Zika Virus RNA Qualitative Real-Time RT-PCR

Quest Diagnostics Infectious Disease, Inc.
(formerly Focus Diagnostics, Inc.)

For use under an Emergency Use Authorization only

Rx Only

Instructions For Use

Table of Contents

Introduction

This document describes the use of Zika Virus RNA Qualitative Real-Time RT-PCR for the *in-vitro* qualitative detection of RNA from Zika Virus in human serum or urine.

Intended Use

Zika Virus RNA Qualitative Real-Time RT-PCR test is a real-time RT-PCR test intended for the qualitative detection of RNA from Zika virus in human serum or urine (collected alongside a patient-matched serum specimen) and is only intended for 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, such as possible sexual transmission). Testing is limited to qualified laboratories designated by Quest Diagnostics Infectious Disease, Inc. in the United States (US) that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests.

Results are for the identification of Zika viral RNA. Zika viral RNA is generally detectable in serum and/or urine during the acute phase of infection and up to 14 days following onset of symptoms, if present. A positive result in either specimen type provides evidence of Zika virus infection, but should always be considered in the context of patient history (symptoms and exposure risk consistent with CDC criteria), in making a final diagnosis and patient management decisions. In high risk patients, e.g. pregnant women, additional testing should be considered. Laboratories are required to report all positive results to the appropriate public health authorities.

Negative results do not rule out 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. For high risk patients, e.g. pregnant women, additional testing should be considered.

The Zika Virus RNA Qualitative 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 Zika Virus RNA Qualitative Real-Time RT-PCR test. The test is only for use under the Food and Drug Administration's Emergency Use Authorization.

Protocol Use Limitations

Testing of clinical specimens using the Zika Virus RNA Qualitative Real-Time RT-PCR test is limited to qualified laboratories designated by Quest Diagnostics Infectious Disease, Inc. in the US.

The Zika Virus RNA Qualitative 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) is not permitted.

Assay Principle

Zika Virus RNA Qualitative Real-Time RT-PCR test utilizes 5' nuclease technology (sometimes known as "Taqman" technology) to detect Zika virus genomic RNA in human serum or urine. The assay is composed of two principal steps: (1) extraction of total nucleic acid from patient specimens using the Roche MagNA Pure Instrument and (2) real-time amplification and detection of two separate 77 nucleotide sequences within the "M" and "env" regions of the Zika virus genome using an Applied Biosystems 7500 Real-Time PCR System. An internal control is included with each specimen to verify nucleic acid extraction and amplification

Specimens

Acceptable Specimens

- Serum
- Urine (collected alongside a patient-matched serum specimen)

Specimen Handling And Storage

- When transporting human specimens, ensure that all applicable regulations for transport of potentially infectious biological specimens are met.
- Serum specimens are stable for 7 days at room temperature (18-26^{o}C).
- Urine specimens are stable for 48 hours at room temperature (18-26^{o}C).
- Serum and urine specimens are stable for 7 days at 2-8°C.
- Long term serum specimen storage at -10°C or lower.
- Transport/ship human serum specimens frozen on dry ice (< -60ºC) or at 2-8°C.
- Transport/ship paired human serum and urine specimens together at 2-8°C.

Warning and 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 or inactivated Zika virus. The controls should be treated as potentially infectious. All controls, serum and urine specimens 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 waste 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 QUEST DIAGNOSTICS INFECTIOUS DISEASE, INC

- **Zika Virus RNA Qualitative Real-Time RT-PCR test**

    - ZV RT-PCR Mix 1 (957uL/tube): contains reaction buffer, primers and probes for Zika virus and internal control.
    - ZV RT-PCR Mix 2 (957uL/tube): contains reaction buffer, primers and probes for Zika virus and internal control
    - Negative Control (0.55mL/tube, single use): normal human serum, with no detectable Zika virus RNA
    - Positive Control (0.55mL/tube, single use,): use one of the following:
        - Zika virus strain FLR diluted in negative control to a level containing 5 genome copies of Zika RNA per µL of serum. CAUTION – Live virus. See Warnings and Precautions.
        - Inactivated Zika virus diluted in negative control to a level containing 5 genome copies of Zika RNA per µL of serum. CAUTION – inactivated virus. See Warnings and Precautions.

    Store RT-PCR Mixes at -10°C or colder in a freezer that is not frost free until the expiration date indicated on the vial. Do not re-freeze. Store Controls at -60°C or colder in a freezer that is not frost free until the expiration date indicated on the vial. Do not re-freeze.

MATERIALS REQUIRED BUT NOT PROVIDED

- Roche Total Nucleic Acid Isolation Kit, Large Volume (Catalog # 03264793001, 192 isolations/kit)
- Life Technologies SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Catalog # 11732-088, 500 reactions/kit)
- Exogenous Internal Control DNA (Internal control), Diasorin Molecular (formerly Focus Diagnostics, Inc.) (Catalog # QMOL 9001, 1 mL/tube)
- Exogenous DNA Primer/Probe Mix (Internal Control Primers/Probe), Diasorin Molecular (formerly Focus Diagnostics, Inc.) (Catalog # QMOL 9005, 0.5 mL/tube)

EQUIPMENT

- Roche MagNA Pure LC System
- ABI 7500 Sequence Detection System

CONSUMABLES

- Sterile screw cap 15 mL conical tubes
- Sterile screw cap 50 mL conical tubes
- P10, P20, P200, P1000 pipettes
- P10, P20, P200, P1000 ART Plugged Tips
- 1.5 mL or 2 mL microcentrifuge tubes
- Nitrile gloves
- Biohazard Absorbent Wipes
- 96-Well Optical Reaction Plate
- Optical Adhesive Cover

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

GENERAL CONSIDERATIONS

- Date and initial all controls upon opening.
- Each container should be labeled with:
  (1) substance name
  (2) lot number
  (3) date of preparation
  (4) expiration date
  (5) initials of technician, and
  (6) any special storage instructions; check for visible signs of degradation.

ASSAY CONTROLS

- Positive Control: one included in each batch of specimens to monitor nucleic acid isolation and detection of Zika virus RNA, consists of cultured (live or inactivated) Zika virus diluted in human serum, and should generally be detected after 30-35 cycles in each reaction mix.
- Negative Control: one included in each batch of specimens to monitor Zika virus contamination, consists of normal human serum, and should have no detectable signal after 40 cycles of amplification in each reaction mix.
- Internal Control: DNA target included in each specimen and control, consists of a portion of *a* plant genome, added to the lysis reagent provided in the MagNA Pure Isolation kit, amplified/detected with proprietary primers and probes, and should generally be detected after 25-30 cycles in each reaction mix. To monitor assay

performance, an internal control is added to each specimen and control during the nucleic acid isolation.

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 should be included in each run.
- Internal control DNA is added to the lysis buffer 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

- MagNA Pure LC Instrument
  Clinical specimens are extracted using the MagNA Pure LC Total Nucleic Acid Isolation Kit Large Volume on the Roche MagNA Pure LC extraction instrument using the Purification Protocol: Total NA_serum_plasma. Follow the manufacturer's instructions, using the following volumes:

  Specimen input volume: 1,000 µL
  Elution volume: 50 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 DNA (ICD) is added to the MagNA Pure Lysis Buffer prior to nucleic acid extraction. A sample can be interpreted as negative only if the ICD 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 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**

  Refer to PLCY entitled “Quality Control Program” for more details on minimum requirements.

  - To establish acceptable performance, all levels of QC controls must be tested with each batch.

  - For additional runs, QC must be incorporated at approximately the following frequency while continuing to ensure that all patient samples are bracketed by QC

Type of Run	Minimum Number of QC samples	QC Percent of Batch Size
Batch	2 QC every batch	Variable

- **Controls Tolerance Limits**

  - Negative Control should be listed as “Undetermined” for both RT-PCR mixes. If the Negative Control is positive (C_T value $\leq$ 39.00) for one or both mixes, 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 or below the acceptable range for both RT-PCR mixes, the Positive Control is considered valid and acceptable. If the Zika Virus Positive Control C_T values are above 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 indeterminate, or Detected 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)**

  - Total Nucleic Acids are extracted from patient specimens and assay controls using the Roche MagNA Pure Total Nucleic Acid Large Volume kit and the Roche MagNA Pure LC Automated DNA Extractor instrument. Refer to TSOP.129.111 for nucleic acid extraction using this kit.

- Under the “Protocol” drop-down menu on the MagNA Pure LC System, select “Total Nucleic Acid Large Volume” and then “Total NA serum plasma.blk” from the list.
- The sample protocol should be “Total NA serum_plasma blk”.
- 1,000 uL should be set for the Sample Volume, and the elution volume should be set at 50 uL.
- The dilution volume should be set at zero for all samples.
- The Post Elution Protocol dropdown menu should be set at “None”.
- In a 50 mL conical tube or equivalent container, prepare Lysis Buffer.
- Based upon the number of specimens (in multiples of 8), combine Lysis Buffer and Exogenous DNA as follows:

# of Specimens	mL LysisBuffer	µL ExogenousDNA
8	15	17
16	25	28
24	35	39
32	50	55

- Mix by repeated inversion. Do not vortex. Transfer the appropriate amount of Lysis Buffer/Exogenous DNA mixture into the appropriate reagent tub.
- Pipet 500 uL of DEPC-Treated water into all required wells of the Sample Cartridge.
- Pipette 500 uL of each specimen or control into the corresponding position in the Sample Cartridge. PIPETTE GENTLY TO AVOID AEROSOLIZATION OR CONTAMINATION.
- Ensure specimen(s) and controls are in the correct position on the Sample Cartridge.
- Visually check the level of samples and controls in the Sample Cartridge to ensure sample(s) were added.
- Transfer the sample cartridge containing samples to the MagNA Pure LC Automated Nucleic Acid extractor and begin the extraction run.
- After nucleic acid extraction is complete, the cartridge containing the extracted controls and patient specimens can be removed from the MagNA Pure. Seal the plate. Store the RNA for up to 15 minutes refrigerated prior to use. RNA should be frozen at <-60°C if longer storage is required before use and may be stored at least 2 weeks at <-60°C.

- **Setting Up Real-time RT-PCR Reactions**
  - In the Reagent Room, thaw one vial of each RT-PCR mix (ZV RT-PCR Mix 1 and ZV RT-PCR Mix 2) containing 957 uL for every 32 samples (specimens plus controls) in the assay run.
  - Centrifuge briefly to deposit all material at the bottom of the tube.
  - If less than 32 total reactions, use 29 uL mix + 1 uL enzyme mix per reaction.
  - Otherwise add 33 uL of Superscript III/Platinum Taq enzyme mix to each 957 uL tube of mix. Vortex gently and centrifuge briefly to deposit all material at the bottom of the tube. Return unused enzyme mix to cold block or freezer immediately.
  - Place the 96-well Optical Reaction Plate in a cooling block that has been pre- cooled to 2-8°C. Dispense 30 uL to each well used for RT-PCR.
  - Transfer the 96-well Optical Reaction Plate containing master mix inside the cooling block to the PCR Set-up Room.
  - Add 20 uL of appropriate extracted Total NA (patient specimens and controls) to the appropriate well of the 96-well Optical Reaction Plate.

    Note: Make sure to follow the Reaction Plate tray map.

Example RT-PCR Reaction Plate Tray Map for Specimens (#1-30) and Controls

	RT-PCR Mix 1					RT-PCR Mix 2						
	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25		1	9	17	25			
B	2	10	18	26		2	10	18	26			
C	3	11	19	27		3	11	19	27			
D	4	12	20	28		4	12	20	28			
E	5	13	21	29		5	13	21	29			
F	6	14	22	30		6	14	22	30			
G	7	15	23	Pos Con		7	15	23	Pos Con			
H	8	16	24	Neg Con		8	16	24	Neg Con			

  - Cover the plate with Optical Adhesive Cover.

    Note: Make sure to handle the Optical Adhesive Cover on the edge only. Do not touch the middle part of the cover.

  - Briefly centrifuge the plate to collect the reactions at the bottom of the wells and to eliminate any air bubbles. Place the reaction plate in the cooling block.

    Take the sealed plate in the cooling block to the ABI 7500 Sequence Detection System. No compression pad is needed for this instrument.

- Use ZIKI (FAM), ZIKII (FAM) and “QICD” (Q670) as the Detectors for the assay run.

- The passive reference should be set at “ROX”.

- Real-time RT-PCR parameters using the ABI 7500 Sequence Detection System:

  50°C 15 minutes
  95°C 2 minutes
  95°C 15 sec; 60°C 60 seconds; 40 cycles
  Sample volume is set at 50 µL
  Choose Standard for correct thermal profile parameters

- **Analyzing the Run Data, Exporting Results and Printing**

	Analyzing the Run Data, Exporting Results and Printing
1.	When the run finishes, click **OK** from the window.
2.	Click on **Analysis** menu and choose **Analysis Settings**. Detectors should be set at “**All Detectors**”. **Manual Ct** should be selected **Manual Baseline** should be selected (Start at 5, Stop at 15) Set the **threshold** at **0.10** for **ZIKI** Set the **threshold** at **0.10** for **ZIKII** Set the **threshold** at **0.02** for **QICD** **Use System Calibration should be selected**
3.	Click the **Analyze** icon (▸) from the toolbar. *Note: Wait approximately one minute for the analysis process to be completed.*
4.	Click the **Results** tab.
5.	Click the **Amplification Plot** tab.
6.	Choose **ZIKI or ZIKII** and **QICD** from the **Detector** window.
7.	Click one well containing a specimen at a time and look at the **Amplification plot** and **Component plot** to check for the accuracy of the result.

8.	Choose **Save** option from the **File** menu after analyzing all the wells.
9.	To export the results to the LIS, start by highlighting the wells containing ZIKA PCR reactions from the plate grid.
10.	From the **File** menu, choose **Export** and then **Results**.
11.	Select **"Taqman on 'samba 2.2.7 (\\lis.focusdx.priv)(M)"** in the **Look in** window.
12.	Type in the name of the plate in the File Name window (e.g.: ZIKAPCR 030816 EXPORT). Note: Make sure to export the files as Text file (Tab-delimited file) and include the word "EXPORT" in the file name to help distinguish exported file from imported file.
13.	Click the Save button. Note: It will take approximately 15 minutes for LIS to download the results. Do not open the exported file while it is being transferred.
14.	To print the results, choose Print from the File menu.
15.	Click Print and then click O.K.

Interpreting Test Results

REPORTING RESULTS AND REPEAT CRITERIA

- Clinical laboratory personnel qualified by applicable state and/or federal regulations (e.g. Clinical Laboratory Scientist licensed by the State of California) 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"). If the result is listed as "Undetermined" it means that no amplification of the target was achieved.

- Amplification plots and component data should be examined for all samples. 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 an increase in fluorescence, the C_T value is the result of non-specific fluorescence (mark the C_T value as "NSF" on the worksheet). "NSF" results are considered equivalent to "Undetermined" results in that no amplification of the target was achieved.
- An internal control DNA (ICD) is included with every sample. The ICD C_T value of all negative samples must have a C_T value within 3 C_Ts of the current ICD mean value. This criterion must be met in order for the result to be valid. A value greater than 3 C_Ts from the current ICD mean value or non-amplification of the ICD 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 $\leq$ 39.00 and has a valid amplification curve) for one or of the two mixes, 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 "Undetermined") for both RT-PCR mixes, then this control is valid and acceptable.

- Zika Virus Positive Control:

  - If the Zika Virus Positive Control C_T values are above the acceptable range for one or both of the RT-PCR mixes, 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 or below the acceptable range for each of the RT-PCR mixes, the Positive Control is considered valid and acceptable.

- **Examination of Patient Specimen Results**

  - Zika Virus RNA DETECTED results must be reported to the appropriate Public Health Authorities.

  - Examination of clinical specimen results should be performed after the Negative Controls have been examined and determined to be valid and acceptable. ZIKI, ZIKII and QICD probe results must be examined for each patient specimen.

    - If the ZIKI and ZIKII C_T values of a patient specimen are listed as "Undetermined" for both RT-PCRs and the QICD C_T values for the specimen are within 3 C_Ts of the current QICD mean C_T value for both of the RT-PCR mixes, the result is reported out as "Not Detected".

    - If the C_T values for a patient specimen is $\leq$ 39.00 for at least one of the two ZIKI or ZIKII RT-PCR mixes and there is a valid amplification curve, the result is reported as "Detected".

    - If the C_T values of a patient specimen are in the range of 39.01- 40 for one or both of the ZIKI or ZIKII RT-PCR mixes and there is a valid amplification curve, the specimen should be retested to verify the positive result. If upon retesting, one or both C_T values is < 40 and there is a valid amplification curve, the result is reported out as "Detected". If upon retesting the C_T value of a patient specimen is listed as "Undetermined and the QICD C_T value of the specimen is within 3 C_Ts of the current QICD mean C_T value for both of the RT-PCR mixes, the result is reported out as "Not Detected".

    - If the C_T values of a patient specimen is listed as "Undetermined" but the QICD C_T values of the specimen are greater than 3 C_Ts above the current QICD mean C_T value for one or both of the ZIKI or ZIKII RT-PCR mixes, 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.”

Assay Limitations

1. This test is for *in vitro* diagnostic use under FDA Emergency Use Authorization only and is limited to the qualified laboratories designated by Quest Diagnostics Infectious Disease, 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.

2. This test is a qualitative test and does not provide the quantitative value of detected organisms present.

3. Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.

4. 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.

5. The performance of this test has not been established for patients without symptoms of Zika virus infection (as per the CDC criteria), or those that do not meet the CDC criteria for epidemiological risk.

6. The performance of this test has not been established for monitoring treatment of Zika virus infection.

7. This test cannot rule out diseases caused by other bacterial or viral pathogens.

8. This assay has not been validated with specimens except serum and urine (collected alongside a patient-matched serum specimen).

9. A patient matched serum specimen, if available, or a serum specimen from a new draw is required for serological follow up testing of negative results, per the CDC testing algorithm. (Found at http://www.cdc.gov/zika/index.html).

10. 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. Repeat testing or additional testing may be indicated in some settings (e.g. high risk patients such as pregnant women).

11. Interpretation of test results must account for the possibility of false-negative results which can arise from:
    - poor sample collection or
    - degradation of the viral RNA during shipping or storage or
    - specimen collection conducted prior to symptom onset

- specimen collection after nucleic acid can no longer be found in the patient (approximately 14 days post-onset of symptoms for serum and/or urine)
- failure to follow the authorized assay procedures
- failure to use authorized extraction kit and platform
- when the infecting organism has genomic mutations, insertions, deletions, or rearrangements or when performed very early in the course of illness
- if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling

12. False-positive results may occur. Repeat testing or additional testing may be indicated in some settings (e.g. high risk patients such as pregnant women). Interpretation of test results must account for the possibility of false-positive results, which can arise from:
    - Cross-contamination of samples

Performance Characteristics

Analytical Sensitivity

Analytical sensitivity Limit of Detection (LOD) studies determine the lowest detectable concentration of Zika virus at which approximately 95% of all replicates test positive. The LOD was initially determined by assaying dilutions of Zika virus synthetic RNA transcripts in buffer and verified by assaying dilutions of cultured (live) Zika virus prepared in serum. In each study, the replicates were tested through the complete assay (nucleic acid isolation and amplification/detection) as described in the procedure sections above.

Cultured Zika virus (strain FLR, GenBank: KU820897.1) was provided by an investigator from a clinical site located in the Southwestern region of the United States. Concentration of the undiluted cultured virus was approximately 10^9 RNA copies/mL. The virus was isolated from a patient that was infected in Colombia in December, 2015. The isolated virus was propagated at the clinical site in mosquito cells. The sequence of the complete virus has recently been added to the NCBI database.

Zika virus genomic RNA was prepared from 0.1 mL of cultured Zika virus FLR using a guanidine lysis and isopropanol precipitation procedure (Trizol LS) and the purified RNA was resuspended in water. Dilutions of the RNA were PCR-amplified to create an 889 bp T7 promoter- Zika virus DNA template that spanned the “Pr”, “M”, and “env” regions and contained the two RT-PCR targets used in this test. Synthetic Zika virus RNA was prepared from this template using the MEGAScript T7 kit, purified by precipitation, and quantitated using a Qubit Fluorometric Quantitation instrument and the Qubit RNA HS kit. After quantitation the RNA was diluted in a buffer consisting of poly(A) RNA and Tris.

A panel of sensitivity standards was prepared from the quantitated Zika virus synthetic RNA transcripts. The sensitivity standards were prepared, aliquotted and stored at -60°C to -90°C until time of testing. The sensitivity standards panel was tested over 5 runs in the complete test. Each run contained 4 replicates of each standard for a total of 20 replicates tested using each RT-PCR mix. The results of testing are shown in Tables 1 and 2. Zika virus synthetic RNA was detected at 100% (20/20 replicates) at 250 RNA copies/mL in both RT-PCR mixes.

Table 1. Sensitivity Results for Detecting Zika Virus Synthetic RNA in Buffer: ZV RT-PCR Mix 1

Zika Virus RNA Copies/mL Specimen	Zika Virus RNA Copies/RT-PCR Reaction	Replicates Tested	Replicates Detected	Detection Success Rate	Mean C_T
1,006	201	20	20	100%	34.29
503	101	20	20	100%	35.18
251	50	20	20	100%	36.58
126	25	20	18	90%	37.13
63	13	20	14	70%	37.66
31	6	20	9	45%	38.66
16	3	20	8	40%	38.60
0*	0	20	0	0%	not detected

* RNA diluent buffer

Table 2. Sensitivity Results for Detecting Zika Virus Synthetic RNA in Buffer: ZV RT-PCR Mix 2

Zika Virus RNA Copies/mL Specimen	Zika Virus RNA Copies/RT-PCR Reaction	Replicates Tested	Replicates Detected	Detection Success Rate	Mean C_T
1,006	201	20	20	100%	35.20
503	101	20	20	100%	35.84
251	50	20	20	100%	37.10
126	25	20	18	90%	38.18
63	13	20	11	55%	38.07
31	6	20	7	35%	38.62
16	3	20	2	10%	38.76
0*	0	20	0	0%	not detected

* RNA diluent buffer

The tentative LOD established using synthetic Zika virus RNA diluted in buffer was confirmed by testing cultured (live) Zika virus diluted in serum. Cultured Zika virus strain FLR was diluted in pooled normal human serum to approximately 25,000 RNA copies/mL and tested in the test in order to accurately determine the concentration of the diluted virus. Four (4) replicates were tested at this concentration and the mean C_T value was calculated from the combined results using both RT-PCR mixes. A calibration curve for the Zika virus RNA RT-PCR was constructed using the mean C_T values (both RT-PCR mixes combined) for the synthetic RNA sensitivity standards and from test results for low and mid positive synthetic Zika virus RNA controls (5,000 and 50,000 RNA copies/mL). This calibration curve was used to assign a copies/mL value to the serum-diluted Zika virus (a mean C_T of 31.01 corresponded to 19,450 RNA copies/mL). The concentration of the serum-diluted Zika virus was used to assign a concentration value to the undiluted viral stock (1.56×10^9 RNA copies/mL).

The LOD of the test in serum was evaluated by testing dilutions of quantitated cultured (live) Zika virus prepared in pooled normal human serum. A volume of diluted virus was prepared at 5,070 copies/mL (ID#SS4). Serial dilutions were prepared from dilution SS4 at 460 copies/mL (ID#SS5) and 40 copies/mL (ID#SS6). A larger volume of diluted Zika virus was prepared from dilution SS4 at the tentative LOD (ID#SS250, 250 copies/mL). Three (3) replicates of dilutions SS4, SS5 and SS6 and 20 replicates of dilution SS250 were tested in a single run. Three (3) replicates of the pooled normal human serum (SS7) were also tested in the same run. The testing results for Zika virus diluted in serum are shown in Table 3 and include the test results from spiked serum (at 250 copies/mL) used as part of the Clinical Accuracy Study Blind Panel (refer to the Clinical Evaluation section for details). The preparation for the Clinical Accuracy Blind Panel was as described above except that the quantitated cultured (live) Zika virus was diluted in 18 individual patient serum samples obtained from sample remnants submitted for Chikungunya (n=10), Dengue (n=6), and West Nile (n=2) virus RT-PCR testing. These serum samples had results of RNA not detected in the corresponding assays. The serum sample remnants used to prepare the Clinical Accuracy Blind Panel were subsequently tested in the Zika Virus RNA Qualitative Real-Time RT-PCR test to rule out the presence of Zika Virus RNA in the unspiked samples. There was insufficient volume to test 3 of the Chikungunya serum remnants that were spiked at the LOD. Zika virus RNA was not detected in the remaining 15 serum remnants that were spiked at the LOD. The LOD of the assay in serum was confirmed to be 250 RNA copies/mL (94.7% detection rate, 36/38 replicates detected at 250 copies/mL).

Table 3. Sensitivity Results for Detecting Cultured (Live) Zika Virus in Serum

Dilution ID	Zika Virus RNA Copies/mL serum	Zika Virus RNA Copies/RT-PCR Reaction	Replicates Tested	Replicates Detected	Detection Success Rate	ZV RT-PCR Mix 1 Mean C_T	ZV RT-PCR Mix 2 Mean C_T
SS4	5,070	1,014	3	3	100%	32.33	33.07
SS5	460	92	3	3	100%	36.40	36.89
Blind Panel	250	50	18	18	100%	37.64	37.96
SS250	250	50	20	18	90%	37.44	38.12
SS6	40	8	3	2	66%	38.55	not detected
SS7*	0	0	3	0	0%	not detected	not detected

* Normal serum diluent

The LOD of the test in urine was evaluated by testing dilutions of quantitated cultured (live) Zika virus prepared in pooled normal human urine. Normal (random) urines were collected from healthy donors from a non-endemic area (California, Sept-Dec 2015), and were presumed Zika negative. Approximately 50% were male urine and 50% female urine, and all normal urines were from adults and de-identified. Pooled normal urine was prepared from 10 of the normal urines (above) and used for the LOD studies. Five male urines and 5 female urines were randomly selected for the pool, and these urines were not used for the subsequent contrived specimen studies. Pooled normal urine used for the LOD studies were confirmed as Zika RNA negative using the assay before spiking. The concentration of the Zika virus stock was determined as previously described in the serum Zika RT-PCR LOD study, using serum-diluted virus tested in parallel with quantitated synthetic Zika viral RNA transcripts. The limit of detection (LOD) in urine was determined in two steps: first by establishing a tentative LOD with limited replicates, and then confirming the LOD with more replicates around the tentative value.

The tentative LOD was determined by spiking pooled normal urine with cultured Zika virus at 6 levels: 10,000, 5,000, 2,000, 1,000, 500, 250 copies/mL, and testing 4 replicates at each level. The LOD was confirmed by testing 20 additional replicates at the tentative LOD, and at concentrations 2-fold above and below the tentative LOD. The detection rate was calculated by dividing the number of positives by the number of valid replicates. The tentative LOD was estimated at 250 copies/mL. The LOD of the assay in urine was confirmed to be 500 RNA copies/mL (95.8% detection rate, 23/24 replicates detected at 500 copies/mL). All replicates tested were valid and were included in calculating the detection rate. The test results for Zika virus diluted in urine are shown in Table 4.

Table 4. Sensitivity Results for Detecting Cultured (Live) Zika Virus in Urine

Dilution ID	Zika Virus RNA Copies/mL urine	Zika Virus RNA Copies/RT-PCR Reaction	Replicates Tested	Replicates Detected	Detection Success Rate	ZV RT-PCR Mix 1 Mean C_T	ZV RT-PCR Mix 2 Mean C_T
1	10,000	2,000	4	4	100%	32.78	33.12
2	5,000	1,000	4	4	100%	33.56	34.08
3	2,000	400	4	4	100%	35.17	35.53
4	1,000	200	4	4	100%	36.02	36.24
5 (u1)	500	100	24	23	96%	37.39	37.39
6 (u2)	250	50	20	20	83%	38.06	38.00
u3	125	25	20	10	50%	38.23	38.41
Unspiked urine	0	0	0	4	0%	-	-

Analytical Sensitivity-FDA Reference Material

An analytical study was performed using FDA Reference Material (S1 and S2) following a standard protocol provided by the FDA. The protocol included range-finding and confirmatory LOD studies to evaluate the analytical sensitivity of the Zika Virus RNA Qualitative Real-Time RT-PCR test. The results of the study are shown in Table 5.

Table 5. LOD Confirmation Results using the FDA Reference Materials

FDA Reference Material	Specimen Type	Confirmed LOD* in RNA NAAT Detectable Units/mL
S1	serum	1,000
S1	urine	1,000
S2	serum	500
S2	urine	1,500

*Study performed according to an FDA issued protocol

Analytical Specificity:

a) Reactivity/Inclusivity: Reactivity of the Zika Virus RNA Qualitative Real-Time RT-PCR was not evaluated by wet testing additional isolates of Zika virus as there were no additional, characterized isolates available at the time of this study. Inclusivity/reactivity was alternatively demonstrated by generating sequence alignments of other Zika virus isolates along with the sequence of the strain used in this study (strain FLR, Genbank KU820897) and the sequences of the test's primers and probes. *In silico* analysis of the Zika Virus RNA Qualitative Real-Time RT-PCR primers and probes sequences was performed to verify sequence homology with the corresponding virus target regions. A total of 21 Zika virus strains representing the Asian lineage associated with the current Zika outbreak were selected for this study.

The results of the *in silico* inclusivity analysis are shown in Table 6. RT-PCR mix 1 primers and probes had generally 100% homology, except for single base mismatches in 3 cases. RT-PCR mix 2 primers had 100% homology, except for one single base mismatch in one case. Probe ZVF1214 (RT-PCR mix 2) had a one base mismatch with all strains except for the strain used for its design (EU545988). The mismatch was present in the strain used for the LOD study and did not appear to reduce the efficiency of the real- time amplification and detection as comparable detection cycle values were obtained using perfectly matched RT-PCR mix 1. Overall, primer and probe sequences showed 95-100% sequence identity with their expected targets, predicting false negative results are not likely to occur.

Table 6. *In Silico* Inclusivity Analysis for the Zika Virus RNA Qualitative Real-Time RT-PCR Primers and Probes:

			Primer/Probe Sequence Identity					
Genbank ID	**Strain**	**Date**	**RT-PCR1-FZV942**	**RT-PCR1-PZVF967**	**RT-PCR1-RZV1018**	**RT-PCR2-FZV1193**	**RT-PCR2-PZVF1214**	**RT-PCR2-RZV1269**
KU820897	Brazil (FLR)	2015	100%	100%	100%	100%	97%	100%
KU922923	Mexico (MEX)	2016	100%	96%	100%	100%	97%	100%
KU820898	China (GZ01)	2016	100%	100%	100%	100%	97%	100%
KU853012	Dominican Republic	2016	100%	100%	100%	100%	97%	100%
KU820899	China (ZJ03)	2016	100%	100%	100%	100%	97%	100%
KU744693	China (VE)	2016	100%	100%	100%	100%	97%	96%
KU497555	Brazil (ZKV2015)	2016	100%	100%	100%	100%	97%	100%
KU707826	Brazil (SSABR1)	2016	100%	100%	100%	100%	97%	100%
KU527068	Brazil (Natal)	2016	100%	100%	100%	100%	97%	100%
KU365777	Brazil (BeH818995)	2015	100%	100%	100%	100%	97%	100%
KU365778	Brazil (BeH819015)	2015	100%	100%	100%	100%	97%	100%
KU365779	Brazil (BeH819966)	2015	100%	100%	100%	100%	97%	100%
KU365780	Brazil (BeH815744)	2015	100%	100%	100%	100%	97%	100%
KU312312	Suriname (Z1106033)	2015	100%	100%	100%	100%	97%	100%
KU321639	Brazil (SPH2015)	2015	100%	100%	100%	100%	97%	100%
AB908162	French Polynesia (ZIKV)	2015	100%	100%	100%	100%	97%	100%
KU509998	Haiti (1225/2014)	2014	100%	100%	100%	100%	97%	100%
KJ776791	French Polynesia (H/PF/2013)	2013	100%	100%	100%	100%	97%	100%
KU681081	Thailand (THA2014)	2014	100%	100%	95%	100%	97%	100%
KU681082	Philippines (PHL2012)	2012	96%	100%	100%	100%	100%	100%
EU545988	Micronesia (YAP)	2007	100%	100%	100%	100%	100%	100%

b) Cross Reactivity: Cross-reactivity of the Zika Virus RNA Qualitative Real-Time RT-PCR test was evaluated by testing additional flaviviruses and/or purified synthetic RNA from additional flaviviruses. Preparations of cultured flavivirus species diluted in human serum and a positive patient specimen were tested. Concentrations of the challenge organisms were determined by testing in the appropriate real-time RT-PCR in-house test. The estimated concentrations of the challenge organisms were between 10^6 and 10^8 RNA copies/mL of specimen (10^5 to 10^7 RNA copies /RT-PCR Reaction). In addition, synthetic viral RNA transcripts consisted of a series of partial transcripts covering multiple regions of the respective virus of the related flavivirus species were purchased from a supplier and tested by adding the purified, synthetic RNA directly into each of the two RT-PCR mixes. Concentrations of the purified synthetic viral RNAs were provided by a supplier (between 10^5 and 10^6 RNA copies/uL). The results of the cross reactivity study are shown in Table 7 below. None of the organisms tested were detected by either of the RT- PCR mixes at the concentrations tested.

Table 7. Cross Reactivity Wet Testing Results for Serum

Organism	Sample Type	Source	Estimated RNA Copies/mL	Estimated RNA Copies/Reaction	Result
Chikungunya	Virus	patient specimen	2.23E+08	4.45E+07	Not Detected
Chikungunya	Virus	high positive stock*	4.11E+07	8.21E+06	Not Detected
Dengue Type 1	Virus	high positive stock*	3.60E+07	7.20E+06	Not Detected
Dengue Type 2	Virus	high positive stock*	4.82E+06	9.64E+05	Not Detected
Dengue Type 3	Virus	high positive stock*	9.35E+06	1.87E+06	Not Detected
Dengue Type 4	Virus	high positive stock*	7.09E+06	1.42E+06	Not Detected
West Nile Virus	Virus	high positive stock*	2.65E+08	5.30E+07	Not Detected
Chikungunya	Synthetic RNA Transcripts	ATCC**	>1.00E+07	>2.00E+06	Not Detected
DengueType 1	Synthetic RNA Transcripts	ATCC**	>1.00E+07	>2.00E+06	Not Detected
DengueType 2	Synthetic RNA Transcripts	ATCC**	>1.00E+07	>2.00E+06	Not Detected
DengueType 3	Synthetic RNA Transcripts	ATCC**	>1.00E+07	>2.00E+06	Not Detected
DengueType 4	Synthetic RNA Transcripts	ATCC**	>1.00E+07	>2.00E+06	Not Detected
St Louis Encephalitis Virus	Synthetic RNA Transcripts	ATCC**	>1.00E+07	>2.00E+06	Not Detected
West Nile Virus	Synthetic RNA Transcripts	ATCC**	>1.00E+07	>2.00E+06	Not Detected

*High positive control stocks supplied by Quest Diagnostics Infectious Disease, Inc., Reference Laboratory Manufacturing Department
**ATCC viral synthetic RNA transcripts were supplied at concentrations > 1.00E+05 RNA copies/uL

Additional wet testing data assessing potential cross-reactivity of the Zika Virus RNA Qualitative Real- Time RT-PCR test were referenced and reviewed from the publication in Emerging Infectious Diseases (2008) 14: 1232-1239 by Robert S. Lanciotti et al., Genetic and Serologic Properties of Zika Virus Associated with an Epidemic, Yap State, Micronesia, 2007, which used the exact same primers and probes as in the ZV RT-PCR Mix 1 and ZV RT-PCR Mix 2 of the Zika Virus RNA Qualitative Real-Time RT-PCR test. In this publication, potential cross-reactivity of the primers and probes was evaluated by wet testing the following viral genomic RNAs, all of which yielded negative results: Dengue-1, Dengue-2, Dengue-3, Dengue-4, West Nile Virus, St. Louis Encephalitis Virus, Yellow Fever Virus, Powassan Virus, Semliki Forest Virus, O'nyong-nyong Virus, Chikungunya, and Spondweni Virus.

Evaluation of the potential cross-reactivity of the Zika Virus RNA Qualitative Real-Time RT-PCR test was also performed through *in silico* analysis of each primer and probe sequence against other common causes of acute febrile illness in humans. BLAST analysis queries of the Zika Virus RNA Qualitative Real-Time RT-PCR test primers and probes were performed against the GenBank public domain nucleotide sequences and showed no significant combined homologies (primer target and probe target) with other conditions that would predict potential false positive results. Conditions and associated causative agents covered in the *in silico* analysis are presented in Table 8 below.

Table 8. *In Silico* Cross Reactivity Analysis Results

	System	RT-PCR Mix 1			RT-PCR Mix 2		
	query name	ZV942	ZV1018	ZVF967	ZV1193	ZV1269	ZVF1214
	query function	F-primer	R-primer	Probe	F-primer	R-primer	Probe
	query length	23	22	27	17	24	31
	strand match	plus/plus	plus/minus	plus/plus	plus/plus	plus/minus	plus/plus
Organism	**tax ID**	**percent match for alignment with highest identity for each analysis**					
Dengue virus 1	11053	48%	95%	44%	94%	50%	none*
Dengue virus 2	11060	48%	91%	37%	100%	46%	39%
Dengue virus 3	11069	43%	64%	41%	37%	54%	32%
Dengue virus 4	11070	43%	77%	44%	65%	46%	29%
Yellow Fever	40005	65%	41%	33%	65%	54%	29%
West Nile Virus	11082	52%	64%	41%	76%	33%	32%
Chikungunya Virus	37124	43%	41%	41%	59%	38%	29%
Mayaro Virus	59301	39%	45%	37%	59%	38%	35%
Parvovirus (B19)	10798	48%	50%	30%	41%	42%	29%
Plasmodium falciparum	5833	65%	68%	41%	71%	58%	35%
St Louis encephalitis	11080	74%	73%	41%	59%	54%	29%
Japanese encephalitis	11071	52%	64%	41%	59%	54%	35%
Spondweni Virus	64318	39%	45%	70%	65%	29%	26%
Hepatitis C Virus	11102	61%	50%	44%	71%	58%	42%
Eastern Equine encep. Virus	11021	52%	45%	33%	59%	54%	35%
Western Equine encep. Virus	11039	43%	36%	33%	47%	38%	35%
Ross River Virus	11029	43%	41%	30%	53%	38%	29%
Barmah Forest Virus	11020	52%	36%	30%	47%	33%	42%
O'nyong-nyong Virus	11027	35%	41%	33%	47%	38%	26%
Measles Virus	11234	43%	36%	41%	65%	42%	29%
Rubella Virus	11041	52%	41%	33%	65%	33%	none*
Enterovirus	12059	65%	55%	48%	71%	50%	42%
Adenovirus B	108098	43%	45%	37%	59%	42%	32%
Adenovirus D	130310	52%	55%	30%	33%	38%	39%
Adenovirus C	129951	43%	50%	33%	71%	38%	39%
Adenovirus B1	565302	43%	41%	37%	59%	42%	32%
Adenovirus 7	10519	43%	32%	30%	53%	42%	32%
Hepatitis B virus	10404	52%	59%	44%	59%	42%	39%
HIV	11676	74%	59%	52%	65%	50%	48%
Varicella Zoster Virus	10335	43%	50%	48%	53%	46%	32%
Cytomegalovirus	10358	61%	45%	37%	59%	42%	45%
Epstein Barr Virus	10376	48%	50%	41%	53%	46%	35%
Rickettsia	780	57%	50%	52%	65%	54%	35%
Borrelia burgdorferi	64895	52%	64%	44%	59%	46%	39%
Group A *Streptococcus*	36470	none*	none*	none*	none*	none*	none*
Plasmodium sp	5820	65%	68%	44%	71%	75%	48%
Leptospira	171	61%	64%	70%	59%	63%	42%
Trypanosoma cruzi	5693	57%	59%	48%	76%	67%	52%
Shistosoma	6181	74%	68%	59%	88%	67%	58%
Escherichia coli	562	74%	59%	56%	71%	54%	61%

*no significant alignment found

Additional *in silico* Cross-reactivity Analysis and Microbial Interference Studies

Cross-reactivity has been further evaluated for primers or probes with >70% homology to potential cross-reactive organism sequences in the database. Additional blast analyses were performed to identify potential amplicon sizes and evaluate primer and probe binding sites for the organism sequences with the highest homology. The smallest predicted amplicon for each primer pair is listed. Predicted amplicons that were greater than 10,000 bases (10 kb) and primers with less than 40% homology were not expected to support exponential amplification. Primers with 40-50% homology but with mismatches at the terminal 3' end were also not expected to support exponential amplification. The results of the additional analyses are shown in Tables 9 and 10. Exponential amplification of these potential cross-reactive organisms was not predicted. Although there was homology for selected organisms with single primers or probes, the primers and probes are provided in sufficient excess (106 to 109 molar excess) in the reaction mixtures relative to clinically relevant levels of potential cross-reactive organisms. The conclusion of these studies was that the presence of these potential cross-reactive organisms in test specimens would not interfere with the ability of the primers and probes to bind to Zika virus nucleic acid or prevent the detection of low levels of Zika virus.

Table 9. Additional i*n-silico* Analysis for RT-PCR Mix 1 Primer and Probe Sequences

Organism	tax ID	Sequence ID	Mix 1 Fow Primer	Mix 1 Rev Primer	Mix 1 Probe	shortest amplicon (bp)	comments*	RT-PCR Mix 1 amplification
Dengue virus 1	11053	KJ649286.1	48%	95%	30%	none	no extension overlap	not predicted
Dengue virus 2	11060	KX380807.1	30%	91%	33%	565	fow primer <40%	not predicted
Dengue virus 4	11070	FJ882588.1	39%	77%	30%	508	fow primer <40%	not predicted
St Louis encephalitis	11080	JQ957869.1	74%	45%	33%	3766	rev primer 45% with 3' mismatch (n=4)	not predicted
St Louis encephalitis	11080	EF158060.1	30%	82%	33%	61	fow primer <40%	not predicted
Spondweni virus	64318	DQ859064.1	35%	45%	70%	2242	fow primer <40%	not predicted
HIV	11676	KC186348.1	74%	41%	26%	1555	rev primer 41% with 3' mismatch (n=11)	not predicted
Leptospira	171	CP015814.1	48%	64%	41%	542527	>10kb, fow primer 3' mismatch (n=12)	not predicted
Shistosoma	6181	LM166548.1	74%	32%	26%	278	rev primer <40%	not predicted
Escherichia coli	562	LT601384.1	74%	50%	52%	428499	>10kb, rev primer 3' mismatch (n=11)	not predicted

* amplicon sizes > 10kb, primers with < 40% homology to the organism sequence, or primers with 3' end mismatches (n=number of bases mismatched) to the target sequence are not predicted to support exponential amplification.

Table 10. Additional *in-silico* Analysis for RT-PCR Mix 2 Primer and Probe Sequences

Organism	tax ID	Sequence ID	Mix 2 Fow Primer	Mix 2 Rev Primer	Mix 2 Probe	shortest amplicon (bp)	comments*	RT-PCR Mix 2 amplification
Dengue virus 1	11053	KR029570.1	94%	42%	23%	63	rev primer 42% with 3' mismatch (n=1)	not predicted
Dengue virus 2	11060	KU529743.1	100%	42%	23%	64	rev primer 42% with 3' mismatch (n=1)	not predicted
West Nile virus	11082	HQ840762.1	76%	29%	none	369	rev primer <40%	not predicted
Plasmodium falciparum	5833	CP017002.1	71%	42%	35%	138461	>10kb, rev primer 42% with 3' mismatch (n=5)	not predicted
Hepatitis C virus	11102	KJ470618.1	71%	29%	29%	742	rev primer <40%	not predicted
Enterovirus	12059	KJ768776.1	71%	29%	26%	195	rev primer < 40%	not predicted
Adenovirus C	129951	FJ025906.1	71%	38%	29%	16465	rev primer < 40%	not predicted
Plasmodium sp	5820	LT594515.1	71%	46%	35%	12950	>10kb, rev primer 46% with 3' mismatch (n=8)	not predicted
Plasmodium sp	5820	XM_004220951.1	41%	75%	26%	none	no extension overlap	not predicted
Trypanosoma cruzi	5693	CP015674.1	82%	46%	35%	15818	>10kb, rev primer 46% with 3' mismatch (n=4)	not predicted
Shistosoma	6181	LL978405.1	88%	29%	26%	none	no extension overlap	not predicted
Escherichia coli	562	CP015074.2	76%	58%	35%	222803	rev primer 58% with 3' mismatch (n=4)	not predicted

* amplicon sizes > 10kb, primers with < 40% homology to the organism sequence, or primers with 3' end mismatches (n=number of bases mismatched) to the target sequence are not predicted to support exponential amplification.

Interference Studies

Interference studies were not performed for the Zika Virus RNA Qualitative Real-Time RT-PCR test. The test uses conventional real-time RT-PCR, a well-established extraction method, and an internal control that is co-purified and co-detected in each specimen tested. Reaction inhibition or interference is not generally expected and can be effectively monitored by the internal control results.

Clinical Evaluation

The performance characteristics of the Zika Virus RNA Qualitative Real-Time RT-PCR test in serum were evaluated in a clinical study testing a Clinical Accuracy Study Blind Panel consisting of 20 Zika virus RNA positive natural clinical serum specimens, 36 contrived positive serum specimens, and, 54 normal human serum specimens. All specimens tested in the clinical evaluation were randomized, blind-labeled, and tested using the Zika Virus RNA Qualitative Real-Time RT-PCR test over a total of 4 runs. The 20 positive natural clinical specimens were obtained from Puerto Rico through collaboration where they were characterized by the CDC Trioplex Real-time RT-PCR assay (Trioplex rRT-PCR). The Trioplex rRT-PCR test results and specimen information that was provided with these specimens are shown in the Table 11 below. Information regarding patient gender, pregnancy status, etc. for these specimens was not provided.

Table 11. CDCTrioplex rRT-PCR Test Results for the 20 Zika RNA Positive Natural Serum Specimens

Sample ID	Date Post Onset	Collection Date	Trioplex Zika Result	Trioplex Zika C_T	Trioplex DENV Result	Trioplex CHIKV Result
#01	4	1/12/2016	Positive	28.2	Negative	Negative
#02	4	1/10/2016	Positive	22.5	Negative	Negative
#03	2	1/14/2016	Positive	30.0	Negative	Negative
#04	4	1/1/2016	Positive	33.7	Negative	Negative
#05	3	1/29/2016	Positive	36.0	Negative	Negative
#06	0	1/31/2016	Positive	37.2	Negative	Negative
#07	4	1/17/2016	Positive	34.4	Negative	Negative
#08	4	2/6/2016	Positive	36.9	Negative	Negative
#09	1	1/27/2016	Positive	35.1	Negative	Negative
#10	4	1/24/2016	Positive	35.2	Negative	Negative
#11	4	2/6/2016	Positive	31.2	Negative	Negative
#12	6	1/7/2016	Positive	33.2	Negative	Negative

Sample ID	Date Post Onset	Collection Date	Trioplex Zika Result	Trioplex Zika C_T	Trioplex DENV Result	Trioplex CHIKV Result
#13	4	2/8/2016	Positive	31.2	Negative	Negative
#14	4	1/29/2016	Positive	34.4	Negative	Negative
#15	4	2/5/2016	Positive	36.1	Negative	Negative
#16	1	2/6/2016	Positive	37.2	Negative	Negative
#17	3	1/13/2016	Positive	30.0	Negative	Negative
#18	4	12/26/2015	Positive	33.5	Negative	Negative
#19	2	12/18/2015	Positive	33.3	Negative	Negative
#20	2	12/27/2015	Positive	31.9	Negative	Negative

The 20 Zika virus RNA positive clinical serum samples (0.3 mL each provided) were supplemented with an additional 0.2 mL of pooled normal human serum to yield sufficient volume for testing in the Zika Virus RNA Qualitative Real-Time RT-PCR test. The 36 contrived positive specimens were prepared in individual negative serum matrix, collected from assumed febrile patients that had submitted serum samples for reference lab testing in the Chikungunya (n=16), Dengue (n=14), and West Nile (n=6) RT-PCR tests between February and March 2016. Half of the contrived positive samples were prepared by spiking quantitated cultured (live) Zika virus at the LOD (250 RNA copies/mL, n=18) and the remaining contrived samples were prepared at 2x LOD (500 RNA copies/mL, n=9) and 4x LOD (1,000 RNA copies/mL, n=9). A total of 54 individual normal human serum samples (assumed non- febrile donors) were used as Zika RNA negative specimens. The normal human serum samples were purchased from a supplier, that were drawn in January 2015, and stored frozen at <-60^{o}C until testing. The normal human serum samples were supplied to Boca Biolistics by Tennessee Blood Services and were collected from healthy blood bank donors.

The results of the clinical performance evaluation for serum are shown in the Table 12. Zika virus RNA was not detected in any of the 54 normal human serum samples. Zika virus RNA was detected in all (36 of 36) contrived positives and 17 out of 20 natural clinical positives. The Trioplex Zika C_T values provided for the 3 non-detected natural clinical specimens indicated low to moderate levels of Zika RNA in these specimens (C_Ts= 34.37, 37.18, 37.23).

Table 12. Clinical Evaluation Results for Detecting Zika Virus RNA in Human Serum

Specimen Category	Number Tested	Zika Virus RNA Qualitative Real-Time RT-PCR Test Result	
		Zika Positive	Zika Negative
Natural Zika Positive Clinical Specimens	20	17/20	3/20
Contrived Zika Positive Clinical Specimens (1XLOD)	18	18/18	0/18
Contrived Zika Positive Clinical Specimens (2XLOD)	9	9/9	0/9
Contrived Zika Positive Clinical Specimens (4XLOD)	9	9/9	0/9
Expected Zika Negative Clinical Specimens	54	0/54	54/54
Positive Percent Agreement	94.6% (53/56) 95% CI: 85.4% to 98.2%		
Negative Percent Agreement	100% (54/54) 95% CI: 93.4% to 100%		

The performance characteristics of the Zika Virus RNA Qualitative Real-Time RT-PCR test in urine were evaluated in a clinical study testing a Clinical Accuracy Study Blind Panel consisting of 10 Zika virus RNA positive natural clinical serum specimens, 10 patient-matched urine specimens collected alongside the positive serum specimens, 60 contrived positive urine specimens, and 60 unspiked normal human urine specimens. The serum/urine specimen pairs were de-identified specimens from an endemic area (Dominican Republic, collected April 2016), serum-positive by the Roche (TIB MOLBIOL) LightMix® Modular Zika Virus (Roche assay), and were obtained from a commercial source. Specimen pairs were selected to cover the detection range (in serum) from 25-35 cycles in the Roche assay. Normal (random) urines were collected from healthy donors from a non-endemic area (California, Sept-Dec 2015), and were presumed Zika negative. Approximately 50% were male urine and 50% female urine, and all normal urines were from adults and de-identified. Each normal urine was split into two sets of aliquots, one set was tested unspiked (60 negatives) and one set was tested spiked with cultured Zika virus (30 urines at 2x LOD=1,000 copies/mL and 30 urines at 5x LOD=2,500 copies/mL). All specimens tested in the clinical evaluation were randomized, blind-labeled, and tested using the Zika Virus RNA Qualitative Real-Time RT-PCR test over a total of 6 runs.

The results for the paired serum and urine study are listed in Table 13. All ten of the natural serum specimens were positive for Zika virus detection with the Zika Virus RNA Qualitative Real-Time RT-PCR test. One natural urine specimen had late detection cycle results in both RT-PCR Mixes (Mix 1 C_T = 39.01, Mix 2 C_T = 39.07) and was repeated according to the standard protocol (repeat if C_T is 39.01 to 40). The repeat results for the natural urine specimen were positive for Zika virus in both ZV RT-PCR mixes (Mix 1 C_T = 34.38, Mix 2 C_T = 36.28) and the results were included in the data

summary. A total of eight urines were positive for Zika virus detection with the Zika Virus RNA Qualitative Real-Time RT-PCR. The percent agreement for the serum urine pairs was 80% (8/10).

Table 13. Clinical Evaluation Results for Paired Serum/Urine Study

Study ID	Gender	days after symptoms onset	Specimen Type	LightMix® Modular Zika Virus (C_T)	Zika Virus RNA Qualitative Real-Time RT-PCR		
					ZV Mix1 (C_T)	ZV Mix 2(C_T)	ZV RT-PCR Test Result
1043-TDS-0123	F	1	Serum	28.49	26.18	27.03	Detected
			Urine	not tested	Undetermined	Undetermined	Not Detected
1043-TDS-0127	M	2	Serum	26.31	22.65	23.29	Detected
			Urine	not tested	39.11	38.73	Detected
1043-TDS-0131	F	1	Serum	34.59	31.08	31.30	Detected
			Urine	not tested	32.12	33.07	Detected
1043-TDS-0141	F	3	Serum	31.88	31.88	31.75	Detected
			Urine	not tested	36.35	36.63	Detected
1043-TDS-0190	F	3	Serum	35.04	30.77	30.80	Detected
			Urine	not tested	35.87	35.30	Detected
1043-TDS-0195	M	4	Serum	30.44	29.00	29.68	Detected
			Urine	not tested	32.08	32.79	Detected
1043-TDS-0208	F	1	Serum	25.67	22.71	23.82	Detected
			Urine	not tested	Undetermined	Undetermined	Not Detected
1043-TDS-0211	F	3	Serum	32.54	31.25	31.21	Detected
			Urine	not tested	33.83	34.62	Detected
1043-TDS-0224	F	1	Serum	31.81	35.04	35.47	Detected
			Urine	not tested	35.14	36.96	Detected
1043-TDS-0254	F	2	Serum	35.14	32.85	32.70	Detected
			Urine	not tested	34.38	36.28	Detected

The results for the contrived urine specimens are summarized in Table 14. There was 100% agreement (60/60) for the spiked urines and 100% agreement for the unspiked urines (59/59). Three unspiked urines had indeterminate results (internal control DNA (ICD) out of range) on initial testing and were repeated once according to the standard protocol. Two of the three urines upon repeat had acceptable ICD detection signals and were included in the data summary. One of the three unspiked urines reproduced the ICD out of range results upon repeat and was excluded from the study. One unspiked urine had a late detection cycle result in ZV RT-PCR Mix 2 on initial testing (Mix 2 C_T = 39.79, Mix 1 C_T = undetermined) and was repeated according to the standard protocol (repeat if C_T is between 39.01 and 40). The repeat results for the unspiked urine were undetermined (Zika not detected) for both mixes and the results were included in the data summary.

Table 14. Clinical Evaluation Results for Detecting Zika Virus RNA in Human Urine

Specimen Category	Number Tested	Zika Virus RNA Qualitative Real-Time RT-PCR Test Result	
		Zika Positive	Zika Negative
Contrived Zika Positive Clinical Urine Specimens (2XLOD, 1,000 copies/mL)	30	30/30	0/30
Contrived Zika Positive Clinical Urine Specimens (5XLOD, 2,500 copies/mL)	30	30/30	0/30
Expected Zika Negative Clinical Urine Specimens	60*	0/59	59/59
Positive Percent Agreement		100.0% (60/60) 95% CI: 94.0% to 100.0%	
Negative Percent Agreement		100.0% (59/59) 95% CI: 93.9% to 100.0%	

*1 unspiked normal urine had an indeterminate result (IPC out of range) and was not included in result totals

Performance of the Zika Virus RNA Qualitative Real-Time RT-PCR test was also referenced and reviewed from the publication in Emerging Infectious Diseases (2008) 14: 1232-1239 by Robert S. Lanciotti et al., Genetic and Serologic Properties of Zika Virus Associated with an Epidemic, Yap State, Micronesia, 2007, which used the exact same primers and probes as in the ZV RT-PCR Mix 1 and ZV RT-PCR Mix 2 of the Zika Virus RNA Qualitative Real-Time RT-PCR test. In this publication, the real-time RT-PCR test consists of two unique sets of primers and probes and was performed on all acute-phase serum specimens obtained during the Yap epidemic (n = 157). Seventeen (17) samples were positive, 10 were equivocal, and 130 were negative. The equivocal designation indicates that a particular sample was positive by only 1 of the 2 primers and probe sets or showed C_T >38.5.

References

Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, et al. (2008) Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis 14: 1232–1239

CDC. Zika virus. Atlanta, GA: US Department of Health and Human Services, CDC; 2016. http://www.cdc.gov/zika/index.html