

# **Zika Virus Detection by RT-PCR**

**ARUP Laboratories**

**For use under Emergency Use Authorization only**

**Rx Only**

Instructions for Use

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## **Introduction**

This document describes the use of the ARUP Laboratories Zika Virus Detection by RT-PCR for the *in-vitro* qualitative detection of RNA from Zika Virus in human serum, plasma, or urine (collected alongside a patient matched serum or plasma specimen).

## **Intended Use**

The ARUP Laboratories Zika Virus Detection by RT-PCR is a real-time RT-PCR test intended for the qualitative detection of RNA from the Zika virus in serum, EDTA plasma and urine (paired with a patient-matched serum or plasma sample) from individuals meeting CDC Zika virus clinical criteria (e.g., clinical signs and symptoms associated with Zika virus infection) and/or CDC Zika virus epidemiological criteria (e.g., history of residence in or travel to a geographic region with active Zika transmission at the time of travel, or other epidemiologic criteria for which Zika virus testing may be indicated). Testing is limited to laboratories designated by ARUP Laboratories 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 virus RNA. Zika virus RNA is generally detectable in serum and or urine during the acute phase of infection and up to 14 days following onset of symptoms, if present. Positive results are indicative of current infection. ARUP Laboratories 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 ARUP Laboratories Zika Virus Detection by RT-PCR is intended for use by trained clinical laboratory personnel at ARUP Laboratories who have received specific training on the use of the Zika Virus Detection by RT-PCR. The test is only for use under the Food and Drug Administration's Emergency Use Authorization.

## **Protocol Use Limitations**

Presently ARUP Laboratories in Salt Lake City, Utah is the only designated laboratory authorized to run this protocol.

ARUP Laboratories' Zika Virus Detection by 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.

## **Test Principle**

The ARUP Laboratories Zika Virus Detection by RT-PCR 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 serum, plasma and urine (collected alongside a patient match serum or plasma specimen) from patients presenting with signs and symptoms of the Zika virus infection and/ or with epidemiological risk factors.

Following patient specimen collection and receipt by the laboratory, nucleic acids are isolated and purified from serum, plasma, or urine (collected alongside a patient-matched serum or plasma specimen) using the Chemagic MSM I extraction platform (Perkin Elmer) using a protocol for total nucleic acid extraction. The purified nucleic acid is reverse transcribed and amplified using a 2X custom Multiplex 1-step RT-qPCR master mix (Quanta Bioscience) with thermal cycling and detection on the QuantStudio 12K Flex real-time PCR instrument (Thermo Fisher). In the process, the probe anneals to a specific target sequence located between the forward and reverse primers generating a fluorescent signal which is measured during the annealing phase of the PCR cycle. With each cycle, additional amplicon is produced, increasing the amount of annealed probe and subsequent fluorescence signal. Fluorescence intensity is monitored at each PCR cycle by the QuantStudio 12K Flex.

## **Specimens**

### **Acceptable Specimens**

- Serum, plasma and urine (collected alongside a patient match serum or plasma sample). A minimum volume of 1.0 mL is required for all sample types.
- Specimens collected in heparin are not acceptable due to potential PCR inhibition.

### **Specimens Handling and Storage**

- Separate serum and plasma specimens according to standard laboratory procedures prior to freezing.
- Specimens should be shipped frozen on dry ice or at refrigerated temperature. If shipped at refrigerated temperature, samples should be received within 5 days.

### **Safety Precautions**

- All specimens should be handled as if infectious.
- Proper biosafety precautions, including personal protective equipment, must be used when handling specimen materials.
- Personal Protective Equipment includes: gloves, laboratory coat, face shield.
- All specimen processing must be performed in a biological safety cabinet.
- Disinfectant procedure includes the use of 10% bleach followed by 70% ethanol.

## Equipment and Consumables

### Materials Required to Perform the Test

1. Components and instrumentation:
  - Chemagic MSM I instrument (Cat# CMG-500), Perkin-Elmer.
  - QuantStudio 12K Flex Real-Time PCR System (Cat# 4471087), Thermo Fisher.
  - Chemagic nucleic acid kit (Cat# CMG-1049), Perkin-Elmer.
  - Zika Virus stock – PRVABC59 virus strain (Cat # VR-1843), ATCC.
  - Escherichia coli bacteriophage MS2 (Cat# 15597-B1), ATCC.
  - Zika virus Detection Reagent (Cat# M400562), EliTech.
  - 2X custom Multiplex 1-step RT-qPCR master mix (Cat# 172-5530), Quanta Bio.
  - Axygen 96-well real-time optical plates (Cat# 10032-806), VWR.
  - ABI Prism Optical Adhesive Cover (Cat# 4311971), Thermo Fisher.
  - Molecular grade water.
2. General
  - Pipettes and Pipette tips
  - PCR workstation (dead air box)
  - Microcentrifuge
  - Plate centrifuge
  - Vortex mixer
  - Microfuge tubes
  - Hamilton STARlet
3. Controls
  - Controls are tested with each batch of patient samples. For details see quality control section

## Quality Control

Quality control and quality assurance procedures should be followed to minimize the risk of false-positive and false-negative results. These include using dedicated laboratory space for sample processing and extraction, master mix preparation, sample inoculation and RT-PCR amplification. In addition, the assay controls are used to verify and assess the performance of the assay. Lot numbers and expiration dates are documented on assay worksheets for all test reagents. Expiration dates and storage conditions are observed for all reagents.

### Test Controls

- No Template Control: A “no template”, amplification control (NTC) is included in each RT-PCR run to ensure that all amplification reagents are free of target RNA or amplicon.

This control is used to demonstrate that detection of Zika virus RNA is unlikely to be due to false positive results caused by contamination of amplification reagents.

- **Zika Virus Negative Extraction Control:** A Zika virus extracted negative control, which is a known-negative sample, is included in each run to ensure that all extraction and amplification reagents are free of Zika RNA or amplicon. This control is used to demonstrate that detection of Zika virus RNA is unlikely to be due to false positive results caused by contamination of extraction and amplification reagents. This control is extracted, amplified, and analyzed in the same manner as patient specimens.
- **Zika Virus Positive Extraction Control:** A Zika virus positive control, consisting of Zika virus (Strain: PRVABC59; GenBank Accession # KU501215; origin – Puerto Rico, 2015), is included in each extraction and RT-PCR run to ensure that the extraction and amplification procedure is performed correctly. The Zika virus positive control is used to demonstrate that the anticipated level of sensitivity has been achieved and that test performance is within specifications. This control is extracted, amplified, and analyzed in the same manner as patient specimens.
- **Internal Control:** A non-competitive RNA internal control (IC), consisting of MS2 bacteriophage, is added to each sample during the extraction process to monitor nucleic acid extraction and the reverse transcription and PCR processes for inhibition. Detection of the MS2 RNA in each extracted sample indicates that the lysis and extraction steps were correctly performed. A delayed crossing threshold or negative result for the internal control may indicate either nucleic acid extraction was not effective or that PCR inhibitors are present in the sample. A positive result is valid even if there is no amplification of the internal control.

## **Nucleic Acid Extraction**

### **Notes on Extraction**


- Serum, plasma or urine (collected alongside a patient match serum or plasma sample) are extracted using the chemagic MSM I.
- Processes for maintaining sample identity, as outlined in lab specific procedures, must be used throughout the extraction process. This includes but is not limited to the use of tray maps and visual sample checks.
- Extraction uses the following volumes:
  - Specimen input volume: 200  $\mu$ L
  - Elution volume: 80  $\mu$ L
- Working lysis buffer should be made immediately before addition to the sample block.

### **Starting the Instrument:**

1. Allow samples and controls to come to room temperature. Lyophilized controls should be allowed to re-hydrate  $\geq$  15 minutes.

- Manual pipetting/manipulation of specimens or controls must be done in a biosafety hood. Clean the hood with 10% sodium hypochlorite (bleach) followed by 70% ethanol prior to and after each use.
2. Complete pre-extraction checklist of daily, weekly, and monthly maintenance.
  3. Check the volume of all reagents and wash buffers to make sure you have enough for a full extraction. Do not refill reagents or wash buffer until the volume is below the minimum fill line.
  4. Wipe the instrument down with 70% EtOH.
  5. Check to see that all reagent and buffer bottles are attached to the chemagic MSM I.
  6. Check tubing to ensure there are no pinched or kinked lines.
  7. Ensure the waste container is below the maximum fill line.
  8. Power on the chemagen MSM I instrument, followed by the computer.



9. Open the chemagic MSMI software:  and select the appropriate username and password.
10. Select “Check Manifolds 1-6 for 96 dispenser.che” from the “Select Protocol” dialog. Press “OK” followed by the green “Start” button. Each dispenser manifold will dispense a small amount of reagent.
  - Watch to see that all 12 channels dispense properly.
  - At the end of the manifold check, the program will ask if a run log is needed. Unless there was an interruption in the protocol, select “No.”

*Note: When a reagent is re-attached to the chemagic MSMI manifold the “Prime Manifolds 1-6.che” program will need to be run.*

  - Twice as much binding buffer is used compared to the other reagents. When necessary, refill the binding buffer bottle and run the Prime Manifold 2 for 96 well.che protocol.
11. Inoculate a 96 deep-well-plate with patient samples and controls, using a new pipette tip for each sample/control. Double-check the orientation of the 96 deep-well plate before inoculation to ensure that sample identity is maintained.

#### Lab Specific Instructions for Preparing the Working Lysis Buffer

- Working lysis buffer should be made immediately prior to adding to the sample block containing the patient samples and controls. It should be added to the block within 10 minutes of being made.
- 467  $\mu$ L of working lysis buffer is added to each well of the sample block.

Table 1. Lysis buffer components and reagent preparation.

<b>Lysis Buffer Component, Reagent Preparation:</b>	
Proteinase K	Add 5.5 mL of molecular grade water to reconstitute
Poly(A) RNA	Add 440 µL of Poly(A) RNA Buffer to the Poly(A) RNA tube
Proteinase K and Poly(A) RNA are stable 4 weeks at 4°C.	

Table 2. Working Lysis buffer preparation.

<b>Working Lysis Buffer Preparation</b>	
<b>Reagent</b>	<b>µL per sample</b>
Lysis Buffer 1 (2X Blood)	450
Proteinase K	10
Poly(A) RNA	4
IC-ms2	1.2
IC-AB	0.872
SPC (added directly to well)	25

Loading the Instrument:

1. Load plates onto the chemagic MSM I with the A1 position located in the upper right hand corner.
  - All disposable plates and blocks must be labeled, oriented correctly, placed in the correct position, and seated flat against the deck to ensure that sample identity is maintained. Labeling deep-well blocks used for wash steps helps troubleshooting processes if needed. Mark well A1 for proper orientation. See Figure 1.



Figure 1. Showing correct plate orientation on the MSM I.



2. Open the chemagic MSM I plate manifold for loading. And load reagents/labware as follows:

Table 3. Reagent/Labware deck position.

Deck Position	Reagent/Labware	Additional Information
1	Disposable tip covers	Unwrap before placing on deck
2	Low-profile plate containing aliquoted magnetic beads.	<ol style="list-style-type: none"> <li>1. Re-suspend the magnetic beads after ensuring the cap is fitted tightly to the top of the bottle, by shaking them vigorously for 30 seconds.</li> <li>2. Pipette 150 <math>\mu</math>L of beads into each well of a low-profile plate.</li> </ol>
3	<p>Deep-well plate with patient sample and working lysis buffer.</p> <p><i>The working lysis buffer degrades once made and must be added to the patient samples and controls within 10</i></p>	<ol style="list-style-type: none"> <li>3. Prepare the working lysis buffer. Vortex well.</li> <li>4. Pipette 467 <math>\mu</math>L of working lysis buffer into each well with patient sample. Use a</li> </ol>

	<i>minutes of preparation.</i>	separate pipette tip for each well. 5. Ensure well A1 is in the upper left hand corner when facing the chemagic.
4	Empty deep-well plate.	chemagic dispenser unit will fill with 500uL of Wash Buffer 3
5	Empty deep-well plate.	chemagic dispenser unit will fill with 500uL of Wash Buffer 4
6	Empty deep-well plate.	chemagic dispenser unit will fill with 500uL of Wash Buffer 5
7	Empty deep-well plate.	chemagic dispenser unit will fill with 500uL of water
8	Elution plate containing aliquoted elution buffer.	1. Pipette the appropriate amount of elution buffer 6 into each well of the final elution plate. 2. The elution plate must be labeled with applicable run information.
9	Empty deep-well plate.	Will be used for discarded tip covers

See Figure 2 for an illustration of the deck position.

Figure 2. Illustration showing the deck positions for each plate.



3. Close the cover of the chemagic MSM I plate manifold.

### Initializing the Extraction

1. Select “Extraction Qiagen Elution” from the drop down menu found next to “Select Protocol”.

2. You will be prompted to check if enough buffer is available, and to check that the buffers are correctly attached to the manifold. Once these conditions are met select “OK”.
3. A map will be presented for prefilling by the chemagic Dispenser units. Most runs should be full blocks. Ensure that “96” is selected under the “Select Number of Samples” dialog and press “OK”.
4. Press the green “Start” button using the mouse.
5. Ensure the 96 head successfully picks up the tip covers before walking away from the instrument. This will allow the run to be aborted before the sample block is compromised in the event of a crash or issue.
6. The extraction program should now be running on the chemagic MSM I. It should take approximately 70 minutes to complete. Check the “Remaining Time” field for an estimation of how much time remains.
7. Open the chemagic MSM I manifold once the run is complete.
8. Remove the elution box carefully from Position #8. It is ready for downstream processing.
9. Clean up by discarding the tip covers from Position #9 into a biohazard container. The deep-well-plate at that position may be reused. Discard all plastics and buffers they contain from Positions #1 through 7.  
*Note: Some of the wells are full of reagents, so be careful when disposing of plastics and make sure the buffers do not splash out of the garbage.*
10. Wipe any reagent spills from the chemagic MSM I manifold using 70% ethanol.
11. Close the chemagic MSM I manifold.
12. At the end of the extraction, the program will ask if a run log is needed. Unless there was an interruption in the protocol, select “No.”
13. Close the software.
14. Turn off the computer followed by the instrument if it will not be in use

## Real Time RT-PCR Test

### Quality Control

- Internal Amplification Control
  - To ensure the absence of non-specific PCR inhibition in a sample, an internal control (IC) RNA is added to the Chemagic Lysis Buffer prior to nucleic acid extraction. A sample can be interpreted as negative only if the IC Ct 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  $\pm 3$  Ct from the mean of the internal control Ct values obtained from 20 replicates (data generated from negative controls over multiple runs). The acceptance ranges will be generated for each lot of Internal Control.

- Controls Use Frequency
  - A Zika virus positive control, which consists of a cultured (live) Zika 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 Controls (and NTC) should be listed as “undetermined” for the RT-PCR reaction. If the Negative Control is positive (Ct value < 50), 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 test run criteria are met.
  - If the Zika virus positive control Ct value is within the acceptable range, the Positive Control is considered valid and acceptable. If the Zika virus positive Control Ct value is above or below the acceptable range, the Positive Control is considered invalid and unacceptable. All patient specimens must be re-tested.
- 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 Detected results.

### **Amplification**

Fill out the amplification worksheet. This can be done by hand or by opening the workbook on the computer and entering the patient names and corresponding PCR ID locations from the extraction tray map. To do this:

1. Open the Excel workbook generated by the MID Extraction Traymap Creator. This workbook is found on the network. It will be named with a date and corresponding run letter (i.e., 031114B).
2. Make manual modifications to the worksheet (i.e., repeat samples).
3. Press the “Done” button in the upper right corner when worksheet is complete. This creates a worksheet for the Hamilton STARlet, and also saves the import spreadsheet for the QuantStudio at the aforementioned address.

NOTE: A prompt will ask for a filename in order to save the worksheet, after which another prompt will let you know the import was successful.

4. Print this worksheet.

NOTE: Once this file has been saved, the chosen filename cannot be used again. If changes must be made to the downloaded file, it will need to be re-named something slightly different.

### Master Mix Preparation

- The Master Mix must be made in a dedicated Area 1 with dedicated pipettes using aerosol resistant pipette tips and sterile tubes.
  - Keep reagents on cold block (or ice) while preparing Master Mix to prevent premature cDNA synthesis.
  - Record lot numbers on the appropriate master mix form.
1. Fill out the Reagent Worksheet contained in the MasterMix Workbook. The Hamilton STARlet worksheet will automatically determine how many reactions to prepare (1 dead volume per 10 samples).
  2. Make reaction mix by adding each reagent to a sterile microcentrifuge tube labeled “Zika” (volume of each reagent calculated in previous step).
  3. Mix by vortexing and spin to remove solution off the sides of the tube.
  4. 40 µL of the Master Mix will be pipetted into each well of a 96-well reaction plate that has been placed in a **cold block**. (Refer to Inoculation below).

### Master Mix Calculation

**Table 4. Master mix calculation**

	<b>Per Rxn.</b>
Zika Detection Reagent (20X)	2.5 µL
custom Multiplex 1-Step RT-qPCR Mix, 2.0X	25 µL
qScript RT enzyme mix, 20X	2.5 µL
Water	10 µL
<b>Total Master Mix per sample</b>	40 µL
<b>Template</b>	10 µL
<b>Reaction total</b>	50 µL

### Inoculation

- Sample inoculation should be performed in the dedicated laboratory space for master mix inoculation.
- Sample inoculation can be done using the Hamilton STARlet (liquid handler) or by manual inoculation.

- 10 µl of eluted RNA is inoculated into the master mix.
1. If using the Hamilton STARlet to perform the Master Mix and elution template inoculation, refer to the Hamilton STARlet Interface and Instrument Operating Procedure. If manually performing the inoculation proceed to step 2.
  2. Using a 5-50 µL pipettor with the corresponding aerosol resistant tips, load 10 µL of each patient sample nucleic acid into the appropriate well of a 96-well reaction plate (containing 40 µL Master Mix).
  3. Using a 5-50 µL pipettor with the corresponding aerosol resistant tips, load 10 µL of each control into the appropriate well.
  4. Seal plate with optical Clear Adhesive Cover.

#### Creating a Plate Document from the Template

- Amplification and Detection should be performed in the Area 3 which is dedicated laboratory space for PCR amplification.
1. Open the QuantStudio software.
  2. Select **Create from Template** found in the **Experiment** box from the **Main Menu**. The **Open** dialog box appears.
  3. Select the **MID Protocol 1** Template file.  
**NOTE:** It is critical that the correct template be selected. If the incorrect template is chosen, the wrong cycling protocol will be run and the samples may need to be re-amplified.
  4. Ensure that once the template opens under the **Experiment Properties** tab, the **Block** is set at **96-well**, **Experiment** setup is set to **Standard Curve**, **Reagent** setup is **Other**, **Instrument Properties** is set at **Standard**, and the **checkbox** for **Include Melt Curve** is checked. These settings should be the default setting associated with the Protocol 1 template file. Ensure the Passive Reference setting is set to **None**.
  5. Type a name for the plate using the current date (ex. 091212A) in the experiment name box near the top of the screen.
  6. Import the sample names to the plate document by clicking the **Import** button at the top of the screen and navigate to the network location containing the plate document for that run.
  7. Select the previously created run and click **Start Import**. Then click **OK**.
  8. Detectors for each well should have automatically been set during the import; if not, select all the wells in use and apply the detectors (Zika [FAM]; IC [AP525]) under the **Assign** tab.
  9. Check that the plate document contains the following cycling conditions in the **Graphical** view under the **Run Method** tab. The following protocol is necessary for hybridization based PCR chemistry.

### **Hold Stage**

10 min; 50°C (RT step)  
2 min; 95°C (activation of Taq polymerase)

### **PCR Stage (50 cycles)**

5 sec; 95°C (denaturation)  
20 sec; 56°C (annealing)  
20 sec; 76°C (extension)

### **Melt Curve Stage**

15 sec; 95°C  
15 sec; 45°C  
15 sec; 80°C

Check that the temperature ramp rates are all set to 1.7°C/s for all ramps, except for the ramps during the melt, which should be set to 1.6°C/s for the ramp from 95°C to 45°C and 0.06°C/s from 45°C to 80°C. Finally, check that data is being collected at only the annealing step 56°C and during the final temperature ramp (between 45°C and 80°C). Data collection is indicated by a small green camera icon under the temperature in question.

### Saving the Plate Document

1. Save the run by clicking the **Save** button in the file menu. The file name will already be entered.
2. Select **Test Document Single files (\*.eds)** from the **Files of type** and select a location for the software to place the new file.
3. Click **Save**. The software saves the plate document as a new file containing detector and method settings identical to those of the template plate document.

### Starting the Assay

1. Check that the instrument tray is in the ready position (outside of the instrument) and does not contain a plate (if the tray is within the instrument, open the instrument). If the tray is not out, click on the red **Eject** button on the instrument touch screen.
2. Seal the optical tape carefully using the supplied tool.
3. Centrifuge the plate briefly to collect the reactions at the bottom of the wells and to eliminate any air bubbles that may be present.

4. Place the plate into the instrument tray.

NOTE: The A1 position is located in the top-left side of the instrument.

5. Click the red Eject button on the instrument touch screen. The instrument tray rotates to the IN position.

6. Click the **RUN** tab. Click the green **Start Run** button and then the Instrument name. The run will start. The results of the run can be analyzed immediately following its completion.

NOTE: Before starting the run, the instrument may pause (up to 15 minutes) to heat the heated cover to the appropriate temperature.

### General Analysis Overview

1. To begin analysis, select all appropriate assay wells in the plate grid. The software outlines the selected wells with a black line.
  2. Under the **Define** tab (Setup menu), check that the Passive Reference is set at 'none'.
  3. From the **toolbar**, click the **Analyze** icon.
  4. Before determining cycle threshold values for individual wells, the baseline and threshold values must be set for all reporter dyes present on the plate. Adjust baseline and threshold values in the **Amplification Plot** tab. Select the detector from the drop down menu. Uncheck the **Auto Threshold** and **Auto Baseline** boxes. Ensure the **Show Threshold** and **Baseline Start** boxes are checked. The software displays the results of the sequence detection run in an amplification plot of normalized reporter fluorescence (Rn) versus threshold cycle (CT). Follow steps 5 and 6 to adjust the settings for each channel.
  5. Baseline: For all assays, set the lower limit of the baseline at cycle 3 for all detectors and assays.
    - Qualitative assays (target channel): Set the upper limit of the baseline at 4 cycles before the earliest CT.
    - All assays (internal control (IC) channel): Set the upper limit of the baseline at 4 cycles before the earliest CT.
  6. Threshold: Set the threshold for each channel above the background as defined by the negative controls (for the target) or water (for IC) and below the plateau and linear regions of the curves present. The geometric phase of the amplification curve is the appropriate location for the threshold.
  7. Click the **Analyze** icon to update the analysis settings.
- Note: The QuantStudio v1.1 software does not update the analysis in real-time. You must click the Analyze icon each time you change baseline or threshold settings.*
8. Visually inspect the Target Channel for each patient and control (see **Target Channel Analysis** section below)
  9. Visually inspect the target dissociation curves for each patient and control (see **Target Dissociation Curve Analysis** section below).
  10. Visually inspect the IC channel for each patient and control (see **IC Analysis** section below).



11. Once the baseline and threshold have been determined for all channels, navigate to the “**Export**” tab. Update the **Export File Location** and **Export File Name** fields. The rest of the settings should be left as default. Export the data using the export button.
12. Make notations as needed on the printed report. Initial and date all handwritten notes.

### **Target Channel(s) Analysis**

All patients and controls should be individually analyzed in the target channel(s). The entire run should also be viewed in order to identify aberrant trends or patterns.

1. Examine the target channel(s) amplification curves for the control(s).
2. The positive control(s) should exhibit amplification and produce a crossing threshold  $\leq 36$ .
3. Record crossing threshold values for the positive controls for quality control purposes.
4. The negative control should exhibit no crossing threshold in the target channel(s).
5. Examine the target channel(s) amplification curves for the patient samples.
6. Ensure that when a sample is assigned a crossing threshold, (as calculated by the SDS software) it also has a curve displayed on the graph with a definite crossing threshold.

*Note: Never go by the number alone. Always look at the graph.*

### **Target Dissociation Curve Analysis**

Each assay is designed to detect a specified sequence in the assay target. The software plots the first derivative of reporter fluorescence against temperature during the melting analysis. There is the possibility that a patient may contain an isolate that contains a polymorphism within the probe-binding sequence. In this case, the corresponding probe may not bind to the amplicon with a normal efficiency. This results in a decreased fluorescent signal and decreased assay sensitivity. However, these amplicons may still generate signal during the melting analysis. For this reason, it is important to examine the dissociation curves for all patient samples, including negatives. Melt analysis is not used to assign a positive or negative result but may aid in surveillance and identification of sequence polymorphisms within the probe binding region.

1. Click the **Dissociation Curve** icon. The software plots the first derivative of reporter fluorescence against temperature during the melting analysis
2. Examine the Dissociation Curves for all target channel(s) for all patients and control(s).
3. A true negative sample should display no melting peak. Any samples that are negative on the amplification plot but display a melting peak on the dissociation plot should be considered for repeat testing.
4. Examine the dissociation curve of each positive sample. All samples with amplification curves present should have a characteristic melt. Consult the Medical Director if a sample

presents with an uncharacteristic melt.

### **IC Analysis**

1. Select the IC channel, and inspect the internal control amplification curve for each well. The internal control should exhibit amplification and produce a crossing threshold  $\leq 36$ .
2. Always ensure that when a sample is assigned a crossing threshold (calculated by the SDS software) it also has a curve displayed on the graph with a definite crossing threshold.

*Note: Never go by the number alone. Always look at the graph.*

### **Interpreting Test Results**

- All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted.
- A Ct will be assigned for each amplification reaction occurring in a well. Ensure that when a sample is assigned a Ct value, it also has an amplification curve displayed on the graph with a definite crossing threshold. The technical supervisor or medical director may be consulted to review any samples that register a Ct, but lack a clear amplification curve.

### **Interpretation of assay controls**

- The Negative control and No Template Control (NTC)
  - The negative control and NTC should yield a negative result in the Zika channel to be valid. If the negative control or NTC produce a Ct in the Zika channel, this control is invalid and suggests possible contamination. Positive specimen results should be repeated. Negative specimen results may be reported if all other test run criteria are met.
  - In addition, the negative control should yield a positive result in the IC channel with a Ct  $\leq 36$  for a Negative control to be valid. If the Negative control does not produce a Ct in the Zika channel and the Ct for the IC is within the acceptable range, the Negative control is considered valid. If the Negative control does not produce a Ct in the Zika channel but the IC Ct  $> 36$ , the result for the Negative control is invalid, positive specimen results must be repeated but the result of the negative specimens may be reported if all other test run criteria are met.
  - The NTC should not yield a Ct in the IC channel.
- The Zika Positive control
  - The Zika Positive control should yield a positive result in the Zika channel with a Ct  $\leq 36$  to be valid. If Zika positive control fails to amplify or the Ct  $\geq 36$ , the run is considered invalid and should be repeated.
  - If the positive control produces a Ct in the Zika channel within the acceptable range, the positive control is considered valid.

### **Interpretation of Patient Specimens**

- Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable.
- Positive Zika virus RNA results must be reported to the appropriate Public Health authorities.
- If the Zika virus results of a patient specimen are listed as “undetermined” and the IC Ct is  $\leq 36$ , the result is reported as “Not Detected”.
- If the Zika virus results of a patient specimen are listed as “undetermined” and the IC Ct is  $> 36$ , then sample inhibition is suspected. Repeat testing to confirm. On retesting, if the IC Ct is  $> 36$ , the result is reported as “Inhibited”. If the IC Ct  $\leq 36$ , the result is reported at “Not Detected”.
- If the Zika virus results of a patient specimen are  $\leq 38$ , the result is reported as “Detected” regardless of the IC Ct value.
- If the Zika virus results of a patient specimen are  $> 38$ , then retesting is required for confirmation. The sample should be re-extracted and amplified in duplicate. If either duplicate produces an amplification curve and Ct  $< 50$ , the result is reported as “Detected” regardless of the IC Ct value. If neither duplicate produces an amplification curve and Ct, the result is reported as “Not Detected”.

Table 5: Results interpretation of patient samples

Ct Result	Interpretation		Action	Confirmation Testing Action
	Zika	IC		
Zika Ct $\leq 38$ IC Ct $\leq 36$	+	+	Report as Zika Virus Detected	
Zika Ct $\leq 38$ IC Ct $> 36$	+	-	Report as Zika Virus Detected	
Zika Ct $> 50$ IC Ct $\leq 36$	-	+	Report as Zika Virus Not Detected	
Zika Ct $> 38$ IC Ct $\leq 36 \geq$	+	+/-	Low positive, repeat extraction and amplification to confirm	If Zika Ct $< 50$ in either replicate, report as Zika Virus Detected, If Zika Ct $> 50$ in both replicates, report as Zika Virus Not Detected
Zika Ct $> 50$ IC Ct $> 36$	-	-	Sample inhibition, repeat extraction and amplification	If Zika Ct $> 50$ and IC Ct $> 36$ , report as Inhibited.

### **Test Limitations**

1. The test is for In vitro diagnostic use under the FDA Emergency Use Authorization (EUA) and is limited to laboratories designated by ARUP Laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests.

2. Interpretation of results must account for the possibility of false-negative and false-positive results. False positive results can be caused by:
  - a. Contamination with previously amplified nucleic acids
  - b. Carry-over from samples processed in the same batch
3. False negative results can be caused by:
  - a. Poor sample collection
  - b. Degradation of viral nucleic acids during shipping or storage
  - c. Specimen collection prior to symptom onset
  - d. Specimen collection after the nucleic acid can no longer be found in the patient specimen
  - e. Failure to follow assay procedures
  - f. Failure to use the authorized extraction method or platform
4. Negative results do not preclude infection and should not be used as the sole basis of patient treatment/management decisions. All results should be interpreted by a trained professional in conjunction with a review of the patient's history, clinical signs, and symptoms.
5. A false negative result may occur when the infecting organisms contains sequence polymorphisms under the PCR primers or probe.
6. Proper collection, storage, and transport of samples are critical for achieving accurate results.
7. Extraction of nucleic acid from clinical specimens must be performed with the specified extraction methods listed in this procedure. Extraction methods not listed in this procedure have not been evaluated for use with this assay.
8. All specimens should be handled as if they are infectious. Proper biosafety precautions must be used.
9. This test has not been validated with specimens other than serum, plasma and urine.
10. **A patient matched serum specimen is required for serological follow up testing of negative results, per the CDC testing algorithm. (Found at <http://www.cdc.gov/zika/index.html>).**
11. This test cannot rule out diseases caused by other bacterial or viral pathogens

## 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.

### LOD Studies

*Limit of Detection (LoD):* Limit of detection studies were done using a Zika virus positive culture isolate (Strain: PRVABC59; GenBank Accession # KU501215; origin – Puerto Rico, 2015) provided by the CDC Arboviral Disease Branch using the entire test system. This material was quantitated by generating a standard curve using log<sub>10</sub> dilutions of RNA transcripts. The quantity of the stock PRVABC59 culture was determined to be 3.4x10<sup>7</sup> copies/reaction (1.3x10<sup>9</sup> copies/mL). The material was then spiked into negative serum, plasma and urine pools. Five-fold dilution series were made and extracted in triplicate. Each extract was amplified in duplicate for a total of 6 replicates at each dilution level. The lowest concentration detected in all 6 replicates was established as the tentative LOD (Tables 3 -5). The LOD was confirmed by extracting and amplifying 20 replicates with a detection rate of 95% (19/20) and can be found in Tables 6 – 8. The LOD for serum, plasma and urine is 160 copies/mL.

**Table 6. Summary of LOD range finding results in serum using Zika strain PRVABC-59.**

Copies/rxn	Copies/mL	Rep 1 Ct	Rep 2 Ct	Rep 3 Ct	Rep 4 Ct	Rep 5 Ct	Rep 6 Ct	Call Rate
500	20,000	30.35	30.53	30.54	30.63	30.50	30.32	6/6
100	4,000	32.52	32.53	32.26	32.19	32.40	32.36	6/6
20	800	33.00	34.39	34.10	35.39	35.15	34.60	6/6
4	160	37.42	36.59	36.98	37.24	36.77	37.12	6/6
0.8	32	Neg	Neg	Neg	40.01	39.36	Neg	2/6
0.2	6	Neg	Neg	Neg	Neg	39.66	Neg	1/6
0.04	2	Neg	Neg	Neg	Neg	Neg	Neg	0/6

**Table 7. Summary of LOD range finding results in plasma using Zika strain PRVABC-59**

Copies/rxn	Copies/mL	Rep 1 Ct	Rep 2 Ct	Rep 3 Ct	Rep 4 Ct	Rep 5 Ct	Rep 6 Ct	Call Rate
500	20,000	30.92	30.53	30.44	30.81	31.14	30.91	6/6
100	4,000	33.17	33.01	32.64	33.22	32.93	32.79	6/6
20	800	35.87	36.16	35.99	36.42	36.08	35.77	6/6
<b>4</b>	<b>160</b>	<b>38.61</b>	<b>38.43</b>	<b>39.38</b>	<b>38.92</b>	<b>38.36</b>	<b>37.63</b>	<b>6/6</b>
0.8	32	Neg	41.05	39.63	Neg	Neg	41.06	3/6
0.2	6	40.57	Neg	40.89	Neg	41.01	41.70	4/6
0.04	2	Neg	Neg	Neg	Neg	Neg	Neg	0/6

**Table 8. Summary of LOD range finding results in urine using Zika strain PRVABC-59**

Copies/rxn	Copies/mL	Rep 1 Ct	Rep 2 Ct	Rep 3 Ct	Rep 4 Ct	Rep 5 Ct	Rep 6 Ct	Call Rate
500	20,000	31.29	33.90	31.77	32.89	32.98	34.41	6/6
100	4,000	33.97	34.68	36.42	33.39	34.66	34.11	6/6
<b>20</b>	<b>800</b>	<b>37.67</b>	<b>36.11</b>	<b>38.41</b>	<b>37.67</b>	<b>39.50</b>	<b>38.56</b>	<b>6/6</b>
4	160	42.04	40.26	41.46	41.56	Neg	42.64	5/6
0.8	32	43.02	Neg	Neg	Neg	42.17	42.44	3/6

0.2	6	Neg	41.19	Neg	43.72	42.00	Neg	3/6
0.04	2	Neg	Neg	Neg	Neg	Neg	Neg	0/6

**Table 9. Limit of detection confirmation in serum using Zika strain PRVBC 59.**

Serum - 800 copies/mL (20 copies/rxn)			Serum - 160 copies/mL (4 copies/rxn)			Serum - 32 copies/mL (0.8 copies/rxn)		
Specimen	Zika CT	IC CT	Specimen	Zika CT	IC CT	Specimen	Zika CT	IC CT
1	36.42	28.97	1	37.43	28.00	1	40.25	28.25
2	37.00	27.94	2	38.65	28.10	2	39.94	28.47
3	36.29	28.00	3	39.31	28.07	3	40.25	28.25
4	36.38	27.97	4	38.71	28.06	4	39.97	28.24
5	36.34	27.97	5	37.92	27.75	5	Neg	28.05
6	37.19	27.86	6	38.82	27.71	6	Neg	28.17
7	37.76	28.60	7	38.60	27.79	7	Neg	28.29
8	36.92	28.69	8	39.09	27.81	8	Neg	28.15
9	36.62	27.93	9	40.06	27.81	9	Neg	28.56
10	36.95	27.92	10	39.26	27.94	10	39.94	27.97
11	36.35	28.03	11	38.74	28.02	11	Neg	28.08
12	35.93	27.95	12	38.50	27.97	12	Neg	28.21
13	36.35	28.44	13	39.38	27.95	13	Neg	28.30
14	36.22	27.74	14	40.33	28.03	14	Neg	28.17
15	36.89	27.83	15	39.07	28.26	15	Neg	28.50
16	36.67	27.76	16	39.15	27.88	16	Neg	28.03
17	36.97	28.06	17	39.34	28.13	17	39.00	27.73
18	36.72	28.08	18	38.35	28.04	18	Neg	27.97
19	36.21	28.14	19	39.20	28.08	19	Neg	27.97
20	36.73	28.20	20	40.60	28.17	20	Neg	27.95
Mean	36.65	28.10	Mean	39.03	27.98	Mean	39.89	28.17
StDev	0.42	0.33	StDev	0.75	0.15	StDev	0.46	0.21
%C.V.	1.15	1.16	%C.V.	1.92	0.53	%C.V.	1.15	0.73
Result	20/20		Result	20/20		Result	6/20	

**Table 10. Limit of detection confirmation in plasma using Zika strain PRVBC 59.**

Plasma - 800 copies/mL (20 copies/rxn)			Plasma - 160 copies/mL (4 copies/rxn)			Plasma - 32 copies/mL (0.8 copies/rxn)		
Specimen	Zika CT	IC CT	Specimen	Zika CT	IC CT	Specimen	Zika CT	IC CT

1	35.43	28.49	1	39.90	27.65	1	Neg	27.94
2	35.28	27.82	2	36.80	27.68	2	Neg	27.83
3	35.14	27.49	3	37.59	27.68	3	38.28	27.98
4	34.92	27.68	4	38.76	27.53	4	Neg	27.79
5	35.63	27.66	5	36.62	28.08	5	Neg	27.79
6	35.75	27.47	6	37.85	27.87	6	Neg	27.74
7	35.31	27.81	7	40.71	27.78	7	Neg	27.77
8	35.46	27.59	8	38.43	27.83	8	Neg	27.75
9	35.53	27.59	9	38.04	27.98	9	Neg	28.45
10	35.54	27.66	10	39.51	27.71	10	Neg	28.14
11	35.67	27.66	11	37.34	27.91	11	39.05	27.95
12	35.62	27.54	12	38.75	27.80	12	39.07	28.07
13	35.75	28.43	13	36.79	27.60	13	Neg	28.07
14	35.23	27.77	14	38.66	27.68	14	Neg	27.97
15	36.21	27.73	15	37.51	27.64	15	Neg	28.26
16	35.31	27.68	16	37.76	27.58	16	38.62	27.75
17	36.57	27.71	17	37.55	28.29	17	Neg	28.01
18	35.93	27.61	18	37.96	28.03	18	40.42	27.98
19	35.66	27.77	19	37.39	27.64	19	Neg	27.76
20	34.87	27.48	20	38.59	28.00	20	39.36	27.68
Mean	35.54	27.73	Mean	38.13	27.80	Mean	39.13	27.93
StDev	0.40	0.27	StDev	1.06	0.20	StDev	0.74	0.20
%C.V.	1.13	0.97	%C.V.	2.78	0.71	%C.V.	1.89	0.71
Result	20/20		Result	20/20		Result	6/20	

**Table 11. Limit of detection confirmation in urine using Zika strain PRVBC 59.**

Urine - 800 copies/mL (20 copies/rxn)			Urine - 160 copies/mL (4 copies/rxn)			Urine - 32 copies/mL (0.8 copies/rxn)		
Specimen	Zika CT	IC CT	Specimen	Zika CT	IC CT	Specimen	Zika CT	IC CT
1	34.75	31.77	1	38.83	31.41	1	Neg	31.50
2	35.25	31.43	2	37.88	31.25	2	41.29	31.43
3	35.58	31.70	3	36.52	31.43	3	39.61	31.33
4	35.81	31.43	4	38.43	31.31	4	40.90	31.52
5	35.45	31.69	5	34.92	31.23	5	39.04	31.53
6	35.82	31.51	6	37.04	31.66	6	38.26	31.59
7	36.60	32.30	7	38.26	31.84	7	41.22	31.42
8	35.15	31.49	8	40.62	31.83	8	39.20	31.44
9	35.93	31.61	9	38.93	32.11	9	Neg	31.72
10	36.95	32.71	10	38.89	31.60	10	38.82	31.49

11	40.27	33.00	11	38.86	31.53	11	38.63	31.35
12	35.22	31.35	12	Neg	31.96	12	40.59	31.63
13	35.16	31.77	13	37.09	31.55	13	44.98	31.84
14	34.51	31.26	14	39.21	31.47	14	Neg	31.98
15	35.42	31.36	15	40.21	32.60	15	39.95	31.37
16	34.99	31.49	16	39.53	31.63	16	Neg	31.67
17	41.05	32.84	17	38.60	31.33	17	39.52	31.94
18	36.63	31.98	18	38.51	31.65	18	Neg	32.13
19	39.21	32.91	19	39.25	32.25	19	Neg	33.07
20	37.11	31.90	20	39.38	31.63	20	Neg	32.01
Mean	36.34	31.87	Mean	38.47	31.66	Mean	40.15	31.70
StDev	1.82	0.56	StDev	1.34	0.35	StDev	1.76	0.40
%C.V.	5.01	1.77	%C.V.	3.48	1.11	%C.V.	4.38	1.26
Result	20/20		Result	19/20		Result	13/20	

### **Analytical Sensitivity – FDA Reference Materials**

An analytical study was performed using reference materials (S1 and S2) and a standard protocol provided by the FDA, which includes a LOD range finding study and a confirmatory LOD study, to evaluate the analytical sensitivity of the ARUP Laboratories Zika Virus Detection by RT-PCR assay. The results are presented in **Table 12** below.

**Table 12. Summary of LOD confirmation results using FDA reference materials**

Reference Materials	Specimen Type	Confirmed LOD* in RNA NAAT Detectable Units/mL
S1	Plasma	3000
S1	Urine	3300
S2	Plasma	1650
S2	Urine	4500

\*Study performed according to an FDA issued protocol

### **Analytical Reactivity/Inclusivity**

In addition to testing Zika strain: PRVABC59, reactivity of the ARUP Laboratories Zika Virus Detection by RT-PCR was evaluated by wet testing the additional isolate of Zika virus (Table 13).

**Table 13. Analytical inclusivity, wet-tested**

Zika Virus Isolate	Source/Sample Type*	Concentration	Ct
MR 766	ATCC VR-1838/Organism	800 copies/mL	39.43
H/PAN/2015/CDC-259359	BEI NR-50219/Organism	500 copies/mL	35.81
FLR	BEI NR-50183/Organism	500 copies/mL	37.47



H/PAN/2016/BEI-259634	BEI NR-50210/Organism	500 copies/mL	37.40
MEX 2-81	BEI NR-50280/Organism	500 copies/mL	37.55
DakArD	ZeptoMetrix 0810522CF/Organism	500 copies/mL	40.67
IbH 30656	ATCC VR-1839/Organism	500 copies/mL	39.31
H/PAN/2015/CDC-259364	BEI NR-50221/Organism	500 copies/mL	39.06
H/PAN/2015/CDC-259249	BEI NR-50220/Organism	500 copies/mL	37.81

**Table 14. *In silico* analysis of Zika virus strains with percent match for each oligo**

GenBank	Strain/Isolate	Year	ZKV3-L1 (For)	ZKV3-FAM1 (Probe)	ZKV3-E1 (Rev)
KU501215	PRVABC59	2015	100%	100%	100%
AY632535	MR 766	1947	100%	100%	94%
KU820897	FLR	2015	100%	100%	100%
KX087102	C1/C2	2051	100%	100%	100%
HQ234500	IbH_30656	1968	100%	100%	100%
KU963574	IbH-30656_SM21V1-V3	1968	100%	100%	100%
KU955592	A.taylori-tc/SEN/1984/41662-DAK	1984	100%	100%	100%
KU758877	17271	2015	100%	100%	100%
KX280026	Paraiba_01	2015	100%	100%	100%
KX262887	103451	2016	100%	100%	100%
KX253996	ZKC	2016	100%	100%	100%
KX247646	COL/UF-1	2016	100%	100%	100%
KX247632	MEX_I_7	2015	100%	100%	100%
KX198135	PAN/BEI-259634_V4	2016	100%	100%	100%
KX197192	Brazil/PE243	2015	100%	100%	100%
KX185891	CN/SZ02	2016	100%	100%	100%
KU937936	ZIKVNL00013	2016	100%	100%	100%
KX156776	PAN/CDC-259364_V1-V2	2015	100%	100%	100%
KX156775	/PAN/CDC-259249_V1-V3	2015	100%	100%	100%
KX156774	PAN/CDC-259359_V1-V3	2015	100%	100%	100%
KX101066	Bahia01	2015	100%	100%	100%
KX117076	Zhejiang04	2016	100%	100%	100%
KX051563	Haiti/1/2016	2016	100%	100%	100%
KU509998	Haiti/1225/2014	2014	100%	100%	100%
KU963796	SZ-WIV01	2016	100%	100%	100%
KU991811	Brazil/2016/INMI1	2016	100%	100%	100%
KU940228	Bahia07	2015	100%	100%	100%
KU940224	Bahia09	2015	100%	100%	100%
KU955589	Z16006	2016	100%	100%	100%
KU870645	FB-GWUH-2016	2016	100%	100%	100%

KU926310	Rio-S1	2016	100%	100%	100%
KU926309	Rio-U1	2016	100%	100%	100%
KU922960	MEX/InDRE/Sm/2016	2016	100%	100%	100%
KU922923	MEX/InDRE/Lm/2016	2016	100%	100%	100%
KU866423	SZ01	2016	100%	100%	100%
KU820898	GZ01	2016	100%	100%	100%
KU740184	GD01	2016	100%	100%	100%
KU853013	Dominican Republic/2016/PD2	2016	100%	100%	100%
KU853012	Dominican Republic/2016/PD1	2016	100%	100%	100%
KU820899	ZJ03	2016	100%	100%	100%
KU729217	BeH823339	2015	100%	100%	100%
KU729218	BeH828305	2015	100%	100%	100%
KU761564	GDZ16001	2016	100%	100%	100%
KU681081	H.sapiens-tc/THA/2014/SV0127- 14	2014	100%	100%	100%
KU744693	VE_Ganxian	2016	100%	100%	100%
KU497555	Brazil-ZKV2015	2015	100%	100%	100%
KU707826	SSABR1,	2015	100%	100%	100%
KU527068	Natal RGN	2015	100%	100%	100%
KU647676	MRS_OPY_Martinique_PaRi	2015	100%	100%	100%
KU501217	8375	2015	100%	100%	100%
KU501216	103344	2015	100%	100%	100%
KU365780	BeH815744	2015	100%	100%	100%
KU365779	BeH819966	2015	100%	100%	100%
KU365778	BeH819015	2015	100%	100%	100%
KU365777	BeH818995	2015	100%	100%	100%
KU312312	Z1106033	2015	100%	100%	100%
KU321639	ZikaSPH2015,	2015	100%	100%	100%
KJ776791	H/PF/2013	2013	100%	100%	100%
KX056898	GZ02/2016	2016	95%	100%	100%
KU955590	Z16019	2016	95%	100%	100%
KU955593	H.sapiens-tc/KHM/2010/FSS13025	2010	100%	100%	100%
KF993678	PLCal_ZV	2013	100%	100%	100%
JN860885	FSS13025	2010	100%	100%	100%
EU545988	-	2007	100%	100%	100%
KU681082	H.sapiens-tc/PHL/2012/CPC-0740	2012	100%	94%	100%
KJ461621	Tahiti	2013	100%	100%	100%
KF383117	ArD128000	1994	100%	100%	100%
HQ234499	P6-740	1966	100%	100%	100%
KX198134	SEN/DAK-AR-41524_A1C1-V2	1984	100%	100%	100%
KU955595	A.taylori-tc/SEN/1984/41671-DAK	1984	100%	100%	100%
KU955591	A.africanus-tc/SEN/1984/41525-DAK	1984	100%	100%	100%

KF383120	ArD142623	2000	100%	100%	100%
KF383116	ArD7117	1968	100%	100%	100%
HQ234501	ArD_41519	1984	100%	100%	100%
KF383121	ArD158095	-	100%	100%	94%
KF383119	ArD158084	2001	100%	100%	94%
KF383118	ArD157995	2001	100%	100%	94%
KF268950	ARB7701	-	100%	100%	100%
KF268949	ARB15076	-	100%	100%	100%
KF268948	ARB13565	1976	100%	100%	100%
KF383115	ArB1362	1968	100%	100%	94%

## Cross Reactivity

Cross-reactivity of the ARUP Laboratories Zika Virus Detection by RT-PCR was evaluated by wet-testing purified nucleic acid from other Flaviviruses and additional pathogens (Table 15).

**Table 15. Analytical specificity – cross-reactivity, wet-tested**

Pathogen	Strain/source	Zika Ct	IC Ct	~ titer tested
Herpes Simples Virus 1	ARUP Laboratories	Neg	30.00	8x10 <sup>4</sup> copies/mL
Herpes Simples Virus 2	ARUP Laboratories	Neg	29.95	8x10 <sup>4</sup> copies/mL
Varicella Zoster Virus	ARUP Laboratories	Neg	29.81	4x10 <sup>6</sup> copies/mL
Cytomegalovirus	ARUP Laboratories	Neg	29.85	9.7x10 <sup>5</sup> IU/mL
Epstein Barr Virus	ARUP Laboratories	Neg	29.85	8x10 <sup>6</sup> copies/mL
HHV 6A	ARUP Laboratories	Neg	29.71	8x10 <sup>6</sup> copies/mL
HHV 6B	ARUP Laboratories	Neg	29.65	1.6x10 <sup>6</sup> copies/mL
West Nile Virus	ARUP Laboratories	Neg	29.20	1x10 <sup>6</sup> copies/mL
Toxoplasma gondii	ARUP Laboratories	Neg	29.78	2x10 <sup>6</sup> copies/mL
HIV	ARUP Laboratories	Neg	29.76	>1,000,000 copies/mL
Hepatitis C Virus	ARUP Laboratories	Neg	29.78	>7.0 Log IU/mL
Hepatitis B Virus	ARUP Laboratories	Neg	29.93	>7.2 Log IU/mL
Dengue virus 1	ATCC VR-1254	Neg	30.00	3.5x10 <sup>6</sup> copies/mL
Dengue virus 2	ATCC VR-345	Neg	30.15	6.4x10 <sup>6</sup> copies/mL
Dengue virus 3	ATCC VR-1256	Neg	29.66	1.7x10 <sup>6</sup> copies/mL
Dengue virus 4	ATCC VR-1257	Neg	29.93	8x10 <sup>6</sup> copies/mL
Yellow Fever, strain 17D	ZeptoMetrix	Neg	29.12	1x10 <sup>6.82</sup> U/mL (TCID <sub>50</sub> )
Borrelia burgdorferi	ATCC 35210	Neg	29.73	4x10 <sup>7</sup> copies/mL
Coxsackievirus A16	ARUP Laboratories	Neg	29.53	4x10 <sup>7</sup> copies/mL
Enterovirus 71	ARUP Laboratories	Neg	29.83	4x10 <sup>8</sup> copies/mL
BK virus	ARUP Laboratories	Neg	29.75	2x10 <sup>7</sup> copies/mL
E. coli	ATCC 35218	Neg	29.47	>1x10 <sup>9</sup> copies/mL
N. meningitides	ATCC 13077	Neg	29.59	>1x10 <sup>9</sup> copies/mL
P. falciparum	ARUP Laboratories	Neg	29.60	1x10 <sup>8</sup> copies/mL
P. vivax	ARUP Laboratories	Neg	29.79	1x10 <sup>5</sup> copies/mL
P. malariae	ARUP Laboratories	Neg	29.77	1x10 <sup>4</sup> copies/mL
P. knowlesi	ARUP Laboratories	Neg	29.74	3x10 <sup>3</sup> copies/mL

P. ovale	ARUP Laboratories	Neg	29.63	2x10 <sup>6</sup> copies/mL
Chikungunya virus	ARUP Laboratories	Neg	29.53	5x10 <sup>9</sup> copies/mL
Parvovirus B-19	ARUP Laboratories	Neg	29.86	3.7x10 <sup>7</sup> IU/mL
Strep. anginosus	ARUP Laboratories	Neg	32.35	>1x10 <sup>9</sup> copies/mL
Strep. pneumoniae	ATCC 49619	Neg	32.27	>1x10 <sup>9</sup> copies/mL
Strep. pyogenes	ATCC 19615	Neg	31.96	>1x10 <sup>9</sup> copies/mL
genomic DNA	ARUP Laboratories	Neg	30.36	111 µg

Additional cross-reactivity of the ARUP Laboratories Zika Virus Detection by RT-PCR primers and probe was evaluated by *in silico* NCBI BLAST analysis to determine if additional organisms have significant combined homology that would predict potential false positive results. Percent homology for each primer and probe is presented in Table 16.

**Table 16. Analytical specificity – cross-reactivity, *in-silico***

Organism	TaxID	ZKV3-L1 (For)	ZKV3-FAM1 (Probe)	ZKV3-E3 (Rev)
Japanese encephalitis virus	11072	70%	56%	61%
St. Louis encephalitis virus	11080	70%	50%	56%
Yellow Fever	11089	50%	56%	56%
Dengue 1	11053	55%	56%	61%
Dengue 2	11060	55%	56%	61%
Dengue 3	11069	55%	56%	61%
Dengue 4	11070	55%	56%	78%
West Nile virus	11802	60%	56%	61%
Chikungunya virus	37124	55%	50%	50%
Mayaro virus	59301	55%	61%	50%
Spondweni virus	64318	70%	50%	50%
Eastern Equine encephalitis virus	11021	55%	61%	61%
Western Equine encephalitis virus	11039	50%	56%	44%
Ross River virus	11029	45%	50%	44%
O'nyong-nyong virus	11027	55%	50%	61%
Hepatitis C virus	11103	60%	83%	37%
Barmah Forest virus	NC_001786	45%	44%	44%
Measles virus	11234	50%	50%	61%
Rubella virus	11041	40%	44%	50%
Enterovirus	12059	80%	61%	78%
Adenovirus	10508	60%	61%	72%
Hepatitis B virus	10407	75%	55%	67%
HIV	11652	75%	67%	72%
Varicella Zoster virus	10335	45%	50%	50%
Cytomegalovirus	10358	55%	61%	61%

Epstein Barr virus	10376	60%	72%	56%
Rickettsia sp	780	65%	67%	67%
Borrelia burgdorferi	64895	65%	61%	72%
Group A Streptococcus	1314	80%	72%	67%
Leptospirosis	1643688	70%	67%	83%
Plasmodium sp	5820	70%	72%	83%
Trypanosoma cruzi	5693	65%	78%	78%
Schistosomiasis	6181	75%	78%	83%
Hepatitis A virus vaccine	208726	35%	44%	50%
Salmonella	590	65%	78%	78%
Salmonella typhi vaccine	527001	55%	67%	72%
Streptococcus	1301	80%	78%	83%
Escherichia coli	562	75%	78%	83%

Hits were evaluated for potential formation of PCR products via binding of primers in close proximity, with the correct orientation to each other and within the same strain. Cross-reactivity is not expected for any organisms in Table 16.

#### Microbial Interference study

In several instances, the primers or probe show  $\geq 70\%$  homology to other organisms based on Blast analysis of the taxon for that pathogen. In several cases, the top hits are for pathogens that do not infect humans but are part of that taxon. Because the amount of primers and probe that are added to the master mix are in excess of  $1 \times 10^{12}$  copies per reaction for each oligo, the presence of a significant co-infection would not impact the ability of the assay to detect a low titer Zika positive. This was also demonstrated by wet-testing Zika virus RNA at 3X LOD concentration in the presence of high titer organisms for Dengue 1-4, Chikungunya, West Nile, Hepatitis B, HIV, Escherichia coli and Plasmodium species : *P. knowlesi*, *P. faciparium*, *P. ovale*, *P. vivax*, and *P. malariae*. These data are shown in Tables 17 and 18.

**Table 17. Microbial interference results**

Pathogen titer	Sample Name	Zika Ct			Mean	Stdev	%CV
		Rep 1	Rep 2	Rep 3			
	Zika	36.75	36.69	36.99	36.81	0.16	0.43
$3.5 \times 10^6$ copies/mL	Zika/Dengue 1	36.49	36.13	36.69	36.43	0.28	0.78
$6.4 \times 10^6$ copies/mL	Zika/Dengue 2	36.79	36.46	36.71	36.65	0.18	0.48
$1.7 \times 10^6$ copies/mL	Zika/Dengue 3	37.06	37.33	36.28	36.89	0.55	1.49
$8 \times 10^6$ copies/mL	Zika/Dengue 4	37.23	36.94	37.22	37.13	0.17	0.45
$5 \times 10^6$ copies/mL	Zika/Chikungunya	36.65	37.66	36.91	37.07	0.52	1.41
$1 \times 10^5$ copies/mL	Zika/West Nile	37.05	36.59	36.54	36.73	0.28	0.75

**Table 18. Microbial interference results**

Pathogen titer	Sample Name	Zika Ct			Mean	Stdev	%CV
		Rep 1	Rep 2	Rep 3			
	Zika	38.31	37.57	39.04	38.31	0.74	1.92
>7.2 Log IU/mL	Zika/Hepatitis B	38.08	38.37	37.65	38.04	0.36	0.96
>1,000,000 copies/mL	Zika/HIV	38.65	36.88	37.59	37.71	0.89	2.36
3x10 <sup>4</sup> copies/mL	Zika/P.knowlesi	39.03	37.85	39.23	38.70	0.75	1.93
1x10 <sup>8</sup> copies/mL	Zika/P.falcip	37.97	38.03	37.46	37.82	0.31	0.83
2x10 <sup>6</sup> copies/mL	Zika/P.ovale	37.99	37.60	40.22	38.60	1.41	3.65
1x10 <sup>5</sup> copies/mL	Zika/P.vivax	37.57	37.58	39.47	38.21	1.09	2.86
1x10 <sup>5</sup> copies/mL	Zika/P.malariae	37.48	38.22	40.70	38.80	1.68	4.34
>1x10 <sup>9</sup> copies/mL	Zika/E.coli	39.23	39.17	38.59	39.00	0.35	0.91

### **Matrix Equivalency between Serum and Plasma**

To demonstrate that the ARUP Laboratories Zika Virus Detection by RT-PCR assay has equivalent performance with serum and plasma specimens, 40 paired serum and plasma samples were tested as part of a matrix equivalency study. Twenty serum and plasma samples were spiked at 1.5X LOD, 10 serum and plasma samples were spiked at 5X LOD and 10 serum and plasma samples were run as unspiked negatives. All samples were blinded and randomized prior to testing. Results of the matrix equivalency study indicate equivalent performance in serum and plasma samples. These results are found in Table 19 and the data is sorted by spiking level.

**Table 19. Results of matrix equivalency study in ARUP Zika Virus Detection by RT-PCR Assay.**

Randomized #	Plasma				Sample ID	Randomized #	Serum			
	Zika CT	IC CT	Spiking Level	Sample ID			Zika CT	IC CT	Spiking Level	Sample ID
60	37.08	29.35	1.5X	P11	22	37.08	29.08	1.5X	S11	
58	37.44	29.10	1.5X	P12	24	36.05	28.52	1.5X	S12	
26	37.47	28.52	1.5X	P13	25	35.76	28.24	1.5X	S13	
28	36.72	28.72	1.5X	P14	27	35.92	28.63	1.5X	S14	
30	36.75	28.72	1.5X	P15	29	37.21	28.59	1.5X	S15	
32	38.08	28.49	1.5X	P16	31	36.11	28.69	1.5X	S16	
34	37.40	29.05	1.5X	P17	33	36.95	29.02	1.5X	S17	
36	36.83	28.90	1.5X	P18	35	36.90	28.94	1.5X	S18	
38	36.99	28.88	1.5X	P19	37	36.75	28.76	1.5X	S19	
40	36.68	29.06	1.5X	P20	39	38.53	29.25	1.5X	S20	
42	37.83	28.59	1.5X	P21	41	37.00	29.04	1.5X	S21	
44	37.76	29.31	1.5X	P22	43	37.30	29.63	1.5X	S22	
46	37.34	29.85	1.5X	P23	45	37.99	29.67	1.5X	S23	

48	37.20	28.97	1.5X	P24	47	37.04	29.25	1.5X	S24
23	36.73	28.90	1.5X	P25	79	37.35	29.07	1.5X	S25
21	36.28	28.74	1.5X	P26	77	36.47	29.20	1.5X	S26
19	37.34	28.52	1.5X	P27	75	36.98	29.35	1.5X	S27
17	37.27	28.21	1.5X	P28	73	36.43	29.33	1.5X	S28
15	37.53	28.90	1.5X	P29	71	36.96	29.93	1.5X	S29
13	37.10	28.68	1.5X	P30	69	37.07	29.28	1.5X	S30
11	35.28	28.58	5X	P31	67	34.99	29.19	5X	S31
9	35.36	28.92	5X	P32	65	34.96	29.07	5X	S32
7	34.94	29.08	5X	P33	63	35.67	29.46	5X	S33
5	35.05	28.97	5X	P34	61	35.15	29.67	5X	S34
3	34.89	29.07	5X	P35	59	35.10	29.37	5X	S35
1	34.90	29.30	5X	P36	57	35.21	29.10	5X	S36
49	35.24	29.13	5X	P37	50	35.46	29.50	5X	S37
51	34.74	28.99	5X	P38	52	34.95	29.08	5X	S38
53	35.26	29.27	5X	P39	54	35.19	29.22	5X	S39
55	34.91	29.19	5X	P40	56	35.61	29.38	5X	S40
80	Neg	28.68	No	P1	2	Neg	28.95	No	S1
78	Neg	29.17	No	P2	4	Neg	28.55	No	S2
76	Neg	28.05	No	P3	6	Neg	28.84	No	S3
74	Neg	29.45	No	P4	8	Neg	29.35	No	S4
72	Neg	29.81	No	P5	10	Neg	28.63	No	S5
70	Neg	29.65	No	P6	12	Neg	28.59	No	S6
68	Neg	29.10	No	P7	14	Neg	28.75	No	S7
66	Neg	29.28	No	P8	16	Neg	28.67	No	S8
64	Neg	29.33	No	P9	18	Neg	28.42	No	S9
62	Neg	29.44	No	P10	20	Neg	28.48	No	S10

### **Interference Studies**

No interference studies were performed as none are required for EUA for assays that use conventional PCR and well established extraction methods. The ARUP Laboratories Zika Virus Detection by RT-PCR assay uses an established nucleic acid extraction method (magnetic bead based extraction via an automated chemagic MSM I platform) followed by real-time RT-PCR.

### **Clinical Evaluation**

#### **Contrived Clinical Specimen Testing:**

Accuracy for the ARUP Laboratories Zika Virus Detection by RT-PCR assay was assessed by testing both contrived and negative plasma and urine samples collected from individual donors. A total of 155 samples (100 urine and 55 plasma) were tested. The urine samples consisted of 50

negative urine specimens and 50 contrived urine specimens spiked at 1.5X LOD (n=25), 5X LOD (n=15) and 10X LOD (n=10). The plasma samples consisted of 30 negative plasma samples and 25 contrived plasma specimens spiked at 1.5X LOD. Zika virus strain PRVABC59 (provided by the CDC Arboviral Disease Branch) was used to spike all contrived specimens. All samples were blinded and randomized prior to testing. Zika virus was detected in 100% of the contrived samples and was not detected in any of the expected Zika negative samples.

Natural Clinical Specimen Testing:

A total of 105 retrospective study samples were collected from patients in Columbia and/or acquired from a commercial vendor. Samples consisted of asymptomatic and suspect cases from high incidence areas. These samples included 75 plasma specimens, 25 urines specimens and 5 serum specimens. Of the 25 urine specimens, 5 had matched serum specimens and the remaining 20 were matched with a plasma specimen. All samples were run in the ARUP Laboratories Zika Virus Detection by RT-PCR assay and an FDA-authorized comparator test. All samples were blinded and randomized prior to testing.

Of the 105 samples included in the comparison study, 36 were Zika positive and 69 were negative by the comparator method. Of the 36 comparator assay Zika virus positive samples, 30 were plasma and 6 were urine; 33 of these samples (28 plasma and 5 urine) were also Zika virus positive or presumptive positive by the ARUP Laboratories Zika Virus Detection by RT-PCR assay. Of the 69 comparator assay Zika virus negative samples (45 plasma, 5 serum and 19 urine), 61 samples (40 plasma, 5 serum and 16 urine) were also negative with the ARUP Laboratories Zika Virus Detection by RT-PCR assay while 8 samples (5 plasma and 3 urine) were Zika virus positive or presumptive positive.

Clinical Performance:

Specimen Category	Number Tested	ARUP Laboratories Zika Virus Detection by RT-PCR assay		
		Zika Positive (Ct ≤ 38)	Zika Presumptive Positive* (38 < Ct ≤ 50)	Zika Negative (Ct > 50)
<b>Plasma</b>				
<b>Natural Zika Positive Clinical Specimens as Determined by FDA Authorized Zika Comparator<sup>b</sup></b>	30	24	4 <sup>a</sup>	2
<b>Contrived Zika Positive Clinical Specimens (1.5 X LoD)</b>	25	25	0	0
<b>Contrived Zika Positive Clinical Specimens (10 X LoD)</b>	20	19	0	1



<b>Natural Zika Negative Clinical Specimens as Determined by FDA Authorized Zika Comparator<sup>b</sup></b>	45	1	4 <sup>a</sup>	40
<b>Contrived Zika Clinical Specimens (Negative)</b>	30	0	0	30
<b>Positive Percent Agreement</b>	<b>90.67% (68/75), 95% CI: 82.0% -95.4%</b>			
<b>Negative Percent Agreement</b>	<b>93.3% (70/75), 95% CI:85.3% - 97.1%</b>			

<sup>a</sup> Per the ARUP results algorithm, samples with Ct values > 38 are presumptive positives and should be re-tested. However, due to limited sample volume, these samples were not re-tested. When calculating PPA presumptive test were treated as negative. When calculating NPA presumptive test were treated as positive.

<sup>b</sup> 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.

Specimen Category	Number Tested	ARUP Laboratories Zika Virus Detection by RT-PCR assay		
		Zika Positive (Ct ≤ 38)	Zika Presumptive Positive* (38 < Ct ≤ 50)	Zika Negative (Ct > 50)
<b>Serum</b>				
<b>Contrived Zika Positive Clinical Specimens (10 X LoD)</b>	20	20	0	0
<b>Matrix Equivalency Study (1.5 x LoD)</b>	20	19	1 <sup>a</sup>	0
<b>Matrix Equivalency Study (5 X LoD)</b>	10	10	0	0
<b>Natural Zika Negative Serum as determine by the FDA Authorized Comparator<sup>b</sup></b>	5	0	0	5
<b>Matrix Equivalency Study (Negative)</b>	10	0	0	10
<b>Positive Percent Agreement</b>	<b>98% (49/50), 95% CI: 89.5% - 99.7%</b>			
<b>Negative Percent Agreement</b>	<b>100.0% (15/15), 95% CI:79.6% - 100.0%</b>			

<sup>a</sup> Per the ARUP results algorithm, samples with Ct values > 38 are presumptive positives and should be re-tested. However, due to limited sample volume, these samples were not re-tested.

<sup>b</sup> 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.

When calculating PPA presumptive test were treated as negative. When calculating NPA presumptive test were treated as positive.

Specimen Category	Number Tested	ARUP Laboratories Zika Virus Detection by RT-PCR assay		
		Zika Positive (Ct ≤ 38)	Zika Presumptive Positive* (38 < Ct ≤ 50)	Zika Negative (Ct > 50)
<b>Urine</b>				
Natural Zika Positive Clinical Specimens as Determined by FDA Authorized Zika Comparator <sup>c</sup>	6	2	3 <sup>a</sup>	1
Contrived Zika Positive Clinical Specimens (1.5 X LoD)	25 <sup>b</sup>	25	0	0
Contrived Zika Positive Clinical Specimens (5 X LoD)	15	15	0	0
Contrived Zika Positive Clinical Specimens (10 X LoD)	10	9	1 <sup>a</sup>	0
Natural Zika Negative Urine as determine by the FDA Authorized Comparator <sup>c</sup>	19	2	1 <sup>a</sup>	16
Contrived Zika Clinical Specimens (Negative)	50	0	0	50
<b>Positive Percent Agreement</b>	<b>91.1% (51/56), 95% CI: 80.7% - 96.1%</b>			
<b>Negative Percent Agreement</b>	<b>95.7% (66/69) 95% CI:87.9% - 96.7%</b>			

<sup>a</sup> Per the ARUP results algorithm, samples with Ct values > 38 are presumptive positives and should be re-tested. However, due to limited sample volume, these samples were not re-tested. When calculating PPA presumptive test were treated as negative. When calculating NPA presumptive test were treated as positive.

<sup>b</sup> Eleven of the initial 25 contrived urines samples had Ct> 38 and were not retested. An additional 11 urine samples were contrived and tested per the ARUP algorithm.

<sup>c</sup> 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.