virus Real-time RT-PCR Result			**Comparator Assay[a] Result**		
Blinded ID	**Sample**	**Specimen**	**ZIKV C_T**	**Result**	**Internal control C_T**	**ZIKV C_T**	**Result**	**Internal control C_T**
17	ZIKV Acc 17	Plasma	ND	Not detected	26.62	36.94	Positive	29.43
20	ZIKV Acc 20	Plasma	38.51	Detected	26.39	ND	Negative	29.61
28	ZIKV Acc 28	Plasma	39.17	Detected	32.77	36.58	Positive	30.14
32	ZIKV Acc 32	Plasma	ND	Not detected	26.2	ND	Negative	30.15
43	ZIKV Acc 43	Plasma	ND	Not detected	27.02	ND	Negative	30.97
44	ZIKV Acc 44	Plasma	34.19	Detected	28.48	33.05	Positive	30.00
45	ZIKV Acc 45	Plasma	40.16	Detected	41.6	34.90	Positive	29.70
48	ZIKV Acc 48	Plasma	36.22	Detected	26.84	34.34	Positive	30.02
50	ZIKV Acc 50	Plasma	25.93	Detected	27.12	25.54	Positive	28.96
54	ZIKV Acc 54	Plasma	30.22	Detected	30.56	28.49	Positive	29.87
55	ZIKV Acc 55	Plasma	36.04	Detected	28.11	34.35	Positive	29.80
59	ZIKV Acc 59	Plasma	29.50	Detected	27.06	28.25	Positive	29.58
70	ZIKV Acc 70	Plasma	ND	Not detected	31.16	ND	Negative	30.05
73	ZIKV Acc 73	Plasma	39.01	Detected	29.54	37.05	Positive	31.17
76	ZIKV Acc 76	Plasma	ND	Not detected	25.58	ND	Negative	30.16
82	ZIKV Acc 82	Plasma	31.22	Detected	32.73	28.79	Positive	30.11
86	ZIKV Acc 86	Plasma	ND	Not detected	25.72	ND	Negative	29.96
87	ZIKV Acc 87	Plasma	ND	Not detected	25.56	ND	Negative	29.21
91	ZIKV Acc 91	Plasma	ND	Not detected	33.61	ND	Negative	29.42
92	ZIKV Acc 92	Plasma	35.15	Detected	26.88	33.35	Positive	29.87
97	ZIKV Acc 97	Plasma	32.79	Detected	28.12	31.83	Positive	30.30
99	ZIKV Acc 99	Plasma	34.05	Detected	25.91	33.60	Positive	29.74
101	ZIKV Acc 101	Plasma	ND	Not detected	30.64	ND	Negative	29.39
112	ZIKV Acc 112	Plasma	40.11	Detected	31.99	ND	Negative	29.69
124	ZIKV Acc 124	Plasma	ND	Not detected	27.02	ND	Negative	29.39
134	ZIKV Acc 134	Plasma	ND	Not detected	26.44	ND	Negative	29.58
136	ZIKV Acc 136	Plasma	32.98	Detected	28.13	32.38	Positive	31.05
144	ZIKV Acc 144	Plasma	ND	Not detected	31.71	ND	Negative	29.49
147	ZIKV Acc 147	Plasma	39.06	Detected	29.32	36.89	Positive	30.22
148	ZIKV Acc 148	Plasma	ND	Not detected	26.97	ND	Negative	29.36

[a]Altona RealStar Zika Virus RT-PCR under an EUA

Table 16. Summary of Zika virus natural clinical samples collected from symptomatic individuals residing in the United States of America

			Viracor-IBT Zika Virus Real-time RT-PCR Result			Comparator Assay[a] Result		
Blinded ID	**Sample**	**Specimen**	**ZIKV C_T**	**Result**	**Internal control C_T**	**ZIKV C_T**	**Result**	**Internal control C_T**
154	ZIKV 50 VIBT	Serum	38.2	Detected	25.28	35.03	Positive	29.55
155	ZIKV 51 VIBT	Serum	36.7	Detected	26.56	36.99	Positive	29.74
156	ZIKV 51 VIBT	Urine	31.2	Detected	28.74	31.24	Positive	30.83

[a]Altona RealStar Zika Virus RT-PCR under an EUA

Table 17. Summary of Zika virus natural clinical samples collected from asymptomatic individuals residing in the United States of America

			Viracor-IBT Zika Virus Real-time RT-PCR Result			Comparator Assay[a] Result		
Blinded ID	**Sample**	**Specimen**	**ZIKV C_T**	**Result**	**Internal control C_T**	**ZIKV C_T**	**Result**	**Internal control C_T**
2	ZIKV Acc 2	Plasma	ND	Not detected	25.6	ND	Negative	29.70
15	ZIKV Acc 15	Plasma	ND	Not detected	25.56	ND	Negative	29.13
47	ZIKV Acc 47	Plasma	ND	Not detected	26.14	ND	Negative	29.49
49	ZIKV Acc 49	Plasma	ND	Not detected	26.16	ND	Negative	29.50
68	ZIKV Acc 68	Plasma	ND	Not detected	25.19	ND	Negative	29.51
74	ZIKV Acc 74	Plasma	ND	Not detected	25.2	ND	Negative	30.07
81	ZIKV Acc 81	Plasma	ND	Not detected	25.38	ND	Negative	29.69
94	ZIKV Acc 94	Plasma	ND	Not detected	25.19	ND	Negative	29.76
96	ZIKV Acc 96	Plasma	ND	Not detected	25.3	ND	Negative	30.22
103	ZIKV Acc 103	Plasma	ND	Not detected	25.38	ND	Negative	29.83
106	ZIKV Acc 106	Plasma	ND	Not detected	25.54	ND	Negative	29.21
115	ZIKV Acc 115	Plasma	ND	Not detected	25.56	ND	Negative	29.05
121	ZIKV Acc 121	Plasma	ND	Not detected	25.44	ND	Negative	29.43
125	ZIKV Acc 125	Plasma	ND	Not detected	25.8	ND	Negative	29.05
127	ZIKV Acc 127	Plasma	ND	Not detected	25.74	ND	Negative	29.06
130	ZIKV Acc 130	Plasma	ND	Not detected	25.92	ND	Negative	28.56
140	ZIKV Acc 140	Plasma	ND	Not detected	25.56	ND	Negative	28.84
143	ZIKV Acc 143	Plasma	ND	Not detected	25.66	ND	Negative	28.81
151	ZIKV Acc 151	Plasma	ND	Not detected	25.69	ND	Negative	28.82
152	ZIKV Acc 152	Plasma	ND	Not detected	25.89	ND	Negative	28.85
9	ZIKV Acc 9	Serum	ND	Not detected	34.95	ND	Negative	29.70
11	ZIKV Acc 11	Serum	ND	Not detected	26.16	ND	Negative	31.27
12	ZIKV Acc 12	Serum	ND	Invalid	35.9	NA	NA	NA
13	ZIKV Acc 13	Serum	ND	Not detected	28.24	ND	Negative	31.08
21	ZIKV Acc 21	Serum	ND	Not detected	27.12	ND	Negative	30.17
33	ZIKV Acc 33	Serum	ND	Not detected	31.62	ND	Negative	29.77
53	ZIKV Acc 53	Serum	ND	Invalid	43.14	NA	NA	NA
56	ZIKV Acc 56	Serum	ND	Invalid	36.81	NA	NA	NA
60	ZIKV Acc 60	Serum	ND	Invalid	35.93	NA	NA	NA
62	ZIKV Acc 62	Serum	ND	Not detected	26.23	ND	Negative	30.88
64	ZIKV Acc 64	Serum	ND	Invalid	37.16	NA	NA	NA

69	ZIKV Acc 69	Serum	ND	Not detected	25.52	ND	Negative	29.69
72	ZIKV Acc 72	Serum	ND	Not detected	32.9	ND	Negative	29.56
75	ZIKV Acc 75	Serum	ND	Not detected	31.04	ND	Negative	29.65
77	ZIKV Acc 77	Serum	ND	Not detected	25.32	ND	Negative	30.03
85	ZIKV Acc 85	Serum	ND	Not detected	25.12	ND	Negative	31.37
95	ZIKV Acc 95	Serum	ND	Not detected	32.4	ND	Negative	29.87
98	ZIKV Acc 98	Serum	ND	Not detected	29.88	ND	Negative	31.03
102	ZIKV Acc 102	Serum	ND	Invalid	35.57	NA	NA	NA
116	ZIKV Acc 116	Serum	ND	Not detected	25.71	ND	Negative	30.59
117	ZIKV Acc 117	Serum	ND	Invalid	35.43	NA	NA	NA
119	ZIKV Acc 119	Serum	ND	Not detected	25.97	ND	Negative	29.75
128	ZIKV Acc 128	Serum	ND	Invalid	41.55	NA	NA	NA
132	ZIKV Acc 132	Serum	ND	Not detected	27.6	ND	Negative	30.06
135	ZIKV Acc 135	Serum	ND	Not detected	25.82	ND	Negative	30.42
137	ZIKV Acc 137	Serum	ND	Not detected	25.76	ND	Negative	30.80
138	ZIKV Acc 138	Serum	ND	Not detected	31.84	ND	Negative	29.38
139	ZIKV Acc 139	Serum	ND	Not detected	29.01	ND	Negative	30.64
141	ZIKV Acc 141	Serum	ND	Invalid	35.95	NA	NA	NA
149	ZIKV Acc 149	Serum	ND	Not detected	26.98	ND	Negative	31.91
1	ZIKV Acc 1	Urine	ND	Not detected	25.26	ND	Negative	30.00
4	ZIKV Acc 4	Urine	ND	Not detected	29.11	ND	Negative	30.25
6	ZIKV Acc 6	Urine	ND	Not detected	29.61	ND	Negative	30.53
16	ZIKV Acc 16	Urine	ND	Not detected	29.03	ND	Negative	30.46
24	ZIKV Acc 24	Urine	ND	Not detected	27.15	ND	Negative	30.48
25	ZIKV Acc 25	Urine	ND	Not detected	24.06	ND	Negative	30.41
26	ZIKV Acc 26	Urine	ND	Not detected	24.55	ND	Negative	30.56
29	ZIKV Acc 29	Urine	ND	Not detected	25.91	ND	Negative	30.57
31	ZIKV Acc 31	Urine	ND	Not detected	28.82	ND	Negative	30.82
39	ZIKV Acc 39	Urine	ND	Not detected	26.03	ND	Negative	30.23
41	ZIKV Acc 41	Urine	ND	Not detected	27.04	ND	Negative	31.16
42	ZIKV Acc 42	Urine	ND	Not detected	25.14	ND	Negative	29.91
46	ZIKV Acc 46	Urine	ND	Not detected	28.57	ND	Negative	29.54
57	ZIKV Acc 57	Urine	ND	Not detected	26.35	ND	Negative	29.24
63	ZIKV Acc 63	Urine	ND	Not detected	25.34	ND	Negative	30.13
78	ZIKV Acc 78	Urine	ND	Not detected	24.26	ND	Negative	29.51
84	ZIKV Acc 84	Urine	ND	Not detected	29.05	ND	Negative	29.88
104	ZIKV Acc 104	Urine	ND	Not detected	26.82	ND	Negative	30.48
105	ZIKV Acc 105	Urine	ND	Not detected	24.09	ND	Negative	29.19
107	ZIKV Acc 107	Urine	ND	Not detected	27.27	ND	Negative	30.09
108	ZIKV Acc 108	Urine	ND	Not detected	26.02	ND	Negative	30.90
110	ZIKV Acc 110	Urine	ND	Not detected	23.95	ND	Negative	29.55
113	ZIKV Acc 113	Urine	ND	Not detected	28.41	ND	Negative	30.26
118	ZIKV Acc 118	Urine	ND	Not detected	23.89	ND	Negative	30.64
120	ZIKV Acc 120	Urine	ND	Not detected	26.4	ND	Negative	30.01
122	ZIKV Acc 122	Urine	ND	Not detected	29.05	ND	Negative	29.62
123	ZIKV Acc 123	Urine	ND	Not detected	27.45	ND	Negative	30.00
131	ZIKV Acc 131	Urine	ND	Not detected	27.17	ND	Negative	29.93
133	ZIKV Acc 133	Urine	ND	Not detected	27.4	ND	Negative	29.96
153	ZIKV Acc 153	Urine	ND	Not detected	27.46	ND	Negative	29.56

[a]Altona RealStar Zika Virus RT-PCR under an EUA

Confirmation of a subset of "detected" results shown in **Tables 14-17** was performed by bidirectional dideoxy (Sanger) nucleotide sequencing of approx. 400 bases from the Zika virus envelop protein gene. Characteristics and the region sequenced for these samples are shown in **Table 18**. A total of 15 positives were tested and all were found to match 100% to the current Asian Zika virus strain circulating in the Americas.

Table 18. Confirmation of positive samples by Sanger Sequencing

Sample Name	Date of collection	Date of symptoms or Asymptomatic (origin)	Days post onset	Specimen Type	ZIKV C_T	Int. Control C_T	Nucleotides Sequenced[1]
ZIKV Acc 8	12/18/2015	12/14/2015	6	Plasma	29.17	27.50	659-1259
ZIKV Acc 22	1/28/2016	1/18/2016	10	Serum	27.90	25.35	659-1259
ZIKV Acc 38	1/28/2016	1/18/2016	10	Serum	31.22	27.01	659-1259
ZIKV Acc 50	12/15/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	25.93	27.12	659-1259
ZIKV Acc 54	12/9/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	30.22	30.56	659-1259
ZIKV Acc 59	12/10/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	29.50	27.06	659-1259
ZIKV Acc 82	12/5/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	31.22	32.73	659-1259
ZIKV Acc 44	12/10/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	34.19	28.48	659-1259
ZIKV Acc 48	12/5/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	36.22	26.84	659-1259
ZIKV Acc 55	12/11/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	36.04	28.11	659-1259
ZIKV Acc 92	12/7/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	31.22	32.73	659-1259
ZIKV Acc 97	12/9/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	35.15	26.88	659-1259
ZIKV Acc 99	12/18/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	32.79	28.12	659-1259
ZIKV Acc 112	12/19/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	34.05	25.91	659-989
ZIKV Acc 136	12/19/2015	Asymptomatic at risk - Surveillance (Colombia)	Unk	Plasma	40.11	31.99	659-1259

[1]Nucleotide positions refer to KU497555 (Brazil, 2015)

A total of 146 samples (61 plasma, 54 serum and 31 urine) from individual subjects were tested by both the Viracor-IBT Zika Virus Real-time RT-PCR assay and the comparator assay (Altona RealStar Zika Virus RT-PCR Kit). (Note that samples which originally returned an "invalid" result by the Viracor-IBT Zika Virus Real-time RT-PCR assay, as shown in Table 20 were not tested by the comparator assay. Additionally, one sample (Acc 129, **Table 14**) was excluded from analysis as there was insufficient sample volume to run on the comparator assay.) All testing was performed in a blinded and randomized manner. A summary of all results is shown in **Table 19**. For the 146 samples tested by both assays, the overall level of agreement was 96.6 % (141 out of 146 samples tested) with four discordant results being Viracor-IBT positive and comparator assay negative; sequencing was successfully performed for two of the four discordant samples (ZIKV acc 38 in **Table 14** and ZIKV acc 112 in **Table 15** above) and Zika virus RNA was determined to be present in these two samples by sequencing. The positive percent agreement (PPA) and negative percent agreement (NPA) of the Viracor-IBT RT-PCR assay compared to the comparator assay was 95.7% and 96.7%, respectively.

Table 19. Summary of results for analysis of individual plasma, serum and urine samples by the Viracor-IBT Zika Virus Real-time RT-PCR and a comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA)

		Comparator result	
		Positive	Negative
Viracor-IBT RT-PCR result	Positive	22	4
	Negative	1	119

Results of testing individual samples by both the Viracor-IBT Zika Virus Real-time RT-PCR and comparator assay (Altona RealStar Zika Virus RT-PCR Kit) for each specimen type represented in **Table 19** are shown in **Tables 20, 21**, and **22** for plasma, serum and urine, respectively. For plasma, the PPA and NPA of the Viracor-IBT Zika Virus Real-time RT-PCR assay compared to the comparator assay was 94.4% and 93.0%, respectively. For serum, the PPA and NPA of the Viracor-IBT Zika Virus Real-time RT-PCR assay compared to the comparator assay was 100% and 98.0%, respectively. For urine, the PPA and NPA of the Viracor-IBT Zika Virus Real-time RT-PCR assay compared to the comparator assay were all 100%.

Table 20. Summary of results for analysis of individual plasma samples by the Viracor-IBT Zika Virus Real-time RT-PCR and a comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA)

		Comparator result	
		Positive	Negative
Viracor-IBT RT-PCR result	Positive	17	3
	Negative	1	40

Table 21. Summary of results for analysis of individual serum samples by the Viracor-IBT Zika Virus Real-time RT-PCR and a comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA)

		Comparator result	
		Positive	Negative
Viracor-IBT RT-PCR result	Positive	4	1
	Negative	0	49

Table 22. Summary of results for analysis of individual urine samples by the Viracor-IBT Zika Virus Real-time RT-PCR and a comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA)

		Comparator result	
		Positive	Negative
Viracor-IBT RT-PCR result	Positive	1	0
	Negative	0	30

Natural Patient Matched/Paired Clinical Specimens Testing:

Paired plasma, serum and urine samples collected concurrently from the same patient were tested by both the Viracor-IBT Zika Virus Real-time RT-PCR assay and a comparator assay (Altona RealStar Zika Virus RT-PCR). Plasma, serum and urine were tested from a total of 12 patients. Paired specimens were collected from Colombia and the Dominican Republic from Zika confirmed-positive patients as determined by clinical presentations and diagnostic testing completed by the repository. A summary of results from individual subjects for both the Viracor-IBT Zika Virus Real-time RT-PCR assay and the comparator assay is shown in **Table 23**. For the Viracor-IBT Zika Virus Real-time RT-PCR assay, all 12 patients had at least one specimen type in which Zika RNA was detected; all three specimen types were "detected" in 6 of 12 patients, two specimen types were detected in 3 of 12 patients, and at least one specimen type was detected in 3 of 12 patients. Urine was the most commonly detected specimen type (11 patients) followed by plasma (9 patients) and serum (7 patients).

Table 23. Summary of paired plasma, serum and urine samples collected concurrently from 12 individual patients.

Patient number	**Specimen Type**	**Viracor-IBT Zika Virus Real-time RT-PCR Result**	**Comparator Assay[a] Result**
1	Plasma	Detected	Positive
	Serum	Detected	Positive
	Urine	Detected	Positive
2	Plasma	Detected	Negative
	Serum	Not detected	Negative
	Urine	Detected	Positive

3	Plasma	Detected	Negative
	Serum	Not detected	Negative
	Urine	Not detected	Negative
4	Plasma	Not detected	Negative
	Serum	Detected	Negative
	Urine	Detected	Positive
5	Plasma	Not detected	Negative
	Serum	Not detected	Negative
	Urine	Detected	Positive
6	Plasma	Detected	Positive
	Serum	Detected	Positive
	Urine	Detected	Negative
7	Plasma	Detected	Positive
	Serum	Detected	Positive
	Urine	Detected	Positive
8	Plasma	Detected	Positive
	Serum	Detected	Positive
	Urine	Detected	Positive
9	Plasma	Not detected	Positive
	Serum	Not detected	Negative
	Urine	Detected	Negative
10	Plasma	Detected	Negative
	Serum	Not detected	Negative
	Urine	Detected	Positive
11	Plasma	Detected	Positive
	Serum	Detected	Positive
	Urine	Detected	Positive
12	Plasma	Detected	Positive
	Serum	Detected	Positive
	Urine	Detected	Positive

[a]Altona RealStar Zika Virus RT-PCR under an EUA

Assay performance for paired plasma, serum and urine samples collected concurrently from the same patient by each specimen type tested is shown in **Tables 24, 25** and **26**. For plasma, the PPA and NPA of the Viracor-IBT Zika Virus Real-time RT-PCR assay compared to the comparator assay was 85.7% and 40%, respectively. For serum, the PPA and NPA of the Viracor-IBT Zika Virus Real-time RT-PCR assay compared to the comparator assay was 100% and 83.3%, respectively. For urine, the PPA and NPA of the Viracor-IBT Zika Virus Real-time RT-PCR assay compared to the comparator assay was 100% and 33.3%, respectively.

Table 24. Summary of results for analysis of individual plasma samples by the Viracor-IBT Zika Virus Real-time RT-PCR and a comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA)

		Comparator result	
		Positive	Negative
Viracor-IBT RT-PCR result	Positive	6	3
	Negative	1	2

Table 25. Summary of results for analysis of individual serum samples by the Viracor-IBT Zika Virus Real-time RT-PCR and a comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA)

		Comparator result	
		Positive	Negative
Viracor-IBT RT-PCR result	Positive	6	1
	Negative	0	5

Table 26. Summary of results for analysis of individual urine samples by the Viracor-IBT Zika Virus Real-time RT-PCR and a comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA)

		Comparator result	
		Positive	Negative
Viracor-IBT RT-PCR result	Positive	9	2
	Negative	0	1

Additionally, the Viracor-IBT Zika virus real-time RT-PCR assay performance by each specimen type relative to the patient infected status is shown in **Tables 27, 28** and **29** for the paired plasma and urine samples. Patient infected status is positive when at least one of the two FDA authorized (under an EUA) paired specimen types (serum and urine) is positive as determined by the comparator assay, and patient infected status is negative when none of the two FDA authorized (under an EUA) paired specimen types (serum and urine) is positive as determined by the comparator assay. For plasma, serum and urine the PPA of the Viracor-IBT Zika virus real-time RT-PCR assay was 80%, 70% and 100%, respectively.

Table 27. Summary of results for analysis of individual plasma samples by the Viracor-IBT Zika Virus Real-time RT-PCR relative to the patient infected status

		Patient infected status	
		Positive	Negative
Viracor-IBT RT-PCR result	Positive	8	1
	Negative	2	1

Table 28. Summary of results for analysis of individual serum samples by the Viracor-IBT Zika Virus Real-time RT-PCR relative to the patient infected status

		Patient infected status	
		Positive	Negative
Viracor-IBT RT-PCR result	Positive	7	0
	Negative	3	2

Table 29. Summary of results for analysis of individual urine samples by the Viracor-IBT Zika Virus Real-time RT-PCR relative to the patient infected status

		Patient infected status	
		Positive	Negative
Viracor-IBT RT-PCR result	Positive	10	2
	Negative	0	0

Furthermore, patient infected status as determined by the Viracor-IBT Zika virus real-time RT-PCR assay testing paired plasma and urine specimens (i.e., detection of Zika virus RNA in serum and/or urine from patients with paired plasma/urine specimens taken is considered as a positive patient, and no detection of Zika virus RNA in both plasma and urine specimens from patients with paired serum/urine specimens taken is considered as a negative patient) was compared against the patient infected status as determined by the comparator assay (as described previously). Results are shown in **Table 30.**

Table 30. Summary of results for comparison of patient the infected status as determined by the Viracor-IBT Zika virus real-time RT-PCR assay (plasma and urine) against the patient infected status as determined by the comparator assay

		Patient infected status – Comparator assay	
		Positive	Negative
Patient infected status - Viracor-IBT RT-PCR Plasma/urine result	Positive	10	2
	Negative	0	0

Patient infected status as determined by the Viracor-IBT Zika virus real-time RT-PCR assay testing paired serum and urine specimens (i.e., detection of Zika virus RNA in serum and/or urine from patients with paired serum/urine specimens taken is considered as a positive patient, and no detection of Zika virus RNA in both serum and urine specimens from patients with paired serum/urine specimens taken is considered as a negative patient) was also compared against the patient infected status as determined by the comparator assay (as described previously). Results are shown in **Table 31.**

Table 31. Summary of results for comparison of patient the infected status as determined by the Viracor-IBT Zika virus real-time RT-PCR assay (serum and urine) against the patient infected status as determined by the comparator assay

		Patient infected status – Comparator assay	
		Positive	Negative
Patient infected status - Viracor-IBT RT-PCR Serum/urine result	Positive	10	1
	Negative	0	1

For all natural clinical specimen testing data combined, the performance of the Viracor-IBT Zika Virus Real-time RT-PCR against the comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA) stratified by specimen type is presented in **Tables 32, 33,** and **34**. The comparator assay was an rRT-PCR assay authorized by FDA for detection of Zika RNA with analytical sensitivity in the range 3162 – 5000 RNA NAAT Detectable Units/mL for serum and 3162 – 5000 RNA NAAT Detectable Units/mL for urine.

Table 32. Summary performance of the Viracor-IBT Zika Virus Real-time RT-PCR against the comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA) - Plasma

Plasma - Specimen Category	**Viracor-IBT Zika Virus Real-time RT-PCR**		
	Number Tested	**Zika RNA Positive**	**Zika RNA Negative**
Natural specimens collected from Zika presumptive-positive patients (as determined by clinical presentation) in Colombia, South America	11	3	7
Natural specimens collected from asymptomatic individuals in Colombia, South America that were considered to be at risk since they were geographically located in mosquito infested areas where Zika is currently endemic	30	17	13
Patient matched/paired specimens collected from Colombia and the Dominican Republic from Zika confirmed-positive patients as determined by clinical presentations and diagnostic testing completed by the repository	12	9	3
Expected Zika RNA negative specimens that were collected from asymptomatic normal donors in the United States	20	0	20
Positive Percent Agreement	**92.0% (23/25); 95% CI (72.5% - 98.6%)**		
Negative Percent Agreement	**87.5% (42/48); 95% CI (74.1% - 94.8%)**		

Table 33. Summary performance of the Viracor-IBT Zika Virus Real-time RT-PCR against the comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA) - Serum

Serum - Specimen Category	Viracor-IBT Zika Virus Real-time RT-PCR		
	Number Tested	**Zika RNA Positive**	**Zika RNA Negative**
Natural specimens collected from Zika presumptive-positive patients (as determined by clinical presentation) in Colombia, South America	32	3	29
Natural specimens collected from Zika presumptive-positive patients (as determined by clinical presentation) in the United States	2	2	0
Natural specimens collected from asymptomatic individuals in Colombia, South America that were considered to be at risk since they were geographically located in mosquito infested areas where Zika is currently endemic	NA	NA	NA
Patient matched/paired specimens collected from Colombia and the Dominican Republic from Zika confirmed-positive patients as determined by clinical presentations and diagnostic testing completed by the repository	12	7	5
Expected Zika RNA negative specimens that were collected from asymptomatic normal donors in the United States.	21	0	21
Positive Percent Agreement	**100% (12/12); 95% CI (69.8% - 100)**		
Negative Percent Agreement	**96.4% (54/56); 95% CI (86.6% - 99.4%)**		

Table 34. Summary performance of the Viracor-IBT Zika Virus Real-time RT-PCR against the comparator assay (Altona RealStar Zika Virus RT-PCR under an EUA) - Urine

Urine - Specimen Category	Viracor-IBT Zika Virus Real-time RT-PCR		
	Number Tested	**Zika RNA Positive**	**Zika RNA Negative**
Natural specimens collected from Zika presumptive-positive patients (as determined by clinical presentation) in Colombia, South America	NA	NA	NA
Natural specimens collected from Zika presumptive-positive patients (as determined by clinical presentation) in the United States	1	1	0
Natural specimens collected from asymptomatic individuals in Colombia, South America that were considered to be at risk since they were geographically located in mosquito infested areas where Zika is currently endemic	NA	NA	NA
Patient matched/paired specimens collected from Colombia and the Dominican Republic from Zika confirmed-positive patients as determined by clinical presentations and diagnostic testing completed by the repository	12	11	1
Expected Zika RNA negative specimens that were collected from asymptomatic normal donors in the United States.	30	0	30
Positive Percent Agreement	**100% (11/11); 95% CI (67.9% - 100%)**		
Negative Percent Agreement	**93.9% (31/33); 95% CI (78.4% - 98.9%)**		

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