Trioplex Real-time RT-PCR Assay

Centers for Disease Control and Prevention

For use under an Emergency Use Authorization only

Instructions for Use

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Introduction

PURPOSE

This document describes the use of real-time (TaqMan®) RT-PCR assays for detection and differentiation of RNA from dengue, chikungunya and Zika viruses in serum, whole blood (EDTA), cerebrospinal fluid (CSF) and for the detection of Zika virus RNA in urine, and amniotic fluid. This protocol has been designed to facilitate simultaneous testing for the presence of dengue, chikungunya and Zika viruses using a single sample.

NOTE: In this assay (Trioplex Real-time RT-PCR) the tests for dengue (DENV), chikungunya (CHIKV) and Zika (ZIKV) viruses may be run in the same rRT-PCR plate well (multiplex). A singleplex reaction option is also presented for use in ZIKV testing of urine and amniotic fluid as well as for ZIKV, CHIKV and DENV testing in other specimen types by laboratories that prefer including only one primer/probe set per well (please see the NOTES in the Real Time RT-PCR Assay section, Page 19, for more details).

INTENDED USE

The Trioplex Real-time RT-PCR Assay (Trioplex rRT-PCR) is intended for the qualitative detection and differentiation of RNA from Zika virus, dengue virus, and chikungunya virus in human sera, whole blood (EDTA) or cerebrospinal fluid (each collected alongside a patient-matched serum specimen), and for the qualitative detection of Zika virus RNA in urine, and amniotic fluid (each collected alongside a patient-matched serum specimen). The assay is intended for use with specimens collected from individuals meeting CDC Zika virus clinical criteria (e.g., clinical signs and symptoms associated with Zika virus infection) and/or CDC Zika virus epidemiological criteria (e.g., history of residence in or travel to a geographic region with active Zika transmission at the time of travel, or other epidemiologic criteria for which Zika virus testing may be indicated as part of a public health investigation). Testing is limited to qualified laboratories designated by the Centers for Disease Control and Prevention (CDC).

Assay results are for the identification of Zika, dengue, and chikungunya viral RNA. Zika virus RNA is generally detectable in serum, whole blood and/or urine during the acute phase of infection and up to 14 days following onset of symptoms, if present. Positive results are indicative of current infection. Laboratories are required to report all results to the appropriate public health authorities. Within the United States and its territories results must be reported to CDC.

Negative Trioplex rRT-PCR results do not rule out dengue, chikungunya and/or Zika virus infections and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

The Trioplex rRT-PCR is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The assay is only for use under the Food and Drug Administration's Emergency Use Authorization.

PROTOCOL USE LIMITATIONS

The Trioplex Real-Time RT-PCR Assay described here has not been extensively tested with clinical specimens. Modifications of these assays (i.e., use of PCR instruments or chemistries other than those described) are not permitted. These assays should not be further distributed without the explicit consent of the CDC.

ASSAY PRINCIPLE

Trioplex Real-Time RT-PCR Assay includes primers and dual-labeled hydrolysis (Taqman®) probes to be used in the *in vitro* qualitative detection of Zika virus RNA isolated from clinical specimens including serum (from serum separator tubes), whole blood (EDTA), CSF, urine, and amniotic fluid. A reverse transcription step produces cDNA from RNA present in the sample. The probe binds to the target DNA between the two unlabeled PCR primers. For the dengue virus-specific probe, the signal from the fluorescent dye (FAM) on the 5' end is quenched by BHQ-1 on its 3' end. For the chikungunya virus-specific probe, the signal from the fluorescent dye (HEX) on the 5' end is quenched by BHQ-1 on its 3' end. For the Zika virus-specific probe, the signal from the fluorescent dye (CAL Fluor Red 610) on the 5'end is quenched by BHQ-2 on its 3' end. During PCR, Taq polymerase extends the unlabeled primers using the template strand as a guide, and when it reaches the probe it cleaves the probe separating the dye from the quencher allowing it to fluoresce. The real-time PCR instrument detects this fluorescence from the unquenched dye. With each cycle of PCR, more probes are cleaved resulting in an increase in fluorescence that is proportional to the amount of target nucleic acid present.

Specimens

ACCEPTABLE SPECIMENS

For Zika, chikungunya and dengue testing:

- Serum (collected in a serum separator tube)
  Tube should be centrifuged prior to shipping to avoid hemolysis
- Whole blood (EDTA)
- Cerebrospinal fluid

For Zika testing only:

- Urine
- Amniotic fluid

NOTE: Serum is the preferred diagnostic specimen. Whole blood (EDTA), CSF, urine and amniotic fluid may only be tested 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.
- Transport/ship human serum, urine, CSF and amniotic fluid specimens in dry ice, if possible; however, using cold-packs is acceptable.
- Store serum, urine, CSF and amniotic fluid specimens at ≤-20°C upon receipt. Thaw sample and keep on ice during sample processing. Store remainder of sample at ≤-70°C for long term storage.
- Transport/ship human whole blood (EDTA) specimens on cold packs.
- Store human whole blood (EDTA) specimens at 2-8°C upon receipt. Testing is recommended within one week of collection.

SAFETY/PRECAUTIONS

Laboratory biosafety guidance for working with Zika virus specimens is provided at http://www.cdc.gov/zika/laboratories/lab-safety.html. It is recommended that laboratories perform a risk assessment when conducting new tests and safety precautions should be based on the laboratory's risk assessment. Dengue and Zika viruses are considered pathogens that can be safely worked with in a biosafety level 2 (BSL-2) laboratory; however, according to the guidelines of the Biosafety in Microbiological and Biomedical Laboratories (BMBL), specimens suspected to contain chikungunya virus should be handled in BSL-3 conditions: http://www.cdc.gov/biosafety/publications/bmbl5/index.htm.

This procedure should be performed with consideration to the potential infectious nature of the specimens involved. The protocol is meant to detect viral genomes; therefore it is assumed that the specimens contain virus. Laboratorians should recognize that chikungunya virus produces high levels of viremia and specimens from suspected chikungunya virus cases should be treated as potentially infectious. Please review CDC guidance for state and local public health laboratories: http://www.cdc.gov/zika/laboratories/lab-safety.html. See the BMBL for additional biosafety information about these viruses and laboratory biosafety practices.

Proper disposable gown, gloves, eye protection, and a biological safety cabinet are recommended for manipulation of clinical specimens. The rRT-PCR assay should be performed in a separate room considered to be free of dengue virus (DENV), chikungunya virus (CHIKV), and Zika virus (ZIKV), or any virus RNA or DNA templates. Likewise, the RNA extraction procedure should be performed in a room different from where the RNA is amplified by rRT-PCR. During the nucleic acid amplification steps, sections of the viral genomes are amplified; therefore all original serum samples and test samples should be maintained separately from the PCR room to avoid contamination of samples.

Equipment and Consumables

DISCLAIMER: Names of vendors or manufacturers are provided as examples of suitable product sources. Use of trade names is for identification purposes only and does not constitute endorsement by CDC or the Department of Health and Human Services.

MATERIALS PROVIDED BY CDC

- CDC Trioplex Real-time RT-PCR Assay Primer and Probe Set (CDC; catalog #KT0166). Refer to product insert for storage and expiration information. Set includes 4 vials with primer and probes for each agent combined in one vial.
  - 1 vial, DENV-F, DENV-R1, DENV-R2, and P
  - 1 vial, CHIKV-F, R and P
  - 1 vial, ZIKV-F, R and P
  - 1 vial, RP-F, R and P (this is a primer/probe set for human RNAse P and is used to verify a successful extraction)
- Trioplex rRT-PCR Assay Positive Control Set (CDC; catalog #KT0167)
  - DENV Positive Control (PC): Inactivated dengue virus
  - CHIKV Positive Control (PC): Inactivated chikungunya virus
  - ZIKV Positive Control (PC): Inactivated Zika virus
  - Human Specimen Control (HSC): extraction control and positive control for RP

MATERIALS REQUIRED BUT NOT PROVIDED

- RNA extraction kits (any of the following may be used - see Nucleic Acid Extraction section for details on appropriate kits for use with each instrument and specimen type):
  - MagNA Pure LC Total Nucleic Acid Isolation Kit (192 reactions) (Roche, catalog #03038505001)
  - MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, catalog #06543588001)
  - MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche, catalog #06374891001)
  - MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche, catalog #03730964001)
  - MagNA Pure Compact (MPC) Nucleic Acid Isolation Kit I – Large Volume (Roche catalog #03730972001)
  - bioMérieux NucliSENS easyMAG automated extraction components:
    Note: CDC has been notified of a product recall for certain lots of easyMAG extraction reagents. Though CDC has experienced no shifts in performance associated with use of these reagents, CDC has not extensively evaluated the impact of these product issues on performance of the easyMAG for extraction of Zika RNA for subsequent testing by Trioplex rRT-PCR. Laboratories should refer to bioMérieux Product Safety Correction Notices for a list of impacted lots and advice for end users. Each lot of affected

reagents should be evaluated at least weekly before use in extraction of diagnostic specimens. Laboratories should also closely monitor for any trend in Ct values of the External Positive Controls and the HSC controls during testing.

- easyMAG Magnetic Silica (bioMérieux catalog #280133)
- easyMAG Disposables (bioMérieux catalog #280135)
- easyMAG Buffer 1 (bioMérieux catalog #280130)
- easyMAG Buffer 2 (bioMérieux catalog #280131)
- easyMAG Buffer 3 (bioMérieux catalog #280132)
- easyMAG Lysis Buffer (bioMérieux catalog #280134)

  - Qiagen QIAamp® Viral RNA Mini kit (Qiagen catalog #52904 or 52906)
  - Qiagen QIAamp® DSP Viral RNA Mini kit (Qiagen catalog #61904)

- rRT-PCR Master mix kits (either may be used):
  - SuperScript® III Platinum® One-Step qRT-PCR Kit (ThermoFisher Scientific catalog #11732088 and/or 11732020)
  - qScript™ One-Step qRT-PCR kit, Low Rox™ (Quanta, catalog # 95059-050 and/or 95059-200)
- Molecular-grade water, nuclease-free

EQUIPMENT

- Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument (ThermoFisher Scientific; catalog #446985 or #4406984);
- QuantStudio™ Dx (QSDx) Real-Time PCR Instrument with 96-Well Fast Block (ThermoFisher Scientific; catalog #4480299)
- Calibration Kit, Fast 96-well (ThermoFisher Scientific; catalog #4432563)
- ROI & Background Calibration Kit, Fast 96-well (ThermoFisher Scientific; catalog #4432638)
- 7500 Fast Real-Time PCR Systems Spectral Calibration Kit II (ThermoFisher Scientific; catalog #4362201)
- Vortex mixer
- Microcentrifuge
- 96-well cold block (or ice)
- Micropipettes (2 or 10 µL, 20 µL, 200 µL and 1000 µL)
- Multichannel micropipettes (5-50 µL)
- Automated RNA extraction instruments (optional):
  - MagNA Pure LC 2.0 instrument (Roche; catalog #05197686001)
  - MagNA Pure 96 Instrument (Roche; catalog #5195322001)
  - MagNA Pure Compact Instrument (Roche; catalog #03731146001)
  - bioMérieux NucliSENS easyMAG (bioMérieux; catalog #280140)

CONSUMABLES

- Acceptable surface decontaminants

- DNA Away (Fisher Scientific; catalog # 21-236-28)
- RNase Away (Fisher Scientific; catalog #21-236-21). This product eliminates RNase and DNA.
- 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
- DNA*Zap*™ (ThermoFisher Scientific; cat. #AM9890) or equivalent.

- Disposable, powder-free gloves and disposable gowns
- Laboratory marking pen
- Aerosol barrier sterile pipette tips for P2/P10, P40, P200, and P1000
- 1.5 mL microcentrifuge tubes
- Racks for 1.5 mL microcentrifuge tubes
- 0.1 mL PCR reaction plates (ThermoFisher Scientific; catalog #4346906 or #4366932) and optical caps (Applied Biosystems; catalog #4323032)
- MicroAmp® Optical Adhesive Film Kit (ThermoFisher Scientific; catalog # 4311971 or #4360954)

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-positive and false-negative results.

GENERAL CONSIDERATIONS

- Personnel must be familiar with the protocol and instruments used.
- Maintain separate areas, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips, gowns and gloves) for
  - assay reagent setup
  - handling of extracted nucleic acids
  - Real-time RT-PCR amplification.
- Work flow must always proceed unidirectionally from the RNA extraction/reagent preparation (clean area) area to the PCR amplification room ("dirty area") in order to avoid contamination of clinical samples with amplified nucleic acids.
- Wear clean, previously unworn, disposable gowns and new, powder-free gloves during assay reagent setup and handling of extracted nucleic acids. Change gloves whenever you suspect they may be contaminated.
- Store primer/probes and enzyme master mix at appropriate temperatures (see package inserts). Do not use reagents beyond their expiration dates.
- Keep reagent tubes and reactions capped as much as possible.
- Use DNA*Zap*™ (or equivalent) or 10% freshly prepared bleach to clean surfaces.
- Do not bring extracted nucleic acid or PCR-amplified material into the assay setup area.
- Use aerosol barrier (filter) pipette tips only.

ASSAY CONTROLS

Assay controls should be run concurrently with all test samples.

Extraction control

Human specimen control (HSC) --- noninfectious cultured human cell material used as an extraction control and positive control for the RNase P primer and probe set (RP) that is **extracted concurrently** with the test samples and included as a sample during rRT-PCR set-up. The HSC should generate negative results with DENV, CHIKV and ZIKV primer and probe sets, but positive results for RP. The HSC is a component of the Trioplex rRT-PCR Positive Control Set (CDC; catalog #KT0167).

Positive controls for agent-specific primer and probe sets

- DENV PC: Inactivated dengue virus
- CHIKV PC: Inactivated chikungunya virus
- ZIKV PC: Inactivated Zika virus

These components of the Trioplex rRT-PCR Positive Control Set (CDC; catalog #KT0167) must be extracted using one of the acceptable RNA extraction methods described herein. Extracted positive nucleic acid should be aliquoted and stored at ≤-20°C until use. Avoid repeated freeze-thaw cycles.

Note: When extracting positive control material, precautions should be taken to prevent cross-contamination. Use caution when opening vials containing inactivated virus. Change or decontaminate gloves between each vial. Similar precautions should be observed when handling extracted positive control material.

RNase P Primer and Probe Set (RP)

All clinical samples and the HSC should be tested for human RNase P gene (using the RP primer and probe set included in the Trioplex rRT-PCR kit) to control for specimen quality and as an indicator that nucleic acid resulted from the extraction process. The RNase P Primer and Probe Set is a component of the Trioplex Real-Time RT-PCR Primer and Probe Set (CDC; catalog #KT0166).

No Template Control (NTC)

NTC reactions include PCR-grade water in place of specimen RNA and must be included for each reaction mixture (one for the ZIKV, CHIKV and DENV reaction and one for the RP reaction) in each run. The NTC is a control for contamination or improper function of assay reagents resulting in false positive results.

Table 1: Overview of positive and negative controls

Control Type	Control Name	Used to Monitor	DENV	CHIKV	ZIKV	RP	Expected C_T Values
Positive	DENV PC	Substantial reagent failure, including primer and probe integrity.	+	-	-	N/A	< 38 C_T
	CHIKV PC		-	+	-	N/A	
	ZIKV PC		-	-	+	N/A	
Negative	NTC	1) Reagent and/or environmental contamination during PCR set-up; and 2) primer and probe set function.	-	-	-	-	None Detected
Extraction	HSC	1) Reagent and/or environmental contamination during extraction; and 2) extraction success.	-	-	-	+	None Detected for DENV, CHIKV, and ZIKV.
							RP C_T < 38

Nucleic Acid Extraction

Notes on Extraction

- Sample extractions must yield RNA or total nucleic acid of sufficient volume to cover all real-time RT-PCR assays.
- Serum, urine, CSF, and amniotic fluid - large volume extraction options (1 mL input volume) are preferred for these specimens as they yield more nucleic acid than the standard volume counterparts. Small volume extractions should only be used for these specimen types if there is insufficient sample volume to perform a large volume extraction or if a large volume option is unavailable.
- **Note**: No Large volume extraction options have been added for manual extractions or MagNA Pure LC 2.0 instrument as data demonstrating superior sensitivity with large volume is not yet available. Only acceptable specimens extracted using one of the prescribed extraction methods may be tested with this assay (see Summary of Extraction Kit Options, Table 2 below).
- HSC should be included in each extraction run as a sample extraction control. For large volume extraction methods (1 mL input volume), 800 µL PCR-grade water should be added to 200 µL re-suspended HSC to create the 1 mL HSC sample for extraction input.
- Retain specimen RNA extracts in a cold block or on ice until testing. If testing will be delayed more than 24 hours, freeze RNA at ≤-20°C immediately following extraction.

Table 2: Summary of Extraction Kit Options

Extraction Instrument	Kits Authorized for Each Specimen Type			
	Serum, Urine, CSF, and Amniotic Fluid Large volume (preferred)	**Serum and Urine Small Volume**	**Whole Blood Small Volume**	**CSF and Amniotic Fluid Small Volume**
Manual (no instrument)	None	QIAamp Viral RNA Mini Kit or QIAamp DSP Viral RNA Mini Kit	None	QIAamp Viral RNA Mini Kit or QIAamp DSP Viral RNA Mini Kit
MagNA Pure 96	MagNA Pure 96 DNA and Viral NA Large Volume Kit	MagNA Pure 96 DNA and Viral NA Small Volume Kit	MagNA Pure 96 DNA and Viral NA Small Volume Kit	MagNA Pure 96 DNA and Viral NA Small Volume Kit
MagNA Pure LC 2.0	None	MagNA Pure LC Total Nucleic Acid Isolation Kit	MagNA Pure LC Total Nucleic Acid Isolation Kit	MagNA Pure LC Total Nucleic Acid Isolation Kit
MagNA Pure Compact	MagNA Pure Compact (MPC) Nucleic Acid Isolation Kit I – Large Volume	MagNA Pure Compact Nucleic Acid Isolation Kit I	MagNA Pure Compact Nucleic Acid Isolation Kit I	MagNA Pure Compact Nucleic Acid Isolation Kit I
easyMAG*	easyMAG reagents are provided individually – see instrument manual and Equipment and Consumables section for a list of required reagents for extraction.	easyMAG reagents are provided individually – see instrument manual and Equipment and Consumables section for a list of required reagents for extraction.	None	easyMAG reagents are provided individually – see instrument manual and Equipment and Consumables section for a list of required reagents for extraction.

*Due to the product recall for certain lots of the bioMérieux easyMAG extraction reagents, each lot of affected reagents should be evaluated at least weekly before use in extraction of diagnostic specimens. Laboratories should also closely monitor for any trend in Ct values of the External Positive Controls and the HSC controls during testing. See Equipment and Consumables section for additional information.

Manual Extraction

Serum, urine, CSF, and Amniotic Fluid specimens may be extracted using either the QIAamp Viral RNA Mini Kit or QIAamp DSP Viral RNA Mini Kit. Follow the manufacturer's instructions, using the following volumes:

Specimen input volume: 140µL
Elution volume: 60µL

Automated Extraction

- MagNA Pure LC 2.0 Instrument
    - Small Volume (Serum, Urine, CSF, Amniotic Fluid) Protocol for MagNA Pure LC

2.0

RNA from serum, urine, CSF, and amniotic fluid clinical specimens may be extracted using the MagNA Pure LC Total Nucleic Acid Isolation Kit-Small Volume. When setting up the instrument for an extraction run, select the protocol for the corresponding specimen input volume:

Specimen input volume: 200 µL
Program: Total NA variable elution volume
Elution Volume: 60 µL

OR with external lysis option:

Specimen input volume: mix 200 µL of specimen with 300 µL of lysis buffer for a total volume of 500 µL before loading into the instrument.
Program: Total NA External lysis
Elution Volume: 60 µL

- Small Volume (Whole Blood) Protocol for MagNA Pure LC 2.0

  RNA from whole blood (EDTA) clinical specimens may be extracted using the MagNA Pure LC Total Nucleic Acid Isolation Kit.
  1. Add 300 µL of LC 2.0 external lysis buffer to 200 µL of the whole blood (EDTA) specimen. Vortex on high and incubate at room temperature for 15 min.
  2. After incubation, vortex on high again and pulse centrifuge to bring the lysate to the bottom of the tube. Load samples into a LC 2.0 sample cartridge.
  3. Load sample plate(s) onto the LC 2.0 extraction instrument and load "small volume" kit reagents.
  4. When setting up the instrument for an extraction run, select the protocol for the corresponding specimen input volume:

     Specimen input volume: 500 µL
     Program: Total NA
     Protocol: Total_NA_external_lysis
     Elution Volume: 100 µL

- MagNA Pure 96 Instrument

  - Large Volume (Serum, Urine, CSF, and Amniotic Fluid) Protocol for MagNA Pure 96

    RNA from serum or urine clinical specimens may be extracted using the MagNA Pure 96 DNA or Viral NA Large Volume Kit. When setting up the instrument for an extraction run, select the protocol for the corresponding specimen input volume:

    Specimen input volume: 1000 µL
    Program: DNA/Viral_NA_LV_2.0.

Protocol: Viral_NA_Universal_LV_1000_3.0.1 or 3.1
Elution Volume: 100 µL

- Small Volume (Serum, Urine, CSF or Amniotic Fluid) Protocol for MagNA Pure 96

  In the case of insufficient serum or urine specimen volume, RNA from serum, urine, CSF and amniotic fluid clinical specimens may be extracted using the MagNA Pure 96 DNA or Viral NA Small Volume Kit. When setting up the instrument for an extraction run, select the protocol for the corresponding specimen input volume:

  Specimen input volume: 200 µL
  Program: DNA/Viral_NA_SV_2.0.
  Protocol: Viral_NA_Universal_SV_3.0 or 3.1
  Elution volume: 100 µL

  OR with external lysis option:

  Specimen input volume: mix 200 µL of specimen with 250 µL of MP96 lysis buffer for a total volume of 450 µL before loading into the instrument.
  Program: DNA/Viral_NA_SV_2.0.
  *Protocol: Viral_NA_Plasma_ext_lys_SV_3.0 or 3.1
  Elution volume: 100 µL

  * "Viral_NA_Plasma_ext_lys_SV_3.0" is the instrument protocol title that cannot be changed; the term "Plasma" used in the program title does not imply that plasma is authorized for use with this assay.

- Small Volume (Whole Blood) Protocol for MagNA Pure 96

  RNA from whole blood (EDTA) clinical specimens may be extracted using the MagNA Pure 96 DNA and Viral NA Small Volume Kit.
  1. Add 250 µL of MP96 external lysis buffer to 200 µL of the whole blood (EDTA) specimen. Vortex on high and incubate at room temperature for 15 min.
  2. After incubation, vortex on high again and pulse centrifuge to bring the lysate to the bottom of the tube. Load samples into a MP96 sample cartridge.
  3. Load sample plate(s) onto the MP96 extraction instrument and load "small volume" kit reagents.
  4. When setting up the instrument for an extraction run, select the protocol for the corresponding specimen input volume:

     Specimen input volume: 450 µL
     Program: DNA/Viral_NA_SV_2.0 kit
     *Protocol: Viral_NA_Plasma_ext_lys_SV_3.0 or 3.1
     Elution Volume: 100 µL

     * "Viral_NA_Plasma_ext_lys_SV_3.0" is the instrument protocol title that cannot be changed; the term "Plasma" used in the program title does not imply

that plasma is authorized for use with this assay.

- MagNA Pure Compact Instrument
  - Large Volume (Serum, Urine, CSF, and Amniotic Fluid) Protocol for MagNA Pure Compact

    RNA from serum or urine clinical specimens may be extracted using the Large Volume MagNA Pure Compact (MPC) Nucleic Acid Isolation Kit I – Large Volume Kit. When setting up the instrument for an extraction run, select the protocol for the corresponding specimen input volume:

    Specimen input volume: 1000 µL
    *Protocol: Total_NA_Plasma_1000_V3_2
    Sample Material: Others
    Internal Control Volume: None
    Elution Volume: 100 µL

    * "Total_NA_Plasma _1000_V3_2" is the instrument protocol title that cannot be changed; the term "Plasma" used in the program title does not imply that plasma is authorized for use with this assay.

  - Small Volume (Serum, Urine, CSF, and Amniotic Fluid) Protocol for MagNA Pure Compact

    RNA from CSF and amniotic fluid clinical specimens may be extracted using the Small Volume MagNA Pure Compact (MPC) Nucleic Acid Isolation Kit I. When setting up the instrument for an extraction run, select the protocol for the corresponding specimen input volume:

    Specimen input volume: 200 µL
    *Protocol: Total_NA_Plasma_100_400_V3_2
    Elution Volume: 100 µL

    * "Total_NA_Plasma_100_400_V3_2" is the instrument protocol title that cannot be changed; the term "Plasma" used in the program title does not imply that plasma is authorized for use with this assay.

  - Small Volume (Whole Blood) Protocol for MagNA Pure Compact

    RNA from whole blood (EDTA) clinical specimens may be extracted using the MagNA Pure Compact Nucleic Acid Isolation Kit I

    1. Add 200 µL of the whole blood (EDTA) specimen to 300 µL of LC external lysis/binding buffer. Vortex on high, pulse centrifuge to bring the lysate to the bottom of the tube, and incubate at room temperature for 15 min.
    2. After incubation, vortex on high again and pulse centrifuge to bring the lysate to the bottom of the tube.
    3. Load sample tubes into the MPC instrument in the sample rack.

4. Follow manufacturer's instructions for setting up instrument reagents/disposables.
5. When setting up the instrument for an extraction run, select the protocol for the corresponding specimen input volume:

   Specimen input volume: 500 µL
   *Protocol: Total_NA_Plasma_external_lysis_V3_2
   Sample Material: Others
   Internal Control Volume: None
   Elution Volume: 100 µL

   * "Total_NA_Plasma_external_lysis_V3_2" is the instrument protocol title that cannot be changed; the term "Plasma" used in the program title does not imply that plasma is authorized for use with this assay.

- bioMérieux NucliSENS easyMAG Instrument
  Note: CDC has been notified of a product recall for certain lots of easyMAG extraction reagents. See Equipment and Consumables section for additional information.

  - Large Volume (Serum, Urine, CSF, and Amniotic Fluid) Protocol for the bioMérieux NucliSENS easyMAG

    RNA from clinical specimens may be extracted using the bioMérieux NucliSENS easyMAG reagents (Buffers 1-3, Lysis buffer, Magnetic Silica, and Disposables). Follow these settings for the off board lysis protocol:

    1. Place fresh disposable cartridges with their barcodes facing forward in the metal carrying rack. Each cartridge contains 8 reaction wells and the easyMAG instrument can process a total of 24 reactions (3 cartridges) per extraction run.
    2. Pipette 2 mL of lysis buffer into each reaction well of the disposable cartridge.
    3. Add 1000 µL of the clinical specimen into its respective well of the disposable cartridge.
    4. Pipette up and down ~5 times to mix.
    5. Incubate at room temperature for 10 minutes.
    6. Add 50 µL of vortexed magnetic silica to each well and mix ~5 times with a 1 mL pipette.
    7. Insert tips and load cartridges onto instrument in the correct order.

    Extraction Request Menu:
    Matrix: Other
    Protocol: Generic 2.0.1
    Volume (mL): 1.0
    Eluate (µL): 100
    Type: Lysed
    Priority: Normal

    Follow manufacturer's instructions to complete the remaining set up and extraction process.

NOTE: Transfer the purified nucleic acids to pre-labeled tubes within 30 minutes of extraction completion to avoid contamination by the magnetic silica stuck to the wall of the sample vessels. If contamination by the beads occurs, collect everything and place into another 1.7 mL tube and place on a magnetic rack to separate the beads from the eluate. Transfer the clean eluate to its pre-labeled tube for storage.

- Small Volume (Serum, Urine, CSF, and Amniotic Fluid) Protocol for the bioMérieux NucliSENS easyMAG

RNA from clinical specimens may be extracted using the bioMérieux NucliSENS easyMAG reagents (Buffers 1-3, Lysis buffer, Magnetic Silica, and Disposables). Follow these settings for the off board lysis protocol:

1. Place fresh disposable cartridges with their barcodes facing forward in the metal carrying rack. Each cartridge contains 8 reaction wells and the easyMAG instrument can process a total of 24 reactions (3 cartridges) per extraction run.
2. Pipette 2 mL of lysis buffer into each reaction well of the disposable cartridge.
3. Add 200 µL of the clinical specimen into its respective well of the disposable cartridge.
4. Pipette up and down ~5 times to mix.
5. Incubate at room temperature for 10 minutes.
6. Add 50 µL of vortexed magnetic silica to each well and mix ~5 times with a 1 mL pipette.
7. Insert tips and load cartridges onto instrument in the correct order.

Extraction Request Menu:
Matrix: Other
Protocol: Generic 2.0.1
Volume (mL): 0.2
Eluate (µL): 100
Type: Lysed
Priority: Normal

Follow manufacturer's instructions to complete the remaining set up and extraction process.

NOTE: Transfer the purified nucleic acids to pre-labeled tubes within 30 minutes of extraction completion to avoid contamination by the magnetic silica stuck to the wall of the sample vessels. If contamination by the beads occurs, collect everything and place into another 1.7 mL tube and place on a magnetic rack to separate the beads from the eluate. Transfer the clean eluate to its pre-labeled tube for storage.

Storage of Nucleic Acid Specimens

Retain specimen RNA extracts in a cold block or on ice until testing.

If testing will be delayed for more than 24 hours, freeze RNA immediately at ≤ -20ºC. Only thaw the number of RNA extracts that will be tested in a single day. Do not freeze or thaw RNA extracts more than once before testing. For long term storage > 7 days, freeze RNA extracts at ≤-20ºC. RNA extracts stored at ≤-20ºC should remain viable for 6 months.

Testing Algorithm

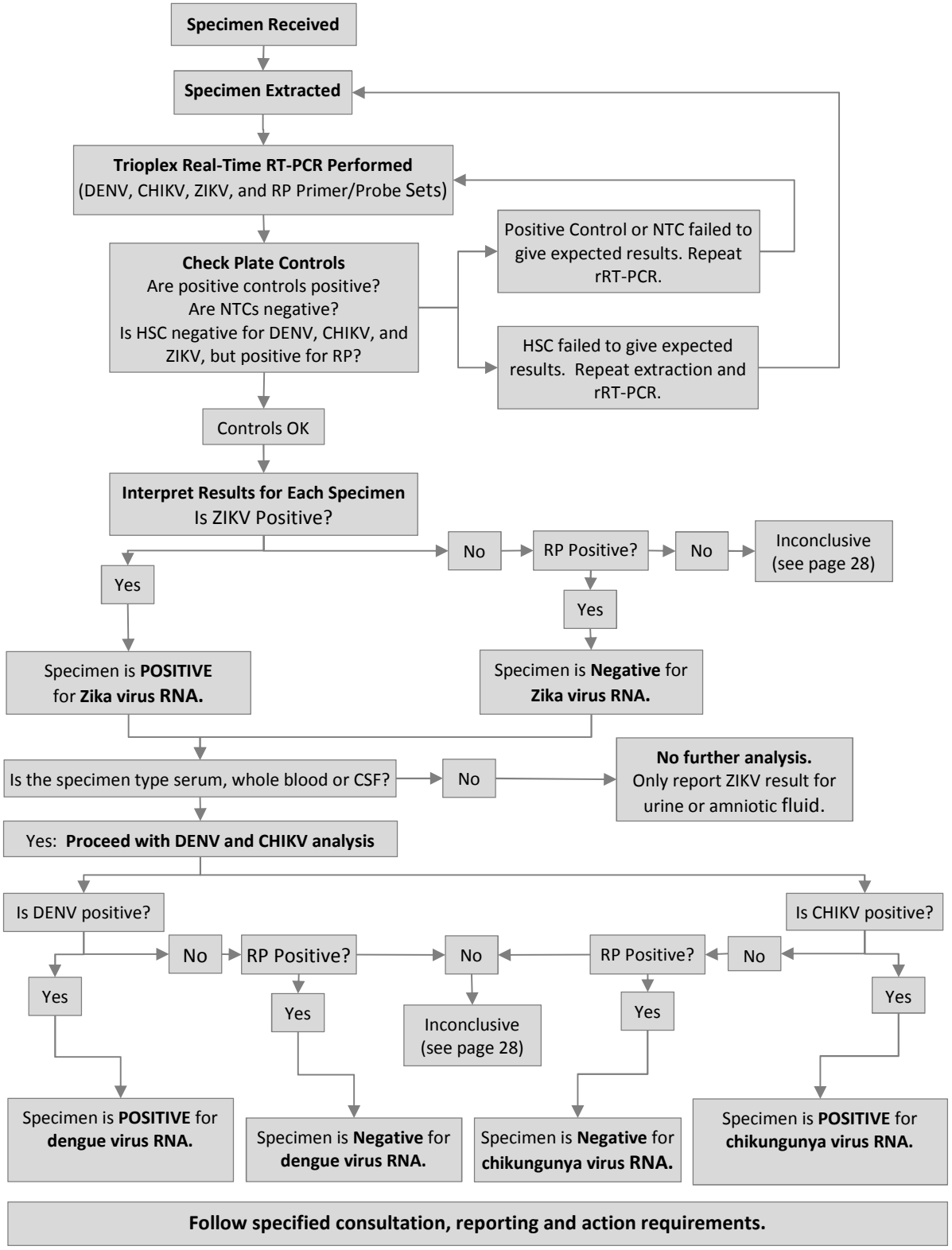


Figure 1: Summary of Test Results Interpretation

Real Time RT-PCR Assay

Stock Reagent Preparation

1. **Preparation of Real-time Primers/Probes**
    - Prior to rehydration, store kits at 2-8°C in the dark.
    - Precautions: These reagents should only be handled in a clean area and stored at appropriate temperatures (see below) in the dark. Freeze-thaw cycles should be avoided. Maintain cold when thawed.
    - Carefully rehydrate lyophilized reagents in 250 µL of 10 mM Tris, pH 7.4 to 8.2 or PCR grade (nuclease free) water and allow to rehydrate for 15 min at room temperature in the dark.
    - Vortex each tube to obtain a uniform mix and aliquot primers/probe mix in 50 µL volumes into 5 pre-labeled tubes.
    - Store rehydrated aliquots of primers and probes at -20°C or below. Do not store in frost-free freezers.
    - Rehydrated primers and probes may be stored frozen for up to 24 months.
    - Thawed aliquots of probes and primers may be stored in the dark up to 4 months at 2-8°C during frequent use.
    - Do not re-freeze thawed aliquots.

Table 3: Primer and Probe Descriptions

Sequence Designator	Part Number	Gene Location
DENV-F	SO3684	5'-UTR
DENV-R1		
DENV-R2		
DENV-P		
CHIKV-F	SO3685	nSP1
CHIKV-R		
CHIKV-P		
ZIKV-F	SO3686	Envelope gene
ZIKV-R		
ZIKV-P		
RP-F	SO3687	Human Ribonuclease P
RP-R		
RP-P		

2. **Assay Controls**
    - Nucleic acid extracted from inactivated dengue virus
    - Nucleic acid extracted from inactivated chikungunya virus
    - Nucleic acid extracted from inactivated Zika virus

3. **No Template Control (NTCs)** (not provided)
   - Sterile, nuclease-free water
   - Aliquot in small volumes
   - Used to check for contamination during specimen extraction and/or plate set-up

4. **HSC extraction control**
   - Human Specimen Control must be extracted and processed with each batch of samples to be tested following the same procedure as with patient samples.
   - Do not dilute extracted RNA prior to testing

5. **Master mix**
   NOTE: Either **SuperScript® III Platinum® One-Step Quantitative RT-PCR System** or **qScript™ One-Step qRT-PCR kit, Low Rox ™** may be used.

   a. **SuperScript® III Platinum® One-Step qRT-PCR System**
      - Place 2X PCR Master Mix and Superscript III RT/Platinum Taq enzyme mix in a cold rack at 2-8°C.
      - Completely thaw the 2X PCR Master Mix vial.
      - Mix the 2X PCR Master Mix by inversion 10 times.

   b. **qScript™ One-Step qRT-PCR kit, Low Rox ™**
      - Thaw all components, except qScript One-Step RT, at room temperature.
      - Mix vigorously.
      - Centrifuge to collect contents to bottom of tube before using.
      - Place all components on ice after thawing.

Equipment Preparation (AB 7500 Fast Dx or QuantStudio™ Dx instrument)

- Turn on instrument and allow the block to reach optimal temperature.
- Prepare experiment, plate set up and select cycling protocol on the instrument of choice, see Table 6 for suggested PCR plate setup.
- For instructions on PCR run setup on the AB 7500 Fast Dx instrument, see PCR Run section below.
- For instructions on programming, calibration and PCR run setup on the QSDx instrument, see Appendix A.

Master Mix and Plate Set-up

NOTES:

- **Plate set-up configuration can vary with the number of specimens and work day organization.**
- **NTCs and assay controls must be included in each run.**
- **Laboratories may elect to run the Trioplex assay as singleplex reactions.**
  - **Amniotic fluid and urine specimens may be tested with ZIKV alone as a singleplex reaction.**

- **Other specimen types should be tested with all three primer and probe sets either as a multiplex reaction or as singleplex reactions in three individual wells. Please note that DENV and/or CHIKV singleplex reactions should not be run alone on any of the specimen types.**
- **All specimens must be tested by RP in each PCR run. RP is always run as a singleplex reaction in a separate well from Trioplex virus-specific primer/probe sets.**
- **Instructions for master mix and plate set-up are identical for both the AB 7500 Fast Dx and QuantStudio™ Dx instruments.**

- In the reagent set-up room clean hood, place primer/probes on ice or cold-block. Keep cold during preparation and use.
- Thaw 2X Reaction Mix (SuperScript III or qScript) prior to use.
- Mix primer/probes by briefly vortexing.
- Briefly centrifuge primers/probes and return to ice or cold block.
- Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the NTC reactions and for pipetting error (see Table 4).
- Use the following guide to determine N:
  - If the number of samples (n) including controls equals 1 through 14, then N = n + 1
  - If the number of samples (n) including controls is greater than 15, then N = n + 2

Prepare reaction mixture according to the following tables (**Tables 4 and 5**). Keep reaction mixture on ice or in cold block.

Table 4: Trioplex rRT-PCR Reaction Mixture

TRIOPLEX Reaction Mix			
Multiplex Option		**Singleplex Option**	
Component	**Quantity/Reaction (µL)**	**Component**	**Quantity/Reaction (µL)**
Water	N x 0.5 µL	Water	N x 1.5 µL
2x PCR Reaction Mix	N x 12.5 µL	2x PCR Reaction Mix	N x 12.5 µL
DENV Mix	N x 0.5 µL	Primer/Probe Mix (DENV, CHIKV **or** ZIKV)	N x 0.5 µL
CHIKV Mix	N x 0.5 µL		
ZIKV Mix	N x 0.5 µL		
Enzyme Mix	N x 0.5 µL	Enzyme Mix	N x 0.5 µL
Subtotal	**N x 15 µL**	**Subtotal**	**N x 15 µL**
Sample RNA	10 µL	Sample RNA	10 µL (per reaction tube)
TOTAL	**25 µL**	**TOTAL**	**25 µL**

NOTE: The same reaction mixture volumes may be used for either the SuperScript III or qScript kit.

Table 5: RP PCR Reaction Mixture

RP Internal Control Reaction Mix	
Component	**Quantity/Reaction (µL)**
Water	1.5 µL
2x PCR Reaction Mix	12.5 µL
RP Mix	0.5 µL
Enzyme Mix	0.5 µL
Subtotal	**15µL**
Sample RNA	10 µL
TOTAL	25 µL

NOTE: The same reaction mixture volumes may be used for either the SuperScript or qScript kit.

PCR Plate Setup

a. In the reagent set-up room clean hood, while maintaining PCR plate on ice (or cold block), add 15 µL of reaction mixture to all wells being utilized.
b. Before moving the plate to the nucleic acid handling area, add 10 µL of nuclease-free water to the NTC wells
c. Loosely apply optical strip caps or optical tape to the tops of the reaction wells and move plate to the nucleic acid handling area on cold block or ice.
d. Remove optical strip caps or optical tape and add 10 µL of extracted sample RNA to each corresponding sample well. **Change tips after each sample addition.**
e. **Add 10 µL of DENV-1-4 Positive Control, CHIKV positive Control, ZIKV Positive Control and HSC (RP positive control) to separate wells as indicated in Table 6.** **Note:** If only running ZIKV as a singleplex reaction, as for amniotic fluid or urine specimens, only the ZIKV Positive Control and HSC are required to be included in the run.

Table 6: Example of Trioplex rRT-PCR plate layout for 3 samples (multiplex option)

Mastermix Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Trioplex	Trioplex	Trioplex	Trioplex	Trioplex							
B	RP	RP	RP	RP	RP							
C												
D												
E												Trioplex
F												Trioplex
G												Trioplex
H												

Template Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	HSC	H2O NTC							
B	S1	S2	S3	HSC	H2O NTC							
C												
D												
E												DENV PC
F												CHIKV PC
G												ZIKV PC
H												

Positive controls: E12-H12

f. Seal plate with optical tape or caps and load plate on Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument.

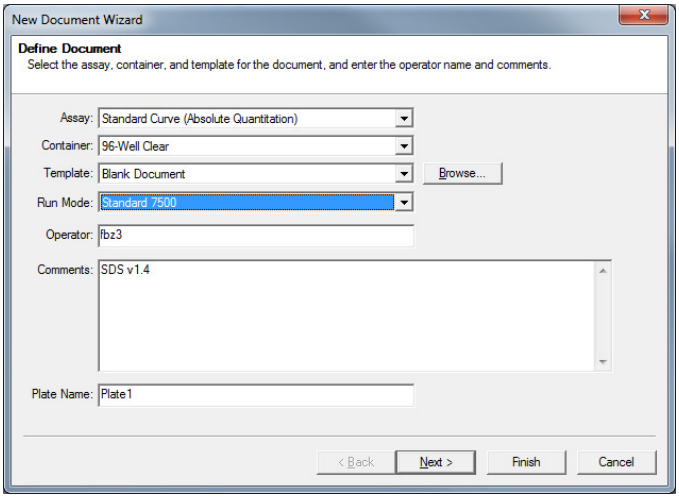
PCR Run

NOTE: Programming instructions for the QuantStudio™ Dx instrument are located in Appendix A. Instructions below are for the the AB 7500 Fast Dx.

g. Launch the AB 7500 software and select **Create new document**.

h. Select **Standard 7500** on the Run Mode menu and click **Next** (**Figure 2**).

Figure 2: Select Run Mode



i. Create a new detector for each target by clicking on **New Detector** (**Figure 3**)**,** name DENV, select reporter dye **FAM** and leave Quencher Dye as **none** (**Figure 4**).

Figure 3: Select Detector

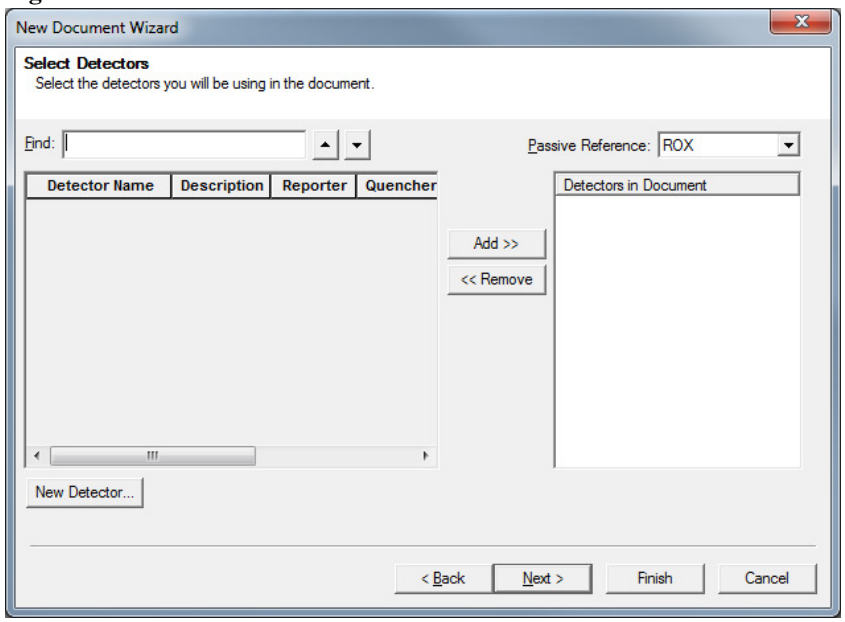
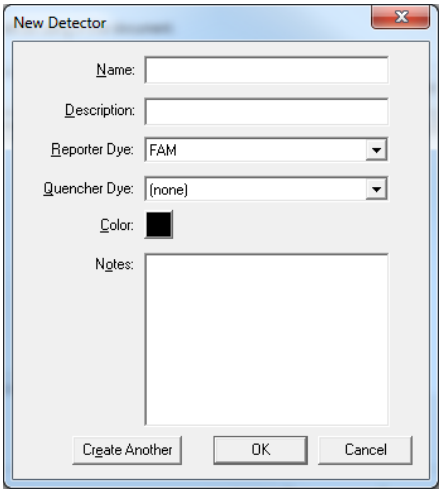


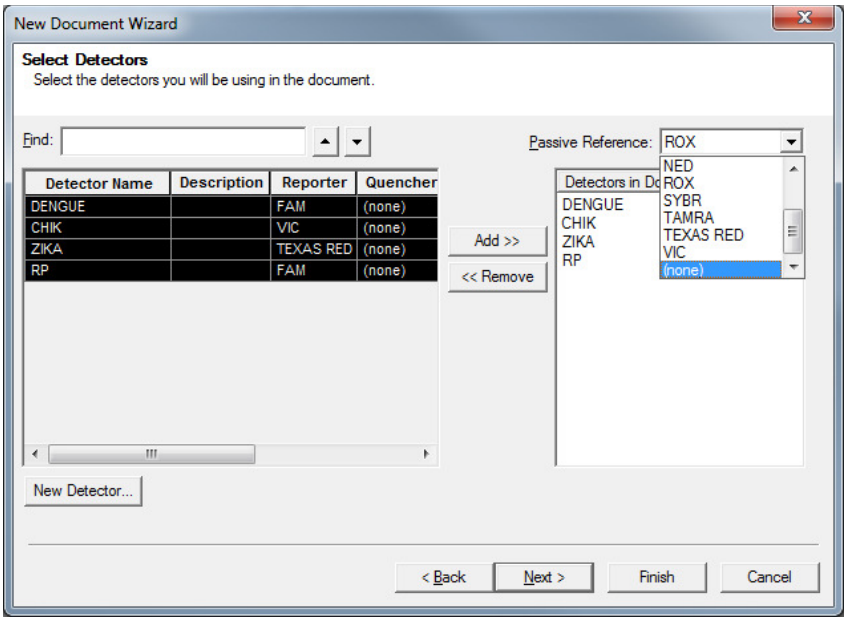
Figure 4: Reporter and Quencher Settings



j. Repeat for CHIKV, select reporter dye **VIC** and leave Quencher Dye **none**.
k. Repeat for ZIKV, select reporter dye **Texas Red** and leave Quencher Dye **none**.
l. Repeat for RP, select reporter dye **FAM** and leave Quencher Dye **none**.
m. On the Select Detectors screen, select **DENV** and click on **Add**.

n. Switch Passive Reference from **ROX** to **none** (Figure 5)**.**

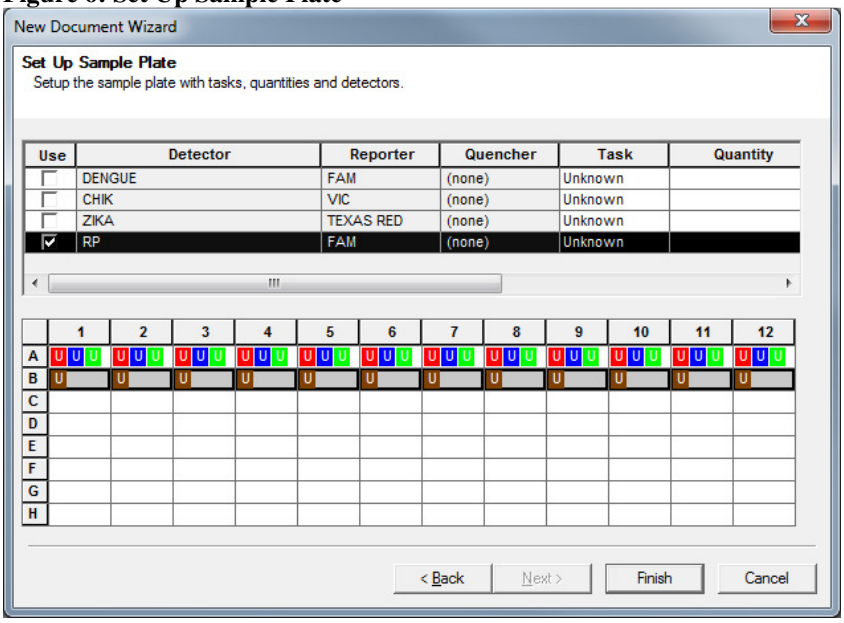
Figure 5: Passive Reference Selection



o. On the Select Detectors screen, select **CHIKV** and click on **Add**.
p. Switch Passive Reference from ROX to **none**.
q. On the Select Detectors screen, select **ZIKV** and click on **Add**
r. Switch Passive Reference from ROX to **none**.
s. On the Select Detectors screen, select **RP** and click on Add
t. Switch Passive Reference from ROX to **none**.
u. On the Set Up Sample Plate window, highlight corresponding wells and select **DENV**, **CHIKV**, and **ZIKV** detectors (**Figure 6**).
Note: If using the singleplex option, you will not be employing all three detectors in all reaction wells:

- Highlight wells containing DENV reactions and select DENV detector.
- Highlight wells containing CHIKV reactions and select CHIKV detector.
- Highlight wells containing ZIKV reactions and select ZIKV detector.

Figure 6: Set Up Sample Plate



v. Double click each well to enter sample name.

w. Select Instrument tab and define thermocycling conditions according to the master mix used:

(1) Stage 1: **30 min** at **50°C**; **1 rep**.

(2) Stage 2:

- SuperScript III: **2 min** at **95°C**; **1 rep**
- qScript: **5 min** at **95°C**; **1 rep**

(3) Stage 3, step 1: **15 sec** at **95°C**

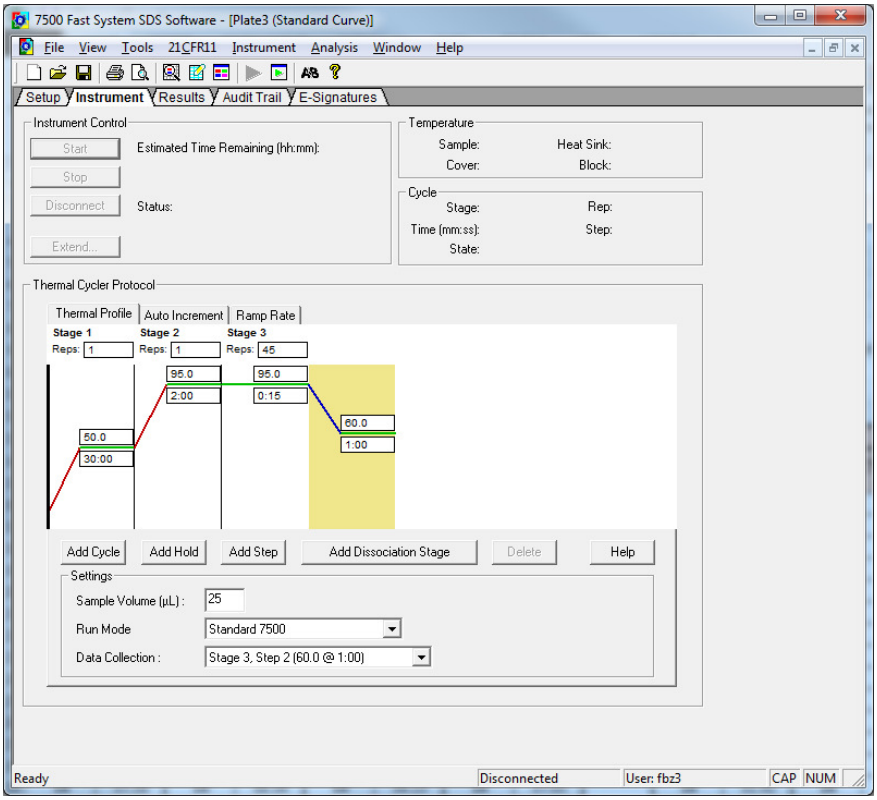
(4) Stage 3, step 2: **1 min** at **60°C**

(5) Stage 3: change reps to **45 cycles** (**Tables below and Figure 7**)

SuperScript III Thermocycling Conditions			
STAGE 1	**STAGE 2**	**STAGE 3 STEP 1**	**STAGE 3 STEP 2**
30 min	**2 min**	15 sec	1 min
50°C	95°C	95°C	60°C
1 rep	1 rep		
		45 cycles	

qScript One-Step Thermocycling Conditions			
STAGE 1	**STAGE 2**	**STAGE 3 STEP 1**	**STAGE 3 STEP 2**
30 min	**5 min**	15 sec	1 min
50°C	95°C	95°C	60°C
1 rep	1 rep		
		45 cycles	

Figure 7: Set Thermal Cycling Conditions



(6) Under **Settings**, change volume to **25 µL**

(7) Under **Settings**, **Run Mode**, select **Standard 7500**

(8) Stage 3, step 2 should be highlighted in yellow indicating data collection

x. Select **Save As**, designate file name and folder

y. Click **Start**. Instrument will initialize and calculate time of run.

Data Analysis

After completion of the run, save and analyze the data following the instrument manufacturer's instructions. Although some versions of the software for the AB 7500 Fast Dx and the QSDx may look similar, data files are not cross-compatible. Follow the same data analysis guide for both instruments. Analyses should be performed separately for each target using a manual threshold setting. Thresholds should be adjusted to fall within the beginning of the exponential phase of the fluorescence curves and above any background signal. The procedure chosen for setting the threshold should be used consistently. CT value results are displayed on the Report tab on the AB 7500 Fast Dx software or the Well Table tab on the QSDx software. Final results can be exported from the AB 7500 Fast Dx as a *.csv file or by copying and pasting from the QSDx Well Table screen into a spreadsheet.

Interpreting Test Results

Regardless of the specimen type being tested, prepare the PCR mix as indicated in "PCR Plate Setup."

TEST VALIDITY DETERMINATION

Before the results can be determined for each clinical specimen, the plate run must be determined to be **valid**. For a test to be valid, the controls must yield the expected results:

- Assay controls (nucleic acid extracted from inactivated DENV, CHIKV, and ZIKV) should be positive and within the expected C_T value range. If assay controls are negative
  - Repeat the plate.
  - If repeat testing generates a negative result from the positive control, contact the LRN helpdesk for consultation.
- NTCs should be negative. If NTCs are positive
  - Clean potential DNA contamination from bench surfaces and pipettes in the reagent setup and template addition work areas.
  - Repeat samples only for the targets that are inappropriately amplified.
  - Extract and test multiple NTCs.
  - Discard working reagent dilutions and remake from fresh stocks.
- HSC (extraction control) should be
  - Positive with RP primer/probe set due to the human DNA in the HSC
  - Negative with virus primer/probe sets. A positive result with the HSC and virus primer/probes would indicate cross-contamination has occurred. If a positive result is obtained, follow the cleaning procedure described above.
- RP Assay for each specimen should be **positive**.
  - If RP Assay for a specimen sample is *negative* and the Trioplex rRT-PCR assays are all *negative* for specimen samples:
    i. Follow the instructions below:

All Specimen Types
1. Repeat rRT-PCR test of specimen using RP and Trioplex assay.
2. Repeat extraction from new specimen aliquot if RP Assay is *negative* for specimens after repeat testing.
3. After repeat extraction and repeat rRT-PCR testing, if DENV, CHIKV, and/or ZIKV is *positive*, consider the result a true *positive* and continue to follow the testing algorithm.
4. If you are unable to resolve the results for a specimen, test other specimens from the patient, if available, or request the collection of additional specimens.
5. Report result as *Inconclusive* to LRN Program Office. LRN member laboratories must report through LRN Results Messenger or LIMS integration.

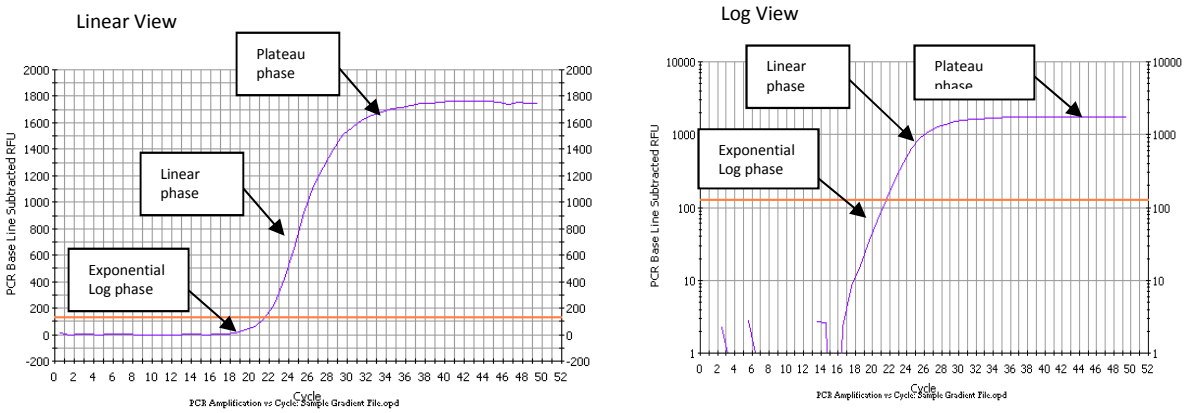
- If RP Assay for a specimen sample is *negative*, but DENV, CHIKV, and/or ZIKV is *positive* for specimen samples:
    - Do not repeat rRT-PCR test and consider the results of the Trioplex rRT-PCR valid.

If all controls have been performed appropriately, proceed to analyze each target.

NOTE: *The following section contains figures that are provided as generic examples. They are not specific to this assay.*

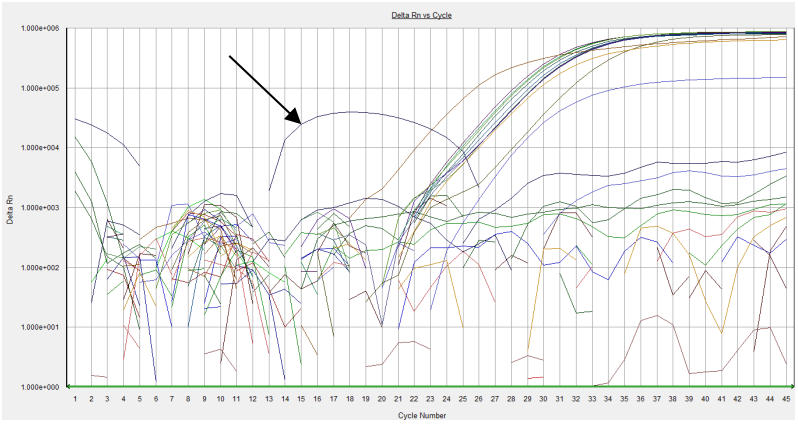
- True positives should produce exponential curves with logarithmic, linear, and plateau phases (**Figure 8**).
  (Note: Weak positives will produce high C_T values that are sometimes devoid of a plateau phase; however, the exponential plot will be seen.)

Figure 8: Linear and log views of PCR curves noting each stage of the amplification plots.



- For a sample to be a true positive, the curve must cross the threshold in a similar fashion as shown in **Figure 8**. It must NOT cross the threshold and then dive back below the threshold.
  Samples containing high concentrations of RNA could generate atypical amplification curves that start to grow exponentially at early cycles (C_T<12) and display atypical patterns (**Figure 9**).

Figure 9: An example of an atypical amplification curve caused by high concentrations of chikungunya virus in the CHIKV assay.

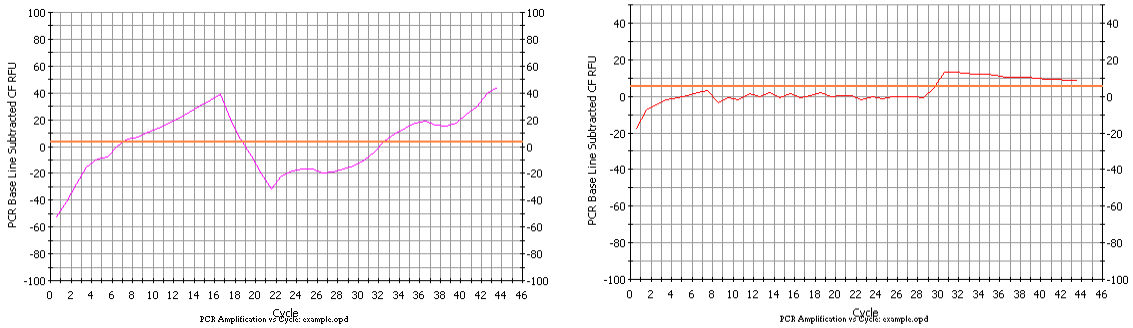


If such amplification curves are obtained, it is recommended that the eluted RNA be diluted 1:10 and 1:100 in nuclease-free water and the PCR test be repeated as indicated above. If true positive curves (exponential curves with logarithmic, linear, and plateau phases [**Figure 8**]) are then observed, the specimen should be deemed positive. NOTE: the C_t obtained will not reflect actual viremia since the sample will have been diluted.

Large bubbles in the PCR plate well could cause similar atypical amplification curve patterns.

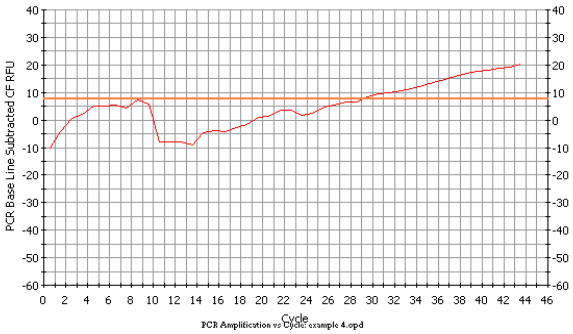
- **Figure 10** shows examples of false positives that do not amplify exponentially.

Figure 10: Examples of false positive curves.



- To better understand and evaluate challenging curves more effectively, use the background fluorescence view (Rn versus Cycle with AB software) to determine if the curve is actually positive. In this view, a sharp increase in fluorescence indicates a true positive while a flat line (or wandering line) indicates no amplification.
  - **Figure 11** shows a curve with a C_T value of 29.2 though it is evident that the sample is negative by looking at the background fluorescence view.
  - **Figure 12** shows an amplification plot with 3 curves: a moderately weak positive with a C_T of 36.6 (black), a very weak positive with a C_T of 42.1 (red), and a negative control (blue). The weak positive (C_T= 42.1) is verified to be positive by the sharp increase in fluorescence seen in the background fluorescence view.

Figure 11: Amplification plot of a sample with a "wandering" curve (left) and the corresponding background fluorescence view (right).



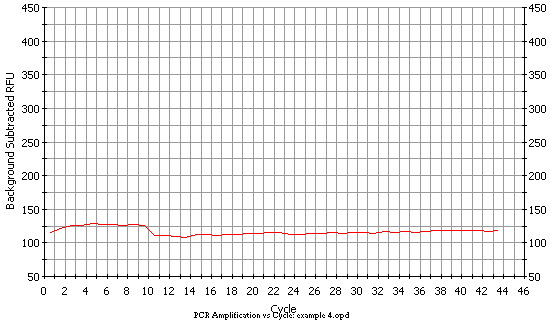
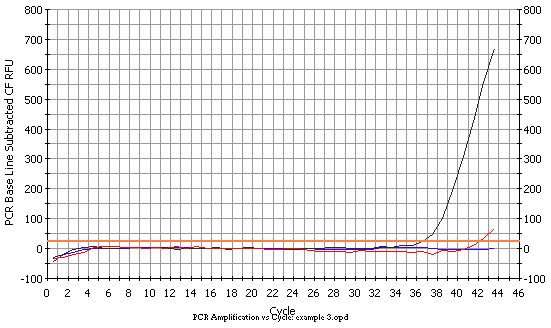
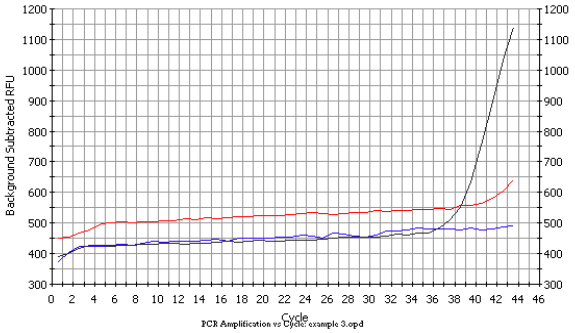


Figure 12: Amplification plot of three samples in the linear view (left) and the corresponding background fluorescence view (right).





- A note on weak positive samples: Weak positives should always be interpreted with caution. Look carefully at the fluorescence curves associated with these results. If curves are true exponential curves, the reaction should be interpreted as positive.
    - If repeat testing of a weak specimen is necessary, it is important to repeat the sample in replicates as a single repeat test run has a high likelihood of generating a discrepant result. The repeat testing should be conducted in singleplex using only primer/probe set(s) giving the weak positive signal.
    - If re-extracting and re-testing the specimen, it may be helpful to elute in a lower volume to concentrate the sample.
    - The LRN helpdesk is available for guidance to help determine if repeat testing may be warranted and to discuss additional testing strategies as appropriate.

SPECIMEN INTERPRETATION AND REPORTING INSTRUCTIONS

All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted.

The result generated for a primer and probe set is interpreted as positive if the reaction generates a fluorescence growth curve that crosses the threshold within (<) 38 cycles.

The result generated for a primer and probe set is interpreted a negative if:

- the reaction generates a fluorescence growth curve that crosses the threshold at or above (≥) 38 cycles, OR
- the reaction fails to generate a fluorescence growth curve that crosses the threshold.

Table 7: Trioplex rRT-PCR Interpretation and Reporting Instructions for Whole Blood (EDTA), Serum and CSF Specimens

ZIKV	DENV	CHIKV	RP	Interpretation	Reporting	Actions
-	-	-	+	Negative	No Zika, dengue, or chikungunya RNA detected by rRT-PCR	Report results to CDC. No further testing required. Note: If date of onset of symptoms is in doubt or if patient is asymptomatic, serological testing may be recommended. Refer to CDC algorithm.*
-	-	-	-	Inconclusive	Specimen inconclusive for the presence of Zika, dengue, and chikungunya RNA by rRT-PCR. An inconclusive result may occur in the case of an inadequate specimen.	Repeat extraction and rRT-PCR. If unable to resolve inconclusive result for a serum specimen, request collection of additional serum from the patient. Report inconclusive results to CDC.
-	+	-	+/-	Positive for DENV, but negative for ZIKV and CHIKV.	Dengue RNA detected by rRT-PCR. No Zika or chikungunya RNA detected.	Report results to CDC. Forward specimen to CDC. Refer to CDC algorithm.*
-	-	+	+/-	Positive for CHIKV, but negative for ZIKV and DENV.	Chikungunya RNA detected by rRT-PCR. No dengue or Zika RNA detected.	
+	-	-	+/-	Positive for ZIKV, but negative for DENV and CHIKV.	Zika RNA detected by rRT-PCR. No dengue or chikungunya RNA detected.	
-	+	+	+/-	Positive for DENV and CHIKV, but negative for ZIKV.	Dengue and chikungunya RNA detected by rRT-PCR. No Zika RNA detected.	
+	+	-	+/-	Positive for ZIKV and DENV, but negative for CHIKV	Zika and dengue RNA detected by rRT-PCR. No chikungunya RNA detected.	
+	-	+	+/-	Positive for ZIKV and CHIKV, but negative for DENV	Zika and chikungunya RNA detected by rRT-PCR. No dengue RNA detected.	
+	+	+	+/-	Positive for ZIKV, DENV, and CHIKV	Zika, dengue, and chikungunya RNA detected by rRT-PCR.	

* CDC Zika laboratory guidance and testing algorithm may be found on CDC's website: http://www.cdc.gov/zika/laboratories/lab-guidance.html
If you have positive specimens to forward to CDC, please notify the LRN Helpdesk (LRN@cdc.gov) and request specimen shipment instructions.

Table 8: Trioplex rRT-PCR Interpretation and Reporting Instructions for Urine, and Amniotic Fluid Specimens

ZIKV	RP	Interpretation	Reporting	Actions
-	+	Negative	No Zika RNA detected by rRT-PCR.	Report results to CDC. Refer to CDC algorithm.*
-	-	Inconclusive	Specimen inconclusive for the presence of Zika RNA by rRT-PCR. An inconclusive result may occur in the case of an inadequate specimen.	Repeat extraction and rRT-PCR. If repeat testing does not resolve inconclusive result, do not test further. Report results to CDC.
+	+/-	Positive	Zika RNA detected by rRT-PCR	Report results to CDC. Forward specimen to CDC. Refer to CDC algorithm.*

*CDC Zika laboratory guidance and testing algorithm may be found on CDC's website: http://www.cdc.gov/zika/laboratories/lab-guidance.html
If you have positive specimens to forward to CDC, please notify the LRN Helpdesk (LRN@cdc.gov) and request specimen shipment instructions.

NOTE: All test results generated using the Trioplex rRT-PCR by LRN laboratories must be sent to CDC using LRN Results Messenger. Please refer to the LRN Data Messaging Policy (found under Documents/LRN Specific Information/LRN Policy Statements on the LRN website). For questions regarding this policy, please contact the LRN Helpdesk at LRN@cdc.gov.

Positive cases must also be reported through ArboNet.

NOTE: Please refer to the **Interpreting Test Results** section for detailed guidance on interpreting weak positives or questionable curves.

Assay Limitations

The Trioplex Real-time RT-PCR Assay is for prescription use only.

Interpretation of rRT-PCR test results must account for the possibility of false-negative and false-positive results. False-negative results can arise from:

- poor sample collection
- degradation of the viral RNA during shipping or storage
- 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, whole blood, and/or urine)
- failure to follow the authorized assay procedures
- failure to use authorized extraction kit and platform

Application of appropriate assay controls that identify poor-quality specimens (such as RNase P) and adherence to CDC guidelines for DENV, CHIKV, and ZIKV testing can help avoid most false-negative results.

The most common cause of false-positive results is contamination with previously amplified DNA.

Liberal use of negative control samples in each assay can help ensure that laboratory contamination is detected and that false positive test results are not reported.

Negative results do not preclude infection with Zika virus and should not be used as the sole basis of a patient treatment/management decision. All results should be interpreted by a trained professional in conjunction with review of the patient's history and clinical signs and symptoms.

Limited information is available about Zika virus RNA shedding patterns in infected individuals across specimen types over time.

This assay is for *in vitro* diagnostic use under FDA Emergency Use Authorization only and is limited to qualified laboratories designated by CDC.

All specimens should be handled as if infectious. Proper biosafety precautions, including personal protective equipment, must be used when handling specimen materials. Additional information on safe handling of Zika virus specimens can be found at: http://www.cdc.gov/zika/laboratories/lab-safety.html.

Proper collection, storage and transport of specimens are essential for correct results.

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.

Performance has only been established with the specimen types listed in the Intended Use. Other specimen types have not been evaluated.

Performance Characteristics

NOTE: All data presented in this section was generated using the AB 7500 Fast Dx instrument unless otherwise stated.

Section 1 - Limit of Detection

Overview

The limits of detection (LoD) for the Trioplex rRT-PCR were established by making 10-fold serial dilutions of stock virus (either ZIKV, DENV, or CHIKV) in matrix. The greatest dilution at which the Trioplex assay could still detect ≥95% of contrived replicates was considered the LoD. The LoD dilution was compared to a previously established concentration standard curve of viral RNA (either ZIKV, DENV, or CHIKV) to determine genome copy equivalents per milliliter (GCE/mL). These LoDs are summarized in Table 9 for the ZIKV primer/probe set and in Table 10 for the DENV and CHIKV primer/probe sets.

- The **ZIKV** LoD for **small volume** automated extraction (**serum and urine**) was determined using the **MagNA Pure LC 2.0** Instrument using SuperScript III (SS III) [Section 1.A.].
- The **DENV** LoD for **small volume** automated extraction (**serum and urine**) was determined

using the **MagNA Pure LC 2.0** Instrument using SS III [Section 1.B.].

- The **CHIKV** LoD for **small volume** automated extraction (**serum and urine**) was determined using the **MagNA Pure LC 2.0** Instrument using SS III [Section 1.C.].
- The **ZIKV** LoD for **large volume** automated extraction (**serum and urine**) was determined using the **MagNA Pure 96** Instrument [Section 4.C.].
- The **ZIKV** LoD for **small volume** automated extraction (EDTA **whole blood**) was determined using the **MagNA Pure 96** Instrument [Section 4.D.].

All bridging studies used the same stock virus that was used in the LoD studies. Automated extraction bridging studies (Section 4) used the LoD dilution of ZIKV (determined in LoD studies) to establish non-inferiority to the automated extraction platforms used in the LoD studies. If the Trioplex assay could detect ≥95% of contrived replicates at the established LoD dilution, the automated extraction platform being used in the bridging study was deemed non-inferior.

The qScript mastermix bridging study (Section 4) used the LoD dilution of ZIKV, DENV, and CHIKV (determined in LoD studies) to establish non-inferiority to the SuperScript mastermix used in the LoD studies. If the Trioplex assay could detect ≥95% of contrived replicates at the established LoD dilution, the qScript mastermix was deemed non-inferior.

Table 9: Overall limit of detection data summary for ZIKV

	Sample Type	Extraction	Mastermix	ZIKV LoD	Refer to Section
Singleplex Option	Serum	MP 96	SS III	Non Inferior to MP LC 2.0 Instrument using SSIII	4H
Multiplex	Serum	MP LC 2.0	SS III	1.93 x 10^4 (GCE/mL)	1A
		QIAamp	SS III	Non Inferior to MP LC 2.0 Instrument using SS III	4E
		MP 96	SS III		1D
		MP LC 2.0	qScript		4A
	Serum Large Volume	MP 96	SS III	2.45 x 10^3 (GCE/mL)	1D
		MP Compact	SS III	Non-inferior to MP 96 Instrument	4C
		EasyMAG	SS III		4D
	Urine	MP LC 2.0	SSIII	5.38 x 10^4 (GCE/mL)	1A
		QIAamp	SS III	Non Inferior to MP LC 2.0 Instrument using SS III	4E
		MP 96	SS III		1D
		MP LC 2.0	qScript		4A
	Urine Large Volume	MP 96	SS III	4.64 x 10^3 (GCE/mL)	1D
		MP Compact	SS III	Non inferior to MP 96 Instrument	4C
		EasyMAG	SS III		4D
	Whole Blood	MP LC 2.0	SS III	2.43 x 10^3 (GCE/mL)	1E
		MP 96	SS III	2.43 x 10^3 (GCE/mL)	
		MP Compact	SS III	2.43 x 10^3 (GCE/mL)	

GCE = genome copy equivalent
MP LC 2.0 = MagNA Pure LC 2.0 Extraction Instrument
MP 96 = MagNA Pure 96 Extraction Instrument
MP Comp = MagNA Pure Compact Instrument
EasyMAG = bioMérieux NucliSENS easyMAG Instrument
QIAamp = QIAamp DSP Viral RNA Mini Kit

Table 10: Overall limit of detection data summary for DENV and CHIKV

	Sample Type	Extraction	Mastermix	DENV LoD	CHIKV LoD	Refer to Section
Multiplex	Serum	MP LC 2.0	SS III	DENV-1 5.82 x 10^4 (GCE/mL) DENV-2 8.25 x 10^4 (GCE/mL) DENV-3 4.36 x 10^4 (GCE/mL) DENV-4 2.68 x 10^4 (GCE/mL)	1.28 x 10^5 (GCE/mL)	1B and 1C
		MP 96	SS III	Non Inferior to MP LC 2.0 Instrument using SS III	Non Inferior to MP LC 2.0 Instrument using SS III	4B
		MP LC 2.0	qScript			4A
	Whole Blood	MP 96	SS III	DENV-2 4.28 x 10^3 (GCE/mL)	4.80 x 10^3 (GCE/mL)	1E

A. ZIKV LoD (Small Volume Extraction with MagNA Pure LC 2.0):
Limit of detection for the ZIKV primer and probe set was evaluated in both normal human serum and in urine using the French Polynesia 2013 strain of Zika virus (live). Five 10-fold serial dilutions were prepared for serum and urine. Each concentration was extracted 20 times using the MagNA Pure LC 2.0 Instrument (200 µL extraction volume) and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Results for serum and urine are summarized in Tables 11 and 12, respectively.

Table 11: ZIKV LoD in serum (small volume extraction – MP LC 2.0) – Trioplex

Dilution	GCE/mL	ZIKV # Positive	Avg. C_T	DENV # Positive	CHIKV # Positive
10^{-3}	1.93 x 10^7	20/20	28.10	0/20	0/20
10^{-4}	1.93 x 10^6	20/20	31.58	0/20	0/20
10^{-5}	1.93 x 10^5	20/20	34.84	0/20	0/20
10^{-6}	1.93 x 10^4	20/20	37.17	0/20	0/20
10^{-7}	1.93 x 10^3	0/20	ND	0/20	0/20

ND = not detected

Table 12: ZIKV LoD in urine (small volume extraction – MP LC 2.0) - Trioplex

Dilution	GCE/mL	ZIKV # Positive	Avg. C_T	DENV # Positive	CHIKV # Positive
10^{-3}	5.38 x 10^7	20/20	26.8	0/20	0/20
10^{-4}	5.38 x 10^6	20/20	30.03	0/20	0/20
10^{-5}	5.38 x 10^5	20/20	33.51	0/20	0/20
10^{-6}	5.38 x 10^4	19/20	36.98	0/20	0/20
10^{-7}	5.38 x 10^3	0/20	ND	0/20	0/20

ND = not detected

B. DENV LoD (Small Volume Extraction - serum):
Limit of detection for the DENV primer and probe set was evaluated in normal human serum using a representative strain from each dengue virus serotype (DENV-1 Puerto Rico 1998, DENV-2 Puerto Rico 1998, DENV-3 Puerto Rico 2004, DENV-4 Puerto Rico 1998).

Five 10-fold serial dilutions of each strain in each matrix were prepared. For each matrix, each concentration was extracted 20 times using the MagNA Pure LC 2.0 Instrument (200 µL extraction volume) and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Results are summarized in Table 13.

Table 13: DENV LoD in serum (small volume extraction – MP LC 2.0) - Trioplex

Dilution		GCE/mL	DENV # Positive	Avg. C_T	ZIKV # Positive	CHIKV # Positive
Serotype 1	1	5.82×10^5	20/20	32.55	0/20	0/20
	2	5.82×10^4	20/20	36.75	0/20	0/20
	3	5.82×10^3	1/20	39.08	0/20	0/20
	4	5.82×10^2	0/20	41.88	0/20	0/20
	5	5.82×10^1	0/20	ND	0/20	0/20
Serotype 2	1	8.25×10^6	20/20	29.18	0/20	0/20
	2	8.25×10^5	20/20	32.17	0/20	0/20
	3	8.25×10^4	19/20	37.03	0/20	0/20
	4	8.25×10^3	0/20	38.94	0/20	0/20
	5	8.25×10^2	0/20	ND	0/20	0/20
Serotype 3	1	4.36×10^6	20/20	29.99	0/20	0/20
	2	4.36×10^5	20/20	33.54	0/20	0/20
	3	4.36×10^4	20/20	37.07	0/20	0/20
	4	4.36×10^3	0/20	39.78	0/20	0/20
	5	4.36×10^2	0/20	ND	0/20	0/20
Serotype 4	1	2.68×10^6	20/20	30.61	0/20	0/20
	2	2.68×10^5	20/20	33.86	0/20	0/20
	3	2.68×10^4	19/20	37.33	0/20	0/20
	4	2.68×10^3	1/20	39.46	0/20	0/20
	5	2.68×10^2	0/20	ND	0/20	0/20

ND = not detected

C. CHIKV LoD (Small Volume Extraction - serum):
Limit of detection for the CHIKV primer and probe set was evaluated in normal human serum using the Puerto Rico 2014 strain of chikungunya virus. Five 10-fold serial dilutions in each matrix were prepared. For each matrix, each concentration was extracted 20 times using the MagNA Pure LC 2.0 Instrument (200 µL extraction volume) and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Results for serum are summarized in Table 14.

Table 14: CHIKV LoD in serum (small volume extraction – MP LC 2.0) - Trioplex

Dilution	GCE/mL	CHIKV # Positive	Avg. C_T	ZIKV # Positive	DENV # Positive
1	1.28×10^7	20/20	31.95	0/20	0/20
2	1.28×10^6	20/20	35.42	0/20	0/20
3	1.28×10^5	19/20	37.22	0/20	0/20
4	1.28×10^4	2/20	38.26	0/20	0/20
5	1.28×10^3	0/20	ND	0/20	0/20

ND = not detected

D. Zika LoD Studies - MagNA Pure 96 Instrument serum and urine small volume (0.2 mL input volume) and large volume extraction (1 mL input volume):
Limit of detection for the ZIKV primer and probe set was evaluated in both normal human serum and in urine using a live French Polynesia 2013 strain of Zika virus. Zika virus was used in this specimen input volume evaluation to represent the three viruses detected by the assay. Four 10-fold serial dilutions of live Zika virus stocks were prepared in serum and urine. For each matrix, each concentration was extracted 20 times using the MagNA Pure 96 Instrument (0.2 mL and 1.0 mL extraction volume) and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Limit of detection was equivalent between using the large and the small (standard) input volumes, however the large input volume generally showed approximately 2 Cts lower than the standard volume, suggesting a slight increase in sensitivity. Thus the large volume extraction method for the MagNA Pure 96 is the recommended method for MagNA Pure 96 extraction of serum and urine for subsequent testing by the Trioplex assay. Results are summarized in Table 15 and Table 16.

Table 15: ZIKV LoD in serum (small volume and large volume extraction – MP 96) -Trioplex

		Serum (Large Volume)			Serum (Small Volume)		
		GCE/mL	ZIKV # Positive	Avg. C_T	GCE/mL	ZIKV # Positive	Avg. C_T
Dilution	10^{-5}	2.45×10^4	20/20	32.42	2.45×10^4	20/20	34.90
	10^{-6}	2.45×10^3	20/20	35.44	2.45×10^3	20/20	37.17
	10^{-7}	2.45×10^2	5/20	37.85	2.45×10^2†	1/20	38.57
	10^{-8}	2.45×10^1	0/20	NA	2.45×10^1	1/20	39.18

Table 16: ZIKV LoD in urine (small volume and large volume extraction – MP 96) - Trioplex

		Urine (Large Volume)			Urine (Small Volume)		
		GCE/mL	ZIKV # Positive	Avg. C_T	GCE/mL	ZIKV # Positive	Avg. C_T
Dilution	10^{-5}	4.64×10^4	20/20	31.63	4.64×10^4	20/20	34.02
	10^{-6}	4.64×10^3	20/20	35.11	4.64×10^3	19/20	37.37
	10^{-7}	4.64×10^2	1/20	38.72	4.64×10^2	3/20	39.10
	10^{-8}	4.64×10^1	0/20	38.88	4.64×10^1	1/20	39.94

E. Zika LoD studies – EDTA whole blood small volume (0.2 mL input volume) extraction (with an external lysis step) on the MagNA Pure 96, the MagNA Pure Compact, and the MagNA Pure LC 2.0 instruments:
Limit of detection for the ZIKV primer and probe set was evaluated in normal human whole blood (EDTA) using a live French Polynesia 2013 strain of Zika virus. Three 10-fold serial dilutions were prepared in EDTA whole blood. Each concentration was extracted 20 times using the MagNA Pure 96 (200 µL extraction volume), the MagNA Pure Compact, and the MagNA Pure LC 2.0 instruments, with an external lysis step, and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Results for EDTA whole blood are summarized in Table 17, Table 18, and Table 19.

Table 17: ZIKV LoD in EDTA whole blood (small volume extraction – MP 96) - Trioplex

Dilution	GCE/mL	ZIKV # Positive	Avg. C_T
10^{-4}	2.43 x 10^4	20/20	35.01
10^{-5}	2.43 x 10^3	20/20	37.21
10^{-6}	2.43 x 10^2	0/20	39.83

Table 18: ZIKV LoD in EDTA whole blood (small volume extraction – MP Comp) - Trioplex

Dilution	GCE/mL	ZIKV # Positive	Avg. C_T
10^{-4}	2.43 x 10^4	20/20	28.03
10^{-5}	2.43 x 10^3	20/20	31.57
10^{-6}	2.43 x 10^2	5/20	37.41

Table 19: ZIKV LoD in EDTA whole blood (small volume extraction – MP LC 2.0) - Trioplex

Dilution	GCE/mL	ZIKV # Positive	Avg. C_T
10^{-4}	2.43 x 10^4	20/20	33.85
10^{-5}	2.43 x 10^3	19/20	37.18
10^{-6}	2.43 x 10^2	0/20	41.92

F. DENV LoD study – EDTA whole blood small volume (0.2 mL input volume) extraction (with an external lysis step) on the MagNA Pure 96 instrument:
Limit of detection for the DENV primer and probe set was evaluated in normal human whole blood (EDTA) using the DENV-2 New Guinea C strain. Three 10-fold serial dilutions were prepared. Each concentration was extracted 20 times using the MagNA Pure 96 Instrument (200 µL extraction volume) and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Results are summarized in Table 20.

Table 20: DENV LoD in EDTA whole blood (small volume extraction – MP 96) - Trioplex

Dilution	GCE/mL	DENV # Positive	Avg. C_T	Zika # Positive	CHIKV # Positive
10^{-4}	4.28 x 10^4	20/20	34.13	0/20	0/20
10^{-5}	4.28 x 10^3	19/20	37.15	0/20	0/20
10^{-6}	4.28 x 10^2	3/20	39.05	0/20	0/20

G. CHIKV LoD study – EDTA whole blood small volume (0.2 mL input volume) extraction (with an external lysis step) on the MagNA Pure 96 instrument:
Limit of detection for the CHIKV primer and probe set was evaluated in normal human whole blood (EDTA) using the CHIKV Puerto Rico 2014 clinical strain. Three 10-fold serial dilutions were prepared. Each concentration was extracted 20 times using the MagNA Pure 96 Instrument (200 µL extraction volume) and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Results are summarized in Table 21.

Table 21: CHIKV LoD in EDTA whole blood (small volume extraction – MP 96) - Trioplex

Dilution	GCE/mL	CHIKV # Positive	Avg. C_T	Zika # Positive	DENV # Positive
10^{-4}	4.80 x 10^4	20/20	33.89	0/20	0/20
10^{-5}	4.80 x 10^3	19/20	37.06	0/20	0/20
10^{-6}	4.80 x 10^2	0/20	39.58	0/20	0/20

H. 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 CDC Trioplex rRT-PCR assay. The study was performed using the MagNA Pure 96 Large Volume Extraction Kit on the MagNA Pure 96 IVD and the SuperScript III Platinum rRT-PCR Kit. The results are presented in Table 22 below.

Table 22: Summary of LoD confirmation results using FDA reference materials

Reference Materials	Specimen Type*	Confirmed LoD** in RNA NAAT Detectable Units/mL
S1	Serum	3,300
S1	Urine	1,000
S2	Serum	1,670
S2	Urine	1,670

*The Trioplex rRT-PCR was also evaluated using the FDA Reference Material panel spiked into whole blood, however, inconclusive results were obtained when spiking heat inactivated Zika virus into the whole blood matrix.

**Study performed according to an FDA-issued protocol.

Section 2 – Inclusivity

Please note that the Trioplex and singleplex assay primers and probes have the same sequences, therefore the following inclusivity and exclusivity evaluations are applicable to both assay formats.

A. DENV inclusivity evaluation:

Inclusivity of the DENV primer and probe set was evaluated using a panel of RNA from 29 international isolates of dengue virus, representing contemporary strains from all clinically relevant genotypes. Testing was conducted using the SuperScript III master mix. A summary of test results are in Table 23.

Table 23: DENV inclusivity across dengue viruses – Trioplex format

Dengue virus serotype	Strains tested	DENV positive results
1	6	100%
2	11	100%
3	6	100%
4	6	100%

B. ZIKV, DENV and CHIKV sequence analysis:
In silico analysis of the Trioplex rRT-PCR primers and probes sequences was performed to verify reagent sequence homology with each corresponding virus and target region. A total of 514 current and historical dengue virus strains including 104 DENV-1, 142 DENV-2, 154 DENV-3 and 114 DENV-4, 206 chikungunya virus strains and 33 Zika virus strains were selected for this study. All primer and probe sequences showed 100% sequence identity with their expected target, predicting no false negative results are likely to occur. Table 24 below contains a summary of these findings.

Table 24: *In silico* inclusivity analysis

			Primer/Probe Sequence Identity									
Virus	**Strain**	**GenBank Acc#**	**DENV-F**	**DENV-R1**	**DENV-R2**	**DENV-P**	**CHIKV-F**	**CHIKV-R**	**CHIKV-P**	**ZIKV-F**	**ZIKV-R**	**ZIKV-P**
DENV-1	Mexico 2012	KJ189368	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-1	Nicaragua 2011	KF973453	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-1	Brazil 2010	JX669466	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-1	Saudi Arabia 2011	KJ649286	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-1	Thailand 2013	KF887994	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-2	Peru 2011	KC294210	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-2	Brazil 2010	JX669477	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-2	Indonesia 2010	KC762679	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-2	Saudi Arabia 2014	KJ830750	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-2	Singapore 2012	KM279577	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-3	Nicaragua 2009	JF937631	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-3	Indonesia 2010	KC762693	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-3	China 2013	KJ622195	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-3	Thailand 2010	HG316483	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-3	Brazil 2009	JF808120	100%	100%	<85%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-4	Venezuela 2007	HQ332174	100%	<85%	100%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-4	Brazil 2010	JN983813	100%	<85%	100%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-4	Pakistan 2009	KF041260	100%	<85%	100%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-4	Singapore 2005	GQ398256	100%	<85%	100%	100%	<20%	<20%	<20%	<20%	<20%	<20%
DENV-4	Cambodia 2008	JN638570	100%	<85%	100%	100%	<20%	<20%	<20%	<20%	<20%	<20%
CHIKV	El Salvador 2014	KR559471	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Jamaica 2014	KR559489	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Trinidad & Tobago 2014	KR046231	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	French Polynesia 2015	KR559473	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Brazil 2014	KR264951	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Guyana 2014	KR559496	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%

			Primer/Probe Sequence Identity									
Virus	**Strain**	**GenBank Acc#**	**DENV-F**	**DENV-R1**	**DENV-R2**	**DENV-P**	**CHIKV-F**	**CHIKV-R**	**CHIKV-P**	**ZIKV-F**	**ZIKV-R**	**ZIKV-P**
CHIKV	Thailand 2013	KJ579186	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	India 2013	KT336782	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Indonesia 2013	KM673291	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	China 2012	KC488650	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Philippines 2013	AB860301	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Rep of Congo 2011	KP003813	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Singapore 2008	FJ445463	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Sri Lanka 2008	FJ513654	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	China 2008	GU199351	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Malaysia 2008	FJ807899	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	India 2008	JN558835	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Mexico 2014	KP851709	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Puerto Rico 2014	KR559474	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
CHIKV	Honduras 2014	KR559488	<20%	<20%	<20%	<20%	100%	100%	100%	<20%	<20%	<20%
ZIKV	China 2016	KU740184	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Brazil 2015	KU527068	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Guatemala 2015	KU501217	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Brazil 2015	KU365778	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	French Polynesia 2013	KJ776791	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Suriname 2015	KU312312	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Puerto Rico 2015	KU501215	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Thailand 2014	KU681081	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Philippines 2012	KU681082	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Martinique 2015	KU647676	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Micronesia 2007	EU545988	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	Haiti 2014	KU509998	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
ZIKV	China 2016	KU744693	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%
IKV	Brazil 2015	KU321639	<20%	<20%	<20%	<20%	<20%	<20%	<20%	100%	100%	100%

Section 3 – Exclusivity

A. Near-neighbor exclusivity evaluation:
Evaluation of the cross-reactivity of each component of the Trioplex rRT-PCR with the viruses targeted by the other components was evaluated extensively as a part of all LoD, bridging and contrived specimen evaluations. No cross-reactivity between the component primers and probes and these three viruses was observed.

Three additional flaviviruses (WNV, YFV and SLEV) were selected to evaluate the specificity of the DENV, ZIKV and CHIKV primer and probe sets. Tissue culture supernatant of WNV (NY99 strain), YFV (17D strain), and SLEV (MSI-7 strain) were extracted with the Roche MagNA Pure LC 2.0 Instrument and tested using the SuperScript III master mix. All three viruses were tested in duplicate at 3 10-fold dilutions. No cross-reactivity was observed. All controls performed as expected.

Table 25: Near neighbor cross-reactivity – Trioplex format

Virus	Strain	ZIKV result	DENV result	CHIKV result
West Nile virus	NY99	No cross-reactivity	No cross-reactivity	No cross-reactivity
yellow fever virus	17D	No cross-reactivity	No cross-reactivity	No cross-reactivity
St. Louis encephalitis virus	MSI-7	No cross-reactivity	No cross-reactivity	No cross-reactivity
Zika virus*	French Polynesia 2013	n/a	No cross-reactivity	No cross-reactivity
dengue virus*	Representatives from all 4 serotypes	No cross-reactivity	n/a	No cross-reactivity
chikungunya virus*	Puerto Rico	No cross-reactivity	No cross-reactivity	n/a

*Cross-reactivity findings for these three viruses were extrapolated from data presented in limit of detection, bridging, archived clinical specimen and contrived specimen evaluations.

B. Non-Arbovirus exclusivity evaluation:
A panel of viruses and organisms known to cause similar signs and symptoms to the viruses detected by the Trioplex rRT-PCR were selected for inclusion in an exclusivity evaluation. The nucleic acid was prepared from quantified stocks of qualified strains of each of the listed organisms. All organisms were tested in triplicate at one high concentration: 100 pg nucleic acid/reaction. No cross-reactivity was observed. All controls performed as expected.

Table 26: Non-Arbovirus cross-reactivity – Trioplex format

Organism		Concentration	Number positive		
			ZIKV	DENV	CHIKV
Bacteria	*Borrelia burgdorferi*	100pg/rxn	0/3	0/3	0/3
Fungus	*Histoplasma*	100pg/rxn	0/3	0/3	0/3
Protozoa	*Plasmodium falciparum*	100pg/rxn	0/3	0/3	0/3
Virus	Cytomegalovirus	100pg/rxn	0/3	0/3	0/3
	HSV-1	100pg/rxn	0/3	0/3	0/3
	Influenza A	100pg/rxn	0/3	0/3	0/3
	Influenza B	100pg/rxn	0/3	0/3	0/3
	VZV	100pg/rxn	0/3	0/3	0/3
	Vaccinia	100pg/rxn	0/3	0/3	0/3
	Adenovirus	100pg/rxn	0/3	0/3	0/3

C. *In silico* evaluation:

Additional evaluation of the analytical specificity of the Trioplex rRT-PCR was 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 Trioplex rRT-PCR 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 rRT-PCR results. Conditions and associated causative agents covered in the *in silico* specificity analysis are presented in Table 27.

Table 27: Organisms evaluated during *in silico* specificity analysis

Organism		Organism (taxid)
Bacteria	*Borrelia burgdorferi*	64895
	Group A Strep	36470
	Salmonella spp.	590
	Leptospira spp.	171
	Rickettsia spp.	780
Trematodes	*Schistosoma spp.*	6181
Fungus	*Histoplasma spp.*	5036
Protozoa	*Plasmodium falciparum*	5833
	Trypanosoma cruzi	5693
Flavivirus	Zika (DENV and CHIKV)	64320
	Dengue (ZIKV and CHIKV)	11052
	WNV	11082
	YFV	40005
	SLEV	11080
	Spondweni virus	64318
	JEEV	11071

Organism		Organism (taxid)
Alphavirus	Chikungunya (ZIKV and DENV)	37124
	EEEV	11021
	WEEV	11039
	Ross River virus	11029
	Barmah Forest virus	11020
	O'nyong-nyong virus	11027
	Mayaro virus	59301
Other virus	Parvovirus (B19)	10789
	Measles virus	11234
	Rubella virus	11041
	Cytomegalovirus	10358
	HSV-1	10298
	HSV-2	10310
	Influenza A	11320
	Influenza B	11520
	VZV	10335
	Vaccinia	10245
	Epstein Barr virus	10376
	HIV	11676
	Hepatitis C	11102
	Enterovirus	12059
	Adenovirus	108098
		130310
		129951
		565302
		10519

Section 4 - Bridging Studies

A. qScript and Superscript III master mix evaluations:
Four pools of material were prepared for evaluation: three pools of serum and one of urine. One pool of serum and one of urine were spiked with French Polynesia 2013 strain of Zika virus at the viral stock dilution factor identified as the LoD for ZIKV with SuperScript III. One pool of serum was spiked with dengue virus (Puerto Rico 1998, serotype 2) at the viral stock dilution factor identified as the LoD for DENV with SuperScript III. And one pool of serum was spiked with chikungunya virus (Puerto Rico 2014) at the viral stock dilution factor identified as the LoD for CHIKV with SuperScript III.

Each pool was extracted using the MagNA Pure LC 2.0 Instrument 20 times. Each resulting RNA sample was tested by the Trioplex rRT-PCR using both the SuperScript III master mix and the qScript master mix. Results show comparable performance between the SuperScript III and qScript master mix.

B. Limit of detection for the DENV primer and probe set was evaluated in normal human serum using a representative strain from each dengue virus serotype (DENV-1 Puerto Rico 1998, DENV-2 Puerto Rico 1998, DENV-3 Puerto Rico 2004, DENV-4 Puerto Rico 1998). Five 10-fold serial dilutions of each strain in each matrix were prepared. Each concentration was extracted 20 times using the MagNA Pure 96 Instrument (200 µL extraction volume) and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Results show comparable performance between using the MagNA Pure 96 and using the MagNA LC 2.0.
C. Limit of detection for the CHIKV primer and probe set was evaluated in normal human serum using the Puerto Rico 2014 strain of chikungunya virus. Five 10-fold serial dilutions in each matrix were prepared. Each concentration was extracted 20 times using the MagNA Pure 96 Instrument (200 µL extraction volume) and tested by the Trioplex rRT-PCR using the SuperScript III master mix. Results show comparable performance between using the the MagNA Pure 96 and using the MagNA LC 2.0.
D. MagNA Pure Compact Instrument serum and urine large volume extraction evaluation: Three 10-fold serial dilutions of live Zika virus (French Polynesia 2013, stock concentration of 2.45×10^9 GCE/mL), were prepared in normal human serum and urine. The virus stock used to prepare the dilution series is identical to that used to prepare the dilutions for the MagNA Pure 96 evaluation above. Concentrations tested here include the dilution factor identified as the limit of detection for the MagNA Pure 96 instrument in serum and urine (10^{-6}). For each matrix, each concentration was extracted 20 times using the MagNA Pure Compact Instrument (1 mL extraction volume) and tested by the Trioplex rRT-PCR using the SuperScript III master mix. As the MagNA Pure Compact was able produce extracted nucleic acid of sufficient quality to allow detection of Zika virus RNA in all 20 reactions at the 10^{-6} dilution factor in both matrices, the instrument is non-inferior to the MagNA Pure 96 and is acceptable for use in this assay.
E. bioMérieux NucliSENS easyMAG Instrument serum and urine large volume extraction evaluation:
The bioMérieux NucliSENS easyMAG Instrument was evaluated to determine non-inferiority in serum and urine large volume extraction in comparison to the MagNA Pure 96. Three 10-fold serial dilutions of live Zika virus, were prepared in normal human serum and urine. The virus stock used to prepare the dilution series is identical to that used to prepare the dilutions for the MagNA Pure 96 evaluation above in Section 1. Concentrations tested here include the dilution factor identified as the limit of detection for the MagNA Pure 96 instrument in serum and urine (10^{-6}). For each matrix, each concentration was extracted 20 times using the bioMérieux NucliSENS easyMAG Instrument (1 mL extraction volume) and tested by the Trioplex rRT-PCR using the SuperScript III master mix. As the MagNA Pure Compact was able produce extracted nucleic acid of sufficient quality to allow detection of Zika virus RNA in all 20 reactions at the 10^{-6} dilution in both matrices, the instrument is non-inferior to the MagNA Pure 96 and is acceptable for use in this assay.

F. QIAamp manual extraction evaluation:
One pool of urine and one pool of serum were spiked using the French Polynesia 2013 strain of Zika virus to the dilution factor of viral stock identified as the LoD for ZIKV with the MagNA Pure LC 2.0 Instrument. Each pool was extracted 20 times using the Qiagen QIAamp DSP Viral RNA Mini Kit and tested with the Trioplex rRT-PCR using the SuperScript III master mix. Results support that the Qiagen QIAamp DSP Viral RNA Mini Kit performs in a non-inferior manner to the MagNA Pure LC 2.0 Instrument in the preparation of nucleic acid for subsequent testing by the Trioplex rRT-PCR and is acceptable for use in the assay.

G. QuantStudio™ Dx Real-Time PCR Instrument evaluation:
The Trioplex rRT-PCR was evaluated in combination with the QuantStudio™ Dx Real-Time PCR Instrument to determine non-inferiority in serum, urine, and whole blood matrices when compared to the AB 7500 Fast Dx instrument. Ten-fold dilutions of Zika (French Polynesia 2013), dengue virus serotypes 1-4 (DENV-1 Puerto Rico 1998, DENV-2 Puerto Rico 1998, DENV-3 Puerto Rico 2004, DENV-4 Puerto Rico 1998), and chikungunya virus (Puerto Rico 2014) stocks were prepared and tested using the Trioplex rRT-PCR assay with both the SuperScript III and qScript master mixes on both PCR instruments. Concentrations tested include those identified as the limit of detection for the AB 7500 Fast Dx instrument. For each matrix, each concentration was extracted 20 times using the MagNA Pure LC 2.0 and MagNA Pure 96 (including large and small volume extraction options for Zika virus in serum and urine) extraction platforms. The QuantStudio™ Dx Real-Time PCR Instrument was able to detect Zika, dengue, and chikungunya viruses in no less than 95% of reactions spiked at the LoD dilution in all three matrices using both the SuperScript III and qScript master mixes, therefore the instrument is non-inferior to the AB 7500 Fast Dx and is acceptable for use with the Trioplex rRT-PCR assay.

H. Singleplex vs. multiplex reaction range-finding comparisons:
A 10-fold dilution series of Zika virus French Polynesia 2013 strain was prepared in normal human serum and tested by the Trioplex assay as a multiplex reaction and by the ZIKV as a singleplex reaction. Each concentration was tested 8 times. Extractions were performed using the MagNA Pure LC 2.0 instrument, small volume protocol following the instructions for use described for this assay. PCR was performed using the Invitrogen SuperScript III master mix. All PCR reactions were performed simultaneously to eliminate run-to-run variation. A summary of results is presented in Table 28 below.

Table 28: Comparison of ZIKV in singleplex verses multiplex in serum (small volume extraction – MP LC 2.0)

	Singleplex (Small Volume)		Multiplex (Small Volume)	
Dilution	ZIKV # Positive	Avg. C_T	ZIKV # Positive	Avg. C_T
10^{-1}	8/8	21.75	8/8	20.94
10^{-2}	8/8	24.86	8/8	23.98
10^{-3}	8/8	28.43	8/8	27.42
10^{-4}	8/8	31.49	8/8	30.49
10^{-5}	8/8	35.56	8/8	34.03
10^{-6}	8/8	36.40	8/8	36.12
10^{-7}	2/8	38.03	4/8	37.99
10^{-8}	0/8	ND	0/8	ND

ND = not detected

A 10-fold dilution series of chikungunya virus Puerto Rico 2014 strain was prepared in normal human serum and tested by the Trioplex assay as a multiplex reaction and by the CHIKV as singleplex reaction. Each concentration was tested 5 times. Extractions were performed using the MagNA Pure LC 2.0 instrument, small volume protocol following the instructions for use described for this assay. PCR was performed using the Invitrogen SuperScript III master mix. All PCR reactions were performed simultaneously to eliminate run-to-run variation. A summary of results is presented in Table 29 below.

Table 29: Comparison of CHIKV in singleplex verses multiplex in serum (small volume extraction – MP LC 2.0)

	Singleplex (Small Volume)		Multiplex (Small Volume)	
Dilution	CHIKV # Positive	Avg. C_T	CHIKV # Positive	Avg. C_T
10^{-1}	5/5	22.04	5/5	22.40
10^{-2}	5/5	25.29	5/5	25.46
10^{-3}	5/5	29.04	5/5	29.48
10^{-4}	5/5	32.19	5/5	32.50
10^{-5}	5/5	35.82	5/5	35.86
10^{-6}	0/5	ND	0/5	ND
10^{-7}	0/5	ND	0/5	ND
10^{-8}	0/5	ND	0/5	ND
10^{-9}	0/5	ND	0/5	ND
10^{-10}	0/5	ND	0/5	ND

ND = not detected

A 10-fold dilution series of dengue virus 4 (Puerto Rico 1998 strain) was prepared in normal human serum and tested by the Trioplex assay as a multiplex reaction and by the DENV as singleplex reaction. Each concentration was tested 8 times for the singleplex reaction and 5 times for the multiplex reaction. Extractions were performed using the MagNA Pure LC 2.0 instrument, small volume protocol following the instructions for use described for this assay. PCR was performed using the Invitrogen SuperScript III master mix. All PCR reactions were performed simultaneously to eliminate run-to-run variation. A summary of results is presented in Table 30 below.

Table 30: Comparison of DENV in singleplex verses multiplex in serum (small volume extraction – MP LC 2.0)

	Singleplex (Small Volume)		Multiplex (Small Volume)	
Dilution	DENV # Positive	Avg. C_T	DENV # Positive	Avg. C_T
10^{-1}	8/8	15.65	5/5	16.55
10^{-2}	8/8	18.47	5/5	18.28
10^{-3}	8/8	21.32	5/5	21.95
10^{-4}	8/8	24.90	5/5	25.30
10^{-5}	8/8	28.82	5/5	30.12
10^{-6}	8/8	34.02	5/5	34.05
10^{-7}	8/8	36.16	2/5	38.32
10^{-8}	0/8	ND	0/5	ND

ND = not detected

I. Zika Singleplex LoD Studies - MagNA Pure 96 Instrument serum small volume (0.2 mL input volume):
Limit of detection for the ZIKV primers and probe in singleplex was evaluated in normal human serum using a live French Polynesia 2013 strain of Zika virus. Four 10-fold serial dilutions of live Zika virus stocks were prepared in serum. Each concentration was extracted 20 times using the MagNA Pure 96 Instrument (0.2 mL extraction volume) and tested by the Trioplex rRT-PCR using the SuperScript III master mix.

Table 31: ZIKV singleplex LoD evaluation in serum (small volume extraction – MP 96)

	Singleplex (Small Volume)		
Dilution	GCE/mL	ZIKV # Positive	Avg. C_T
10^{-5}	1.35×10^4	20/20	35.72
10^{-6}	1.35×10^3	20/20	37.02
10^{-7}	1.35×10^2	3/20	39.88
10^{-8}	1.35×10^1	0/20	39.77

Section 5 - Clinical evaluation

A. Clinical performance of Trioplex rRT-PCR:
From the archival collection of the CDC Dengue Branch in Puerto Rico, 130 serum specimens were selected to evaluate the performance of the Trioplex rRT-PCR. Forty-eight (48) specimens from dengue cases (12 from each serotype), 12 from chikungunya cases, 20 from Zika cases and 50 negative specimens from symptomatic individuals were included in this specimen set. Upon removal from the archive (-70 ºC), specimens were tested with the Trioplex rRT-PCR (using SuperScript III and the MagNA Pure LC 2.0 Instrument), the FDA-cleared DENV 1-4 rRT-PCR, singleplex in-house developed and validated Zika NS3 and chikungunya nSP1 rRT-PCR assays. Results of testing with the DENV 1-4 rRT-PCR, in-house Zika and chikungunya rRT-PCR assays matched the previous determination associated with all but one of the repository specimens.

One archived Zika specimen generated negative results with the in-house Zika NS3 assay (C_T 38.45, assay positive cutoff <38) and positive Zika result with the Trioplex rRT-PCR. Due to the in-house Zika NS3 assay result, the archived Zika specimen was re-classified as a negative specimen and the Trioplex result analyzed as a false positive. Trioplex rRT-PCR results of testing are compared to this archival specimen category in the table below.

Table 32: Trioplex rRT-PCR performance with archived clinical serum specimens

Specimen category	Tested	Trioplex component result		
		ZIKV positive	DENV positive	CHIKV positive
Zika	19*	19/19	0/19	0/19
Dengue	48	0/48	47/48**	0/48
Chikungunya	12	0/12	0/12	12/12
Negative	51*	2/51	0/51	1/51
Positive percent agreement		**100%** (19/19) 95% CI: 83.2% - 100%	**97.9%** (47/48) 95% CI: 89.1% - 99.6%	**100%** (12/12) 95% CI: 75.8% - 100%
Negative percent agreement		**98.2%** (109/111) 95% CI: 93.7% - 99.5%	**100%** (82/82) 95% CI: 95.5% - 100%	**99.2%** (117/118) 95% CI: 95.4% - 99.9%

* One archived Zika specimen, when tested upon retrieval from archive, gave a C_T value just above the cutoff for the in-house Zika NS3 assay. Thus the specimen was re-classified as a negative specimen. The specimen gave a Zika positive result with the Trioplex assay, and is presented as a false positive result.
**One dengue specimen (serotype 4) generated a C_T of 39.87, which is a negative result. DENV 1-4 assay test result for the specimen was positive for serotype 4.

B. Clinical performance of ZIKV, CHIKV, and DENV when run in singleplex (separate wells):
In section 5A (clinical performance of Trioplex rRT-PCR), samples were tested with

all 3 primer/probe sets in a single test well (multiplex). The same 130 samples tested in section 5A were tested again, but with ZIKV, CHIKV, and DENV, in separate wells (singleplex) to compare their performance when run in singleplex versus multiplex. Upon removal from the archive (-70 ºC), specimens were tested with ZIKV, CHIKV, and DENV separately using SuperScript III and the MagNA Pure LC 2.0 Instrument. They were also tested with the FDA-cleared DENV 1-4 rRT-PCR as well as singleplex in-house developed and validated Zika NS3 and chikungunya nSP1 rRT-PCR assays. Results of testing with the DENV 1-4 rRT-PCR, in-house Zika and chikungunya rRT-PCR assays matched the previous determination associated with all but one of the repository specimens.

One archived Zika specimen generated negative results with the in-house Zika NS3 assay (C_T 38.45, assay positive cutoff <38) and positive Zika result with the ZIKV primer and probe set. Due to the in-house Zika NS3 assay result, the archived Zika specimen was re-classified as a negative specimen and the ZIKV result analyzed as a false positive. Results of individual primer/probe set testing are compared to this archival specimen category in the table below.

Table 33: ZIKV, CHIKV, and DENV singleplex performance with archived clinical serum specimens

Specimen category	Tested	Results of Singleplex reactions		
		ZIKV positive	DENV positive	CHIKV positive
Zika	19*	19/19	0/19	0/19
Dengue	48	0/48	48/48	0/48
Chikungunya	12	0/12	0/12	12/12
Negative	51*	1/51	0/51	0/51
Positive percent agreement		**100%** (19/19) 95% CI: 83.2% - 100%	**100%** (48/48) 95% CI: 92.6% - 100%	**100%** (12/12) 95% CI: 75.8% - 100%
Negative percent agreement		**99.1%** (110/111) 95% CI: 95.1% - 99.8%	**100%** (82/82) 95% CI: 95.5% - 100%	**100%** (118/118) 95% CI: 96.9% - 100%

* One archived Zika specimen, when tested upon retrieval from archive, gave a C_T value just above the cutoff for the in-house Zika NS3 assay. Thus the specimen was re-classified as a negative specimen. The specimen gave a Zika positive result with the ZIKV reaction, and is presented as a false positive result.

C. Detection of Zika RNA in Case-Matched Clinical Specimens:
Serum, urine and whole blood (EDTA) were collected within the first week following onset of symptoms from 175 patients diagnosed with Zika virus infection in Puerto Rico during the 2016 outbreak. As part of the study, all three specimens were collected from the patient on the same day and were submitted for analysis. Serum specimens were tested using the CDC Zika MAC-ELISA according to the EUA protocol. Serum, urine and whole blood were tested with the CDC Trioplex rRT-PCR

using the small volume MagNA Pure 96 extraction protocol (200 µL input volume) with an external lysis step, and the Invitrogen SuperScript III master mix. In addition, 65% of these patients were recaptured to give a second serum sample after 6 days DPO. This second serum sample was tested using the CDC Zika IgM MAC-ELISA according to the EUA protocol.

For the purpose of data presentation for this study, these 175 cases were determined as true Zika virus infected cases if at least one of the diagnostic tests was positive or equivocal in one or more of the samples. A summary of results across specimen types is presented in Table 34. During the first 7 days post onset of symptoms, Zika RNA was generally detectable across patient specimens.

Table 34: Percent positive results by days post onset of symptoms (DPO) across assays and specimen types – Trioplex format

DPO	Serum Zika IgM		Serum Trioplex (ZIKV)		Urine Trioplex (ZIKV)		Whole Blood Trioplex (ZIKV)	
0	1/11	9.1%	8/11	72.7%	4/11	36.4%	10/11	90.9%
1	7/40	17.5%	27/40	67.5%	16/40	40%	38/40	95%
2	4/26	15.4%	20/26	76.9%	9/26	34.6%	25/26	96.2%
3	4/22	18.2%	19/22	86.4%	14/22	63.6%	21/22	95.5%
4	30/39	76.9%	24/39	61.5%	24/39	61.5%	36/39	92.3%
5	16/22	72.7%	11/22	50%	13/22	59.1%	20/22	90.9%
6	10/14	71.4%	7/14	50%	10/14	71.4%	13/14	92.9%
7	1/1	-	0/1	-	0/1	-	0/1	-
All	73/175	41.7%	116/175	66.3%	90/175	51.4%	163/175	93.1%

D. Detection of Dengue and Chikungunya RNA in Archived Patient-Matched Serum and Whole Blood Specimens

A panel of whole blood (EDTA) specimens were selected from the CDC archive collection to include whole blood collected from 60 individuals diagnosed with dengue virus infection and 56 individuals diagnosed with chikungunya virus infection in Puerto Rico. Specimens were collected and archived prior to the current Zika virus outbreak in Puerto Rico. Dengue diagnoses were made based on rRT-PCR testing of the serum specimen using the FDA-cleared CDC DENV 1-4 Real-time RT-PCR Assay. Chikungunya virus diagnoses were made based on serum testing including the Lanciotti et al. in-house chikungunya virus nSP1 rRT-PCR assay. All serum and whole blood specimens were stored frozen.

All serum and whole blood specimens were extracted using the corresponding MagNA Pure 96 method described in the Instructions for Use. Invitrogen SuperScript III was used for Trioplex rRT-PCR testing.

All archived whole blood specimens and all but one archived serum specimens generated Trioplex rRT-PCR results in both specimens that agreed with the qualitative diagnostic results generated with the DENV 1-4 rRT-PCR and CHIV in-house rRT-PCR in the serum specimen prior to archiving. Whole blood Trioplex rRT-PCR Ct values were generally 2-3 Ct. lower for DENV and CHIKV in whole blood than in the

patient-matched serum specimen.

None of the 116 patient whole blood or serum specimens tested positive for Zika virus RNA using the Trioplex assay, demonstrating no cross-reactivity and 100% negative percent agreement with the expected Zika virus RNA negative results (95% CI: 96.8% - 100%). Data are summarized in Table 35 below.

Table 35: Detection of dengue and chikungunya RNA in archived sera and whole blood – Trioplex format

Archive Group	Comparator Assays (serum testing)		Trioplex Serum Testing			Trioplex Whole-Blood Testing		
	DENV 1-4 pos	CHIKV nSP1 pos	DENV pos	CHIKV pos	ZIKV pos	DENV pos	CHIKV pos	ZIKV pos
Dengue Cases	60/60	na	59/60*	0/60	0/60	60/60	0/60	0/60
Chikungunya Cases	na	56/56	0/56	56/56	0/56	0/56	56/56	0/56

[1] All comparator assay testing was conducted before the specimens were placed into archive freezer.
[2] One serum specimen generated a Trioplex DENV Ct value of 40.05. Ct. value for DENV 1-4 assay prior to archival was 36.11, in the range of Ct.values generally observed at the limit of detection for the assay.

E. Taking into consideration study results from both clinical studies described in Section 5B and Section 5C above, performance of the Trioplex rRT-PCR assay testing EDTA whole blood specimens for Zika virus RNA was assessed against "Patient Zika Infected Status" as determined by the Trioplex rRT-PCR assay Zika virus results testing patient-matched serum and urine specimens, or expected Zika RNA negative results. "Patient Zika Infected Status" is considered as positive when patient-matched serum and/or urine specimens test Zika RNA positive by the Trioplex rRT-PCR assay. "Patient Zika Infected Status" is considered as "indeterminate" when both patient-matched serum and urine specimens test Zika RNA negative by the Trioplex rRT-PCR assay. A total of 23 patients (23/175) with "indeterminate" "Patient Zika Infected Status" are excluded from this additional performance analysis.

Table 36: Performance of the Trioplex rRT-PCR Assay Testing Whole Blood Specimens for Zika Virus RNA against "Patient Zika Infected Status" or "Expected Zika RNA Results"

Whole Blood (WB) - Specimen Category	CDC Trioplex rRT-PCR		
	Number Tested	Zika RNA Positive	Zika RNA Negative
Natural specimens collected from symptomatic patients in Puerto Rico where Zika virus is currently endemic	175	163	12
Expected Zika RNA negative natural specimens that were collected from symptomatic patients in Puerto Rico prior to the current Zika virus outbreak. These patients were diagnosed with Dengue virus infection using an FDA-cleared Dengue NAAT test	60	0	60
Expected Zika RNA negative natural specimens that were collected from symptomatic patients in Puerto Rico prior to the current Zika virus outbreak. These patients were diagnosed with CHIKV infection using a CHIKV NAAT test developed and validated by the CDC	56	0	56
Positive Percent Agreement (PPA)	**96.1% (146/152)* 95% CI (91.7% - 98.2)**		
Negative Percent Agreement (NPA)	**100% (116/116) 95% CI (96.8% - 100%)**		

*2/6 "false negative" subjects tested positive for Zika RNA by the CDC Trioplex test in patient matched serum specimens only; 3/6 "false negative" subjects tested positive for Zika RNA by the CDC Trioplex test in patient matched urine specimens only; 1/6 "false negative" subjects tested positive for Zika RNA by the CDC Trioplex test in both patient matched serum and urine specimens

F. Secondary specimen data:

Two urine specimens collected from symptomatic female patients suspected of Zika virus infection during the current Zika outbreak were submitted to the CDC laboratory in Puerto Rico for analysis. These specimens were tested with the Trioplex rRT-PCR using the MagNA Pure LC 2.0 Instrument and SuperScript III master mix. Specimens were also tested with the ZIKV primer and probe set run singleplex and with an in-house Zika NS3 rRT-PCR assay. Each specimen was tested alongside a patient-matched serum specimen collected the same day the urine was collected. Results are presented in Table 37.

Table 37: Urine specimen data – Trioplex format

		Trioplex			ZIKV singleplex	Zika NS3 rRT-PCR
Case 1	DPO	DENV	CHKV	ZIKV		
Serum	3	Neg	Neg	32.51	33.21	34.6
Urine	3	Neg	Neg	29.34	28.56	31.56

		Trioplex			ZIKV singleplex	Zika NS3 rRT-PCR
Case 2	DPO	DENV	CHKV	ZIKV		
Serum	2	Neg	Neg	29.23	28.56	31.05
Urine	2	Neg	Neg	27.45	27.12	29.6

DPO = days post onset of symptoms

Amniotic Fluid and CSF Specimens:
Four amniotic specimens and two CSF specimens, all with Zika virus results from matched serum specimens, were evaluated with rRT-PCR analysis. The results from rRT-PCR analysis of amniotic and CSF specimens matched the serum Zika virus results in all cases.

G. Contrived specimen evaluation:
Testing was conducted in two rounds. For the first round, 50 negative human serum specimens were used to prepare contrived specimens to evaluate the performance of the Trioplex rRT-PCR. Each specimen was aliquoted into 3 tubes. One aliquot from each specimen was not spiked (50 of specimen group 15). The remaining aliquots (n=100) were distributed into subgroups and spiked with whole virus as outlined in Specimen groups 1-13 in Table 38 below. For the second round of testing, an additional 25 contrived serum specimens were prepared: 15 as defined for specimen group 14, and 10 more negatives (specimen group 15) to mix in with them.

Low spiking level for Zika (French Polynesia 2013) was approximately 1.5-3 x LoD, moderate was approx. 100 x LoD, and high was approx. 1000 x LoD. For dengue (serotype 2, Puerto Rico 1998) and chikungunya (Puerto Rico 2014), low spiking level was 5-10 x LoD, high was 100-150 x LoD.

Aliquots were blinded and passed on to an operator for testing by the Trioplex rRT-PCR. Extraction was performed using the MagNA Pure LC 2.0 Instrument and rRT-PCR was conducted with the SuperScript II master mix. Results of testing are summarized in Table 39. Agreement between expected results and testing results for all three primer and probe sets was 100%.

Table 38: Spiking plan for contrived specimen study – Trioplex format

Specimen group #	N	Zika	dengue	chikungunya
1	5	Moderate	-	-
2	5	-	Low	-
3	5	-	-	Low
4	5	High	-	-
5	5	-	High	-
6	5	-	-	High
7	10	Moderate	High	-
8	10	Moderate	-	High
9	10	High	Low	-
10	10	-	Low	High
11	10	High	-	Low
12	10	-	High	Low
13	10	High	High	High
14	15	Low	High	High
15	60	-	-	-

Table 39: Contrived specimen summary of results – Trioplex format

	High Positive		Moderate Positive		Low Positive		Negative	
	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
Zika	35	35	25	25	15	15	100	0
Dengue	50	50			25	25	100	0
Chikungunya	50	50			25	25	100	0

Procedure Notes

Send comments, suggestions and questions on this procedure to LRN@cdc.gov

References

- Lanciotti RS, Kosoy OL, Laven JJ, Panella AJ, Velez JO, Lambert AJ, Campbell GL. 2007. Chikungunya virus in US travelers returning from India, 2006. Emerg Infect Dis. May; 13(5):764-7
- Lanciotti RS, Kosoy OL, Laven JJ, velez JO, lambert AJ, Johnson AJ, Stanfield SM, Duffy MR. 2008. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. Aug; 14(8):1232-1239
- CDC DENV-1-4 Real Time RT-PCR Assay for detection and serotype identification of dengue virus. Package Insert cat# KK0128. 2013. http://www.cdc.gov/dengue/clinicalLab/realTime.html
- Lustig Y, Mendelson E, Paran N, Melamed S, Schwartz E. 2016. Detection of Zika virus RNA in whole blood of imported Zika virus disease cases up to 2 months after symptom onset, Israel, December 2015 to April 2016. Euro Surveill. 2016 Jun 30; 21(26).
- Rios, M., Daniel, S., Chancey, C., Hewlett, I.K., Stramer, S.L. West Nile Virus Adheres to Human Red Blood Cells in Whole Blood. Clin. Infect. Dis. 2007; 45; 181-186.
- http://www.cdc.gov/zika/laboratories/lab-guidance.html
- http://www.cdc.gov/zika/laboratories/lab-safety.html

Appendix A: Programming the QuantStudio™ Dx PCR Instrument

Equipment Preparation

QuantStudio™ Dx Real-Time PCR Instrument

The manufacturer provides initial installation of the QuantStudio™ Dx (QSDx) instrument including calibration for detection of FAM, VIC and ROX fluorophores. In order to run the CDC Trioplex Real Time RT-PCR Assay, the user must calibrate the instrument to detect the CAL Fluor Red 610 fluorophore using the ThermoFisher Fast Real-Time PCR Systems Spectral Calibration Kit II (cat # 4362201, purchased separately).

Instrument Calibration

The QSDx includes two modes of operation: *In vitro* diagnostic use mode (IVD) and research use only mode (RUO). Under the EUA, the CDC Trioplex rRT-PCR is considered an IVD and not an RUO assay, however the assay can only be run in the RUO mode of the QSDx instrument.

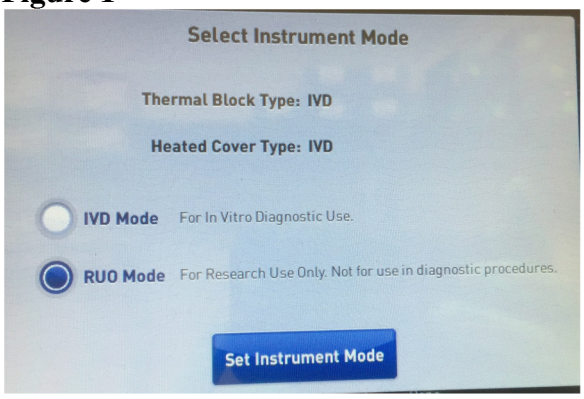
NOTE: The QSDx instrument will require verification and/or calibration every 6 months. Furthermore, background calibrations must be performed monthly. Please refer to the QSDx Instructions for Use manual for specifics.

1. Power on the instrument and the associated computer. Log in if necessary.
2. Verify status of current ROI, Background, Uniformity, Dye and Normalization calibration.
3. Perform calibrations where required.
4. To add CAL Fluor Red 610 detection, remove the Fast Real-Time PCR Systems Spectral Calibration Kit II from the freezer, remove the Texas Red calibration plate from the kit and allow the plate to reach room temperature, according to manufacturer's instructions. Return the remaining calibration plates to the freezer.
5. Open the sealed pouch and centrifuge the plate briefly.
6. Refer to the Dye Calibration section of the QSDx user manual and follow the manufacturer's calibration protocol.

PCR Run

1. Power on the instrument and the associated computer. Log in if necessary.
2. On the instrument touch screen panel, select **RUO Mode** and select the **Set Instrument Mode** button **(Figure 1)**.

Figure 1



3. Launch the QuantStudio™ Test Development software icon on the computer.
4. Confirm that the instrument is communicating properly with the computer by selecting the **Instrument Console** icon under the **Run** menu **(Figure 2)**. If the instrument is communicating properly, the QSDx instrument icon under **My Instruments** will display a green check mark **(Figure 3)**. Close tab once communication is confirmed.

Figure 2

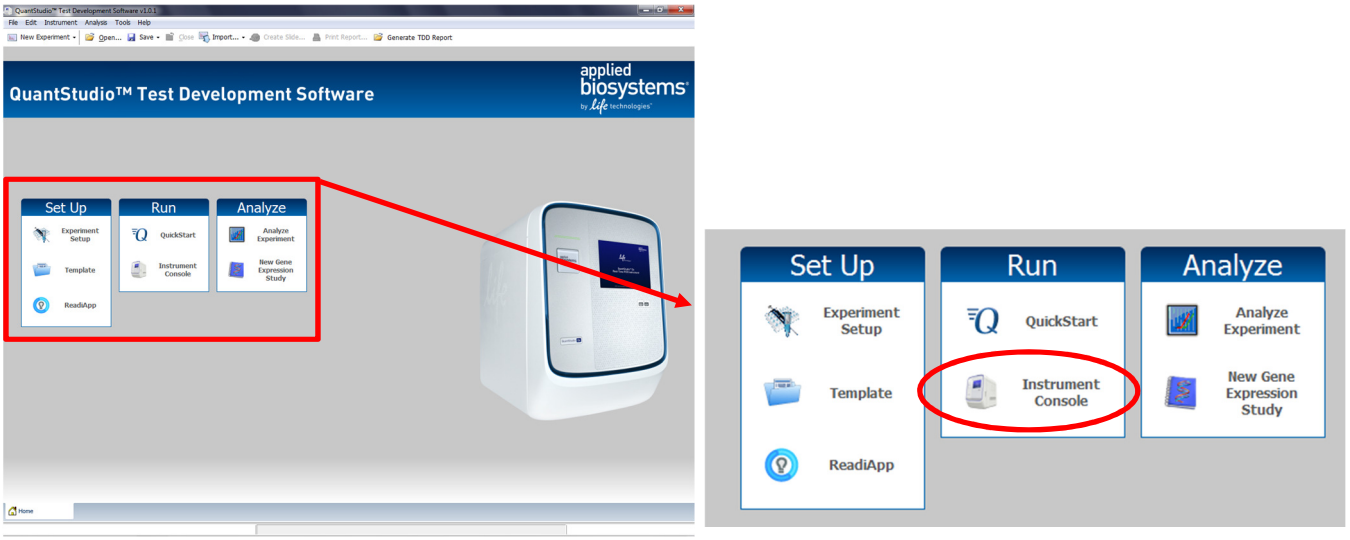
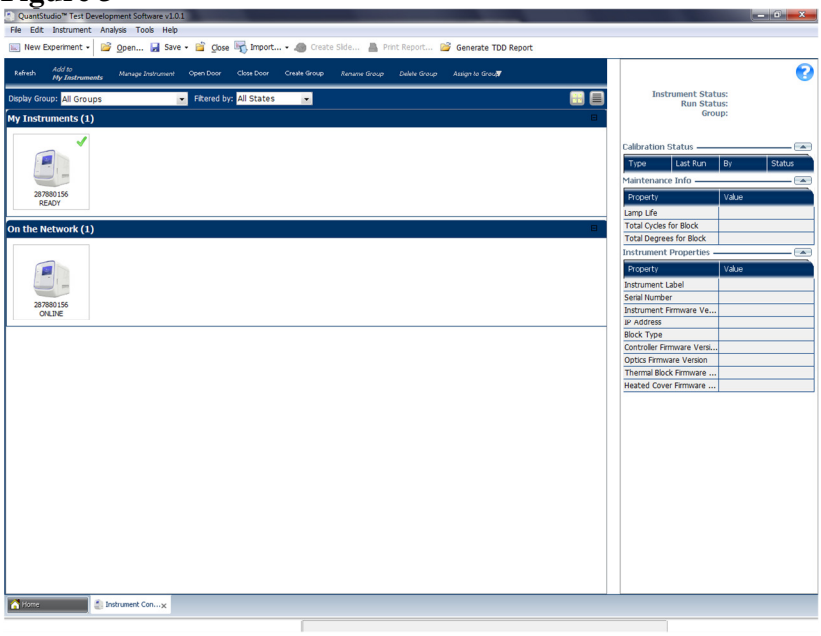
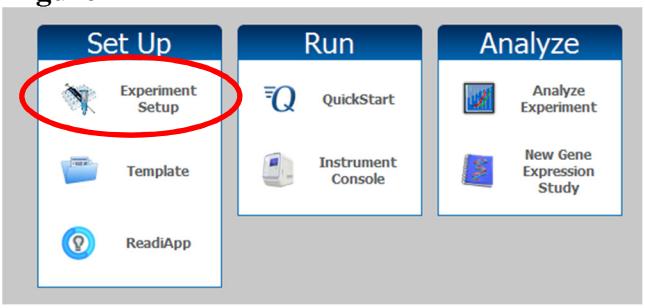


Figure 3



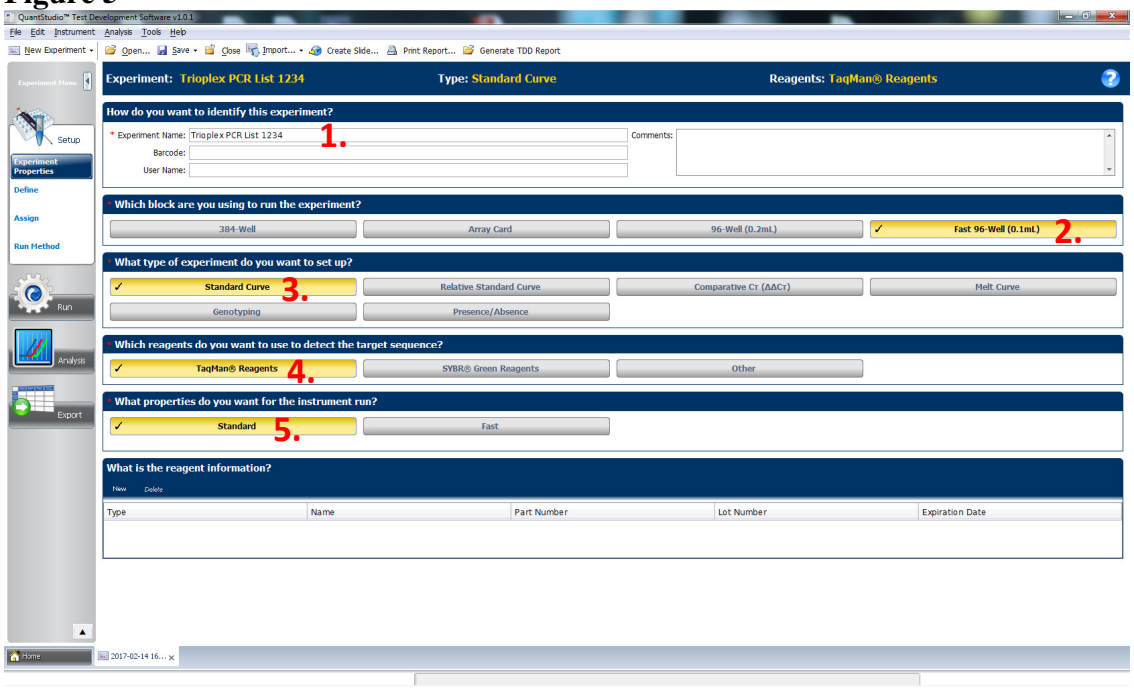
5. To start a new PCR run, select the **Experiment Setup** icon under the **Set Up** menu **(Figure 4)**.

Figure 4



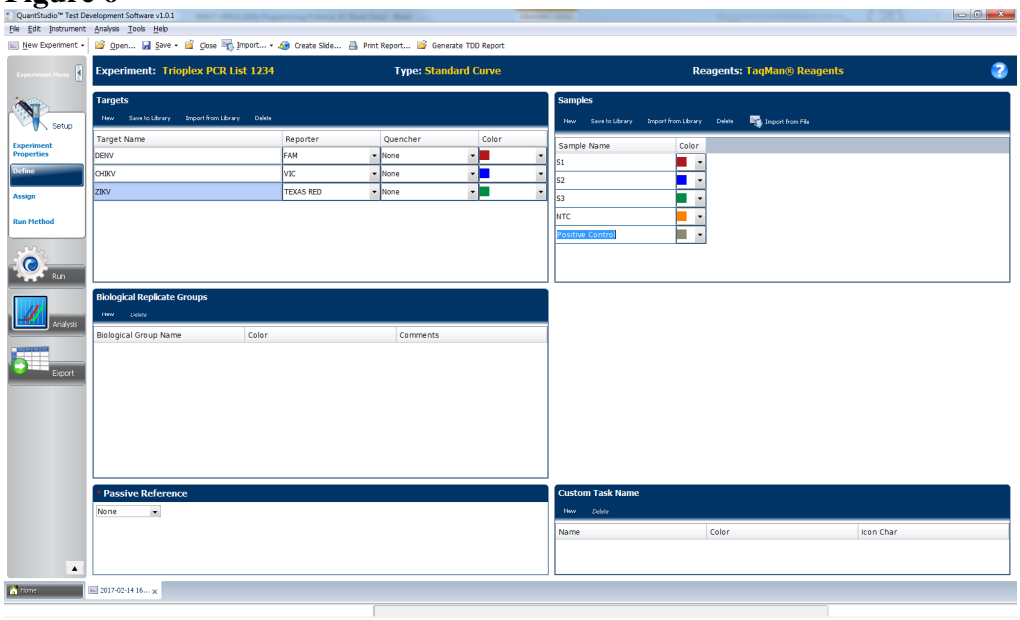
6. Under the **Experiment Properties** section, select the following options (**Figure 5**):
   a) Name experiment using a file name with appropriate information **(1)**
   b) Select Fast 96-well (0.1mL) block **(2)**
   c) Select Standard Curve experiment type **(3)**
   d) Select TaqMan® Reagents **(4)**
   e) Select Standard instrument run **(5)**

Figure 5



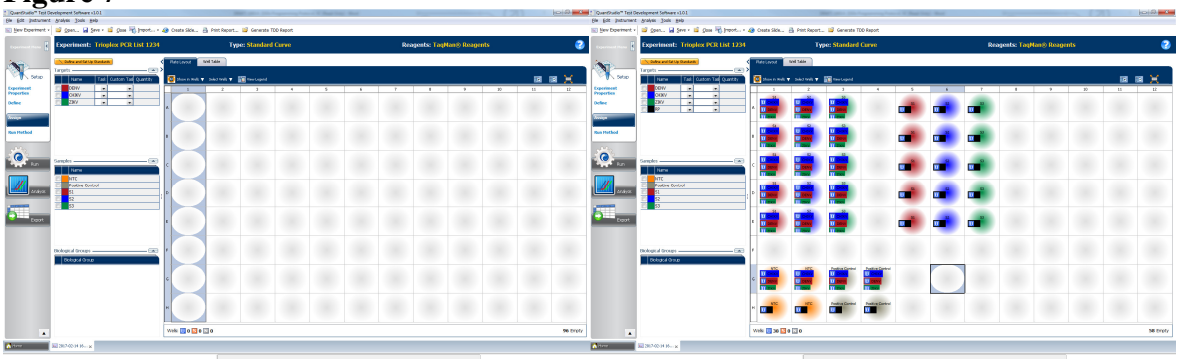
7. Under the **Define** section, add targets corresponding to each virus **(Figure 6)**:
   a) Select **New** and define each target with the corresponding **Target Name** (DENV, CHIKV, ZIKV), **Reporter** (FAM, VIC, Texas Red, accordingly), **Quencher** (none), and color of choice (default). Targets can be saved to the Library for future use.
   b) Samples can be labeled individually in the **Sample** section by selecting **New.**
   c) Under the **Passive Reference** section, select **None**

Figure 6



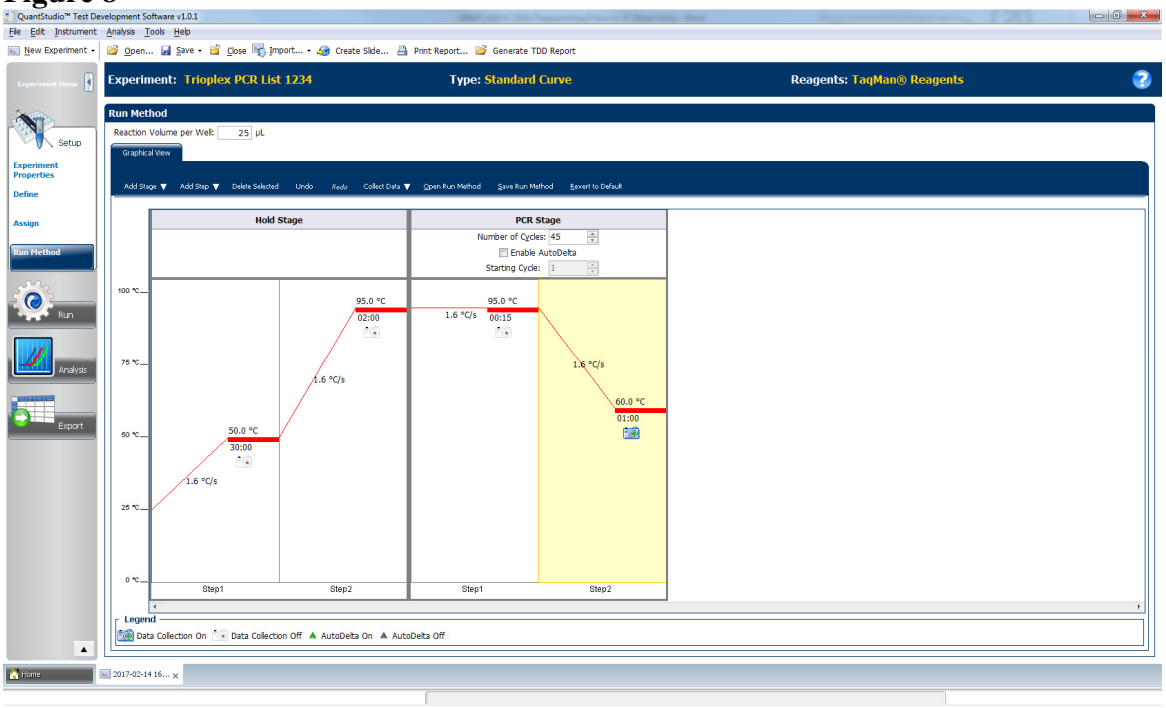
8. Under the **Assign** section, indicate where in the PCR plate the samples are located **(Figure 7)**.
   a) Highlight the wells in the **Plate Layout** section
   b) Under the **Targets** menu, check all the targets that will be detected in the highlighted PCR plate wells
   c) Sample labels can be added to the highlighted PCR wells by checking the corresponding label under the **Samples** menu

Figure 7



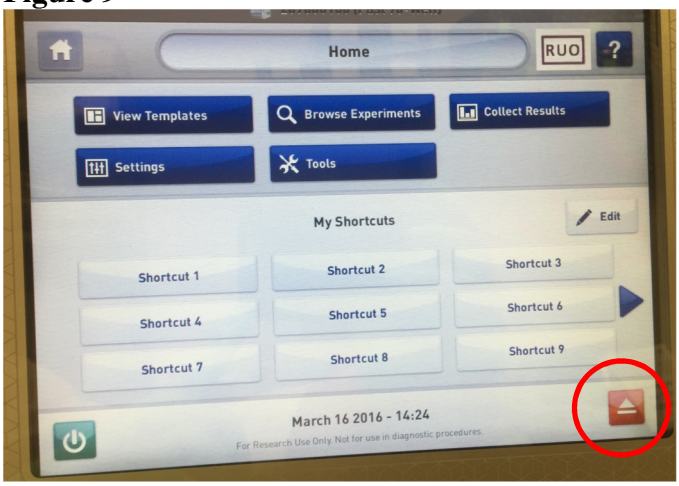
9. Under the **Run Method** section, enter the thermocycling parameters according to the PCR master mix to be used (Invitrogen SuperScript III or Quanta qScript) (**Figure 8**):
   a) Set **Reaction Volume per Well** to 25 µL
   b) Enter Step 1 Hold Stage: 50.0°C for 30:00 minutes
   c) Enter Step 2 Hold Stage: 95.0°C for 2:00 minutes (SuperScript III) or 5:00 minutes (qScript)
   d) Enter Step 1 PCR Stage: 95.0°C for 0:15 minutes
   e) Enter Step 2 PCR Stage: 60.0°C for 1:00 minute (collect fluorescence data at this step)
   f) Change number of cycles to 45

Figure 8



10. Load the PCR plate sealed with appropriate optical sealing tape into the instrument by selecting the Open/Close button on the instrument touch screen (**Figure 9**).

Figure 9

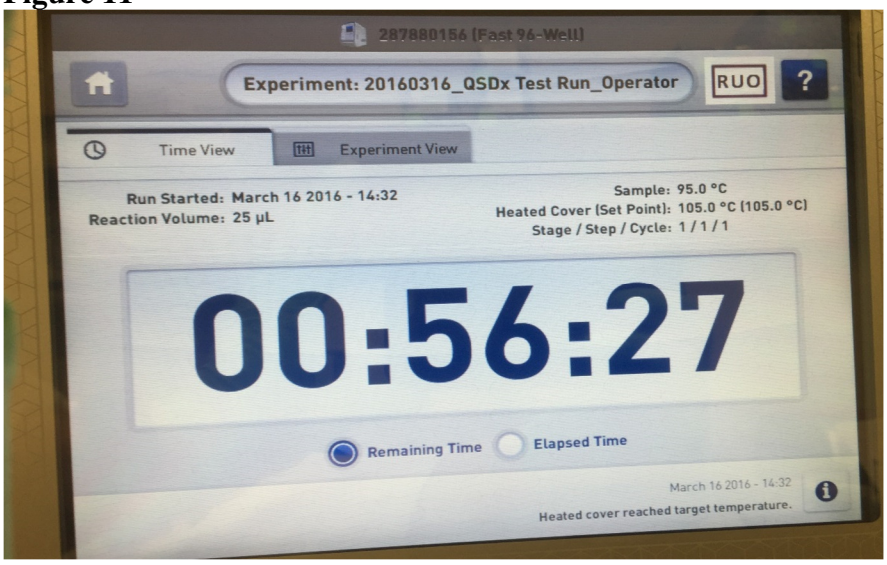


11. The plate arm will open to the side of the instrument. Confirm that the plate arm contains the FAST 96 well Plate insert and that the A1 well of the PCR plate is aligned with the A1 symbol on the upper left corner of the plate insert (**Figure 10**). Close the plate arm.

Figure 10

12. To start run, click on the **Run** menu on the left and click on the **Start Run** button. A window will pop up asking to Save File. Assign appropriate file name and the run data will be saved with a .eds file extension in a pre-designated data folder. If run does not start at this point, click **Start Run** again and run will start. The time window will appear on the touch screen of the instrument once the protocol has started (**Figure 11**).

Figure 11



Data Analysis

After completion of the run, save and analyze the data following the software instructions and the data interpretation guidelines provided in this manual. The same data analysis guidelines provided for the AB 7500 Fast Dx instrument apply to the QuantStudio™ Dx instrument. Analyses should be performed separately for each target using a manual threshold setting. Thresholds should be adjusted to fall within the beginning of the exponential phase of the amplification curve and above the background signal. The procedure chosen for setting the threshold should be used consistently from run to run.