VELA DIAGNOSTICS

Sentosa® SA ZIKV RT-PCR Test (4x24)

Instructions for Use

Version 1.0

For use under an Emergency Use Authorization (EUA) only.

IVD

Rx Only

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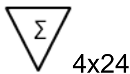
REF 300615



4x24 tests

MAT PS103106A

Consult instructions for use

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Intended use

The *Sentosa*® SA ZIKV RT-PCR Test is a real-time RT-PCR test intended for the qualitative detection of RNA from the Zika virus in serum, EDTA plasma or urine (collected alongside a patient-matched serum or plasma specimen) from individuals meeting Centers for Disease Control and Prevention (CDC) Zika virus clinical criteria (e.g., clinical signs and symptoms associated with Zika virus infection) and/or CDC Zika virus epidemiological criteria (e.g., history of residence in or travel to a geographic region with active Zika transmission at the time of travel, or other epidemiological criteria for which Zika virus testing may be indicated). Testing is limited to U.S. laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests, and similarly qualified non-U.S. laboratories.

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

Negative results do not preclude Zika virus infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

The *Sentosa*® SA ZIKV RT-PCR Test is intended for use by trained clinical laboratory personnel who have received specific training on the use of the *Sentosa*® SA ZIKV RT-PCR Test. The test is only for use under the Food and Drug Administration's Emergency Use Authorization.

The *Sentosa*® SA ZIKV RT-PCR Test is configured for automated workflow using the *Sentosa*® SX101 instrument, in conjunction with the Applied Biosystems® 7500 Fast Dx Real-Time PCR instrument (ABI 7500 Fast Dx) or the *Sentosa*® SA201. The *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24) is used for extraction.

Safety information

- When working with samples and chemicals, always wear a suitable laboratory coat, disposable gloves, and protective goggles. For more information on *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24) and *Sentosa*® SA ZIKV RT-PCR Test, please refer to the respective material safety data sheet (MSDS) available online in PDF format at www.veladx.com.
- For more safety information on the instruments, please refer to the relevant instrument user manual.
- Discard samples and waste according to the local safety regulations.

Pathogen information

Zika virus (ZIKV) is a single-stranded RNA virus that belongs to the flavivirus genus. Similar to dengue and chikungunya, the primary mode of transmission is through the bite of an infected *Aedes* species mosquito. Viral transmission can be further mediated through vertical transmission, sexual contact and possibly blood transfusion. Patients infected with ZIKV may develop mild symptoms, which include rash, fever, joint pain and red eyes that last 2-7 days after infection[1]. However, the majority of infected individuals remain asymptomatic[2].

Outbreaks of ZIKV have been reported in many South and Central American countries as well as tropical Africa, Southeast Asia and the Pacific Islands[1]. A ZIKV outbreak is especially significant in Brazil where an estimate of 1.3 million suspected cases were reported. In parallel, an increase in the number of babies born with microcephaly of approximately twenty-fold has been observed. ZIKV virus infection in pregnancy is a cause of microcephaly. Among fetuses and infants infected with ZIKV prior to birth, absence or poor development of brain structures, hearing, eye defects and impaired growth have been detected. ZIKV has also been correlated with Guillian-Barre syndrome - damaging of the peripheral nervous system by the immune system, resulting in muscle weakness[1].

Assay Principle

The *Sentosa*® SA ZIKV RT-PCR Test comprises a ready-to-use kit for the detection of ZIKV RNA by PCR on the ABI 7500 Fast Dx Real-Time PCR instrument or *Sentosa*® SA201, with nucleic acid extraction and PCR assay set-up using the *Sentosa*® SX101 instrument. The ZIKV master mix contains reagents and enzymes for reverse transcription and specific amplification of a 103 base pair (bp) fragment of the NS4A gene within the open reading frame (ORF) of the ZIKV. The master mix also contains specific primers/probe for the direct detection of ZIKV amplicons in the fluorescence channels Cycling Green of the ABI 7500 or *Sentosa*® SA201.

Detection of the targets occurs in two channels: green and red on the ABI 7500 Fast Dx Real-Time PCR instrument or *Sentosa*® SA201. Output is recorded as the increase of fluorescence over time in comparison to background signal. Monitoring the fluorescence intensities during the PCR run allows the detection of the accumulating product without having to re-open the reaction tubes after the PCR run.

In addition, the *Sentosa*® SA ZIKV RT-PCR Test contains a second set of primers / probes designed to detect an extraction control (EC1) target in the fluorescence channel Cycling Red. This extraction control is used as a control for the nucleic acid extraction procedure and as a PCR inhibition control. The EC1 amplification does not compromise the detection limit of the analytical ZIKV PCR. The test also contains a negative control (NC1) and a positive control (ZIKV PC) that allow the user to assess whether the PCR reaction has been performed properly.

Pathogen detection by PCR is based on the amplification of specific regions of the

pathogen genome. In real-time PCR, the amplified products are detected via fluorescent dyes linked to oligonucleotide probes that bind specifically to the target sequences. Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Real-time monitoring of the fluorescence intensities during the PCR run allows the detection of the accumulating products[3].

Kit contents

Kit name and item no.	Item	Cap color	Description	Quantity (tube)	Volume /tube
***Sentosa*® SA ZIKV RT-PCR Test v2.0 (4x24) 300615**	ZIKV M1 (24)	Green	Primer and probe mix	4	150 µL
	RNA4 M2 (24)	Orange	Amplification mix	4	325 µL
	RNA4 M3 (96)	Pink	Reverse transcriptase	1	330 µL
	NC1	Yellow	Negative control (NC)	4	300 µL
	ZIKV PC	Blue	Positive control (PC)	4	300 µL
	EC1	Red	Extraction control (EC)	4	200 µL

Storage

The components of the *Sentosa*® SA ZIKV RT-PCR Test are stable for 12 months (until the expiration date stated on the label) when stored at −20°C. They should not be subjected to repeated freeze-thaw cycles as this may compromise assay performance. Each set of reagents (ZIKV M1, RNA4 M2, NC1, ZIKV PC and EC1) is meant for single-use and not recommended for reuse.

RNA4 M3 is designed for maximum of four runs. RNA4 M3 is an enzyme in liquid state under −20°C storage conditions. RNA4 M3 should be used directly out of the freezer or kept on ice during reagent preparation. Handle it carefully to avoid contamination and store the remaining RNA4 M3 at −20°C immediately after use for subsequent runs.

Materials required but not provided in the kit

Table 1. List of items to be supplied by user.

Equipment / software	Description / use	Vela item no.
Pipettes[i] (adjustable)	For pipetting buffers, reagents and / or samples	N/A
Vortex mixer	To mix reagents	N/A
Bench top centrifuge[i]	To spin down reagents and remove any bubbles	N/A
Sentosa® SX101 instrument[i]	Automated liquid handling system	400089
Sentosa® SX software	To operate *Sentosa*® SX101 instrument	460018
MPS 1000 Mini Plate Spinner, 120V or Eppendorf Centrifuge 5430 / 5430R with Rotor FA-45-24-11[ii]	For PCR plate centrifugation	N/A
Sentosa® SA201 Real-Time PCR Instrument[ii]	Real-time and end-point thermal cycling using PCR, detection and analysis	400125
Sentosa® SA201 Series Software	To operate *Sentosa*® SA201 Real-Time PCR Instrument and to perform PCR data analysis.	460012
Alternative PCR Instrument and Software option		
ABI 7500 Fast Dx Real-Time PCR instrument	Real-time and end-point thermal cycling using PCR, detection and analysis	N/A
ABI 7500 Fast Dx SDS Software	To operate ABI 7500 Fast Dx Real-Time PCR instrument and to perform PCR data analysis	N/A

[i] Ensure that the instruments have been checked and calibrated according to the manufacturer's recommendations.

[ii] MPS 1000 Mini Plate Spinner, 120V from Labnet (Cat. No. C1000) or Eppendorf Centrifuge 5430 / 5430R with Rotor FA-45-24-11 from Eppendorf (Cat. No. 022654047) are recommended.

Accessory / consumable / reagent	Description / use	Vela item no.
Sentosa® SX Virus Total Nucleic Acid Kit v2.0 (4x24)	4x24 tests	300353
Sentosa® SX qPCR Starter Kit	Contains labware used for qPCR applications	400107
Sentosa® SX Magnetic Separator	1 piece	400024
Sentosa® SX Safe-Lock Tubes[iii] (1000)	1000 pieces	400031
Sentosa® SX Partition 50 µL Filter Tips (960)	10 boxes of 96 tips	400026
Sentosa® SX Partition 1000 µL Filter Tips (960)	10 boxes of 96 tips	400025
Sentosa® SX 100 mL Reservoir (50)	Set of 50 reservoirs	400027
Sentosa® SX 30 mL Reservoir (50)	Set of 50 reservoirs	400028
Sentosa® SX Deepwell Plate 96/2000 µL (20)	Set of 20 plates	400068
Sentosa® SX Microplate 96/V (80)	Set of 80 plates	400030
Sentosa® SX PCR Foil, adhesive (100)	100 pieces	400032
Sentosa® SX Biohazard Bag (100)	100 pieces	400033
Sentosa® SX Thermoblock PCR 96[iv]	Base for *Sentosa*® SA 96-Well Optical Plate during nucleic acid extraction and PCR set up on *Sentosa*® SX101 instrument	400079
Sentosa® SX Dispensing tool TM 50	Pipetting tool for *Sentosa*® SX101 instrument	400060
Sentosa® SA 96-Well Optical Plates	Set of 20 plates	400149
Sentosa® SA Optical Adhesive Seals	100 pieces	400146
MicroAmp® Fast Optical 96-Well Reaction Plate (0.1mL)[v]	Set of 20 plates	N/A
MicroAmp™ Optical Adhesive Film[vi]	100 pieces	N/A
MicroAmp® Splash-free Support Base[vii] (optional)	To hold *Sentosa*® SA 96-Well Optical Plate securely during plate sealing	N/A
MicroAmp® Adhesive Film Applicator[viii]	For applying adhesive film	N/A
Sterile pipette tips with filters	For pipetting buffers, reagents and / or samples	N/A

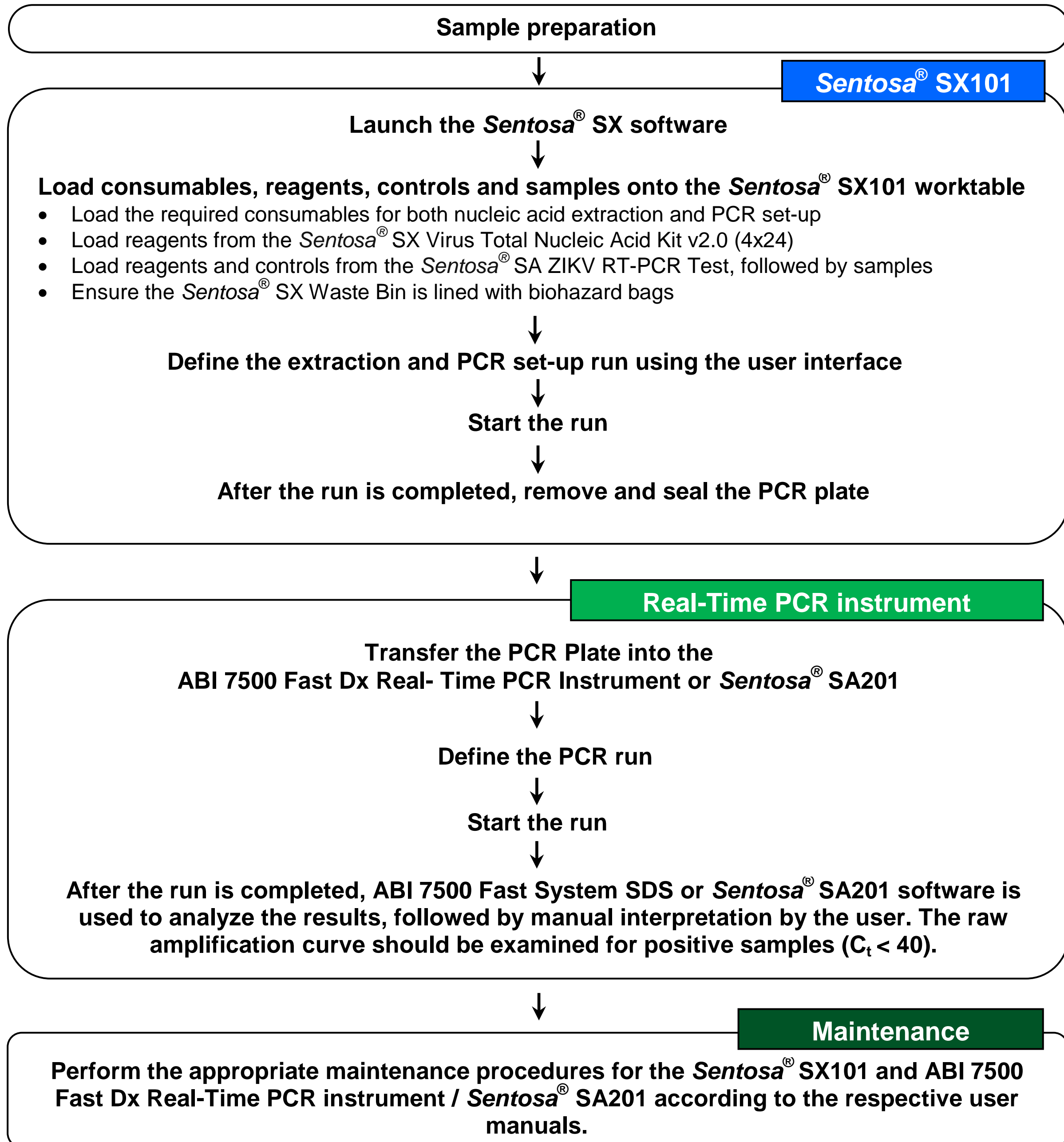
[iii] 1.5 mL Sarstedt tubes from Sarstedt AG & Co. (Cat. No. 72.692.005) have also been validated.
[iv] *Sentosa*® SX Thermoblock PCR 96 must be used.
[v] MicroAmp® Fast Optical 96-Well Reaction Plate (0.1mL) from Applied Biosystems (Cat. No. 4346906) must be used.
[vi] MicroAmp™ Optical Adhesive Film from Applied Biosystems (Cat. No. 4311971) must be used.
[vii] MicroAmp® Splash-free Support Base from Applied Biosystems (Cat. No. 4312063) is recommended.
[viii] MicroAmp® Adhesive Film Applicator from Applied Biosystems (Cat. No. 433183) must be used.

Workflow overview

The *Sentosa*® SA ZIKV RT-PCR Test workflow starts with sample lysis followed by the automated extraction of nucleic acids from serum / EDTA plasma / urine samples on the *Sentosa*® SX101 instrument using the *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24). After extraction, the instrument will set up PCR with the extracted nucleic acids automatically in a *Sentosa*® SA 96-Well Optical Plate or MicroAmp® Fast Optical 96-Well Reaction Plate. Subsequently, the PCR Plate is sealed and transferred to the ABI 7500 Fast Dx Real-Time PCR Instrument or *Sentosa*® SA201 for PCR amplification, followed by data analysis and manual result interpretation.

An overview of a Workflow is provided (refer to **Flowchart**). The workflow can process up to 22 samples with one negative control (NC1) and one positive control (ZIKV PC) in each run. For more information, please refer to the "Protocol: Automated nucleic acid extraction, PCR set-up and detection on the *Sentosa*® SX101 and the ABI 7500 Fast Dx Real-Time PCR instrument or *Sentosa*® SA201" section, page 9.

Flowchart. *Sentosa*® workflow using ABI 7500 Fast Dx or *Sentosa*® SA201.



Warnings and Precautions

General precautions

- For *In Vitro* Diagnostic Use.
- Use sterile pipette tips with filters.
- During manual steps, ensure that the tubes are closed when possible to avoid contamination.
- **Do not mix components from kits with different lot numbers.**
- Proceed continuously from one part of the workflow to the next. Do not exceed 30 minutes of transfer time between the *Sentosa*® SX101 and the ABI 7500 Fast Dx Real-Time PCR or *Sentosa*® SA201 instruments.

Laboratory procedures

- All samples and waste should be considered potentially infectious. Clean and disinfect all work surfaces thoroughly with disinfectants recommended by local authorities.
- Do not eat, drink or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats and eye protection when handling samples and kit reagents.
- Clean and decontaminate work area and instruments, including pipettes, with commercially available decontamination products.
- Avoid microbial and nuclease contamination of reagents when removing aliquots from reagent bottles. Use sterile disposable pipette tips.
- To avoid environmental contamination from amplicons, do not remove the PCR seal after amplification.
- Wash hands thoroughly after handling samples and kit reagents.

Storage of nucleic acids

Nucleic acids are extracted from serum, EDTA plasma or urine samples and prepared for PCR automatically with the *Sentosa*® SX101 instrument.

The remaining extracted nucleic acids are kept in the *Sentosa*® SX Microplate 96/V. If storage is required, seal the microplate using the *Sentosa*® SX PCR Foil. Keep the extracted nucleic acids at −20°C for long-term storage. Repeated freeze-thaw of extracted nucleic acids should be minimized.

It is not recommended to store the remaining PCR reaction mix after PCR set-up.

Protocol: Automated nucleic acid extraction and RT-PCR set-up on the *Sentosa*® SX101 instrument followed by real-time RT-PCR amplification and detection on the ABI 7500 Fast Dx Real-Time PCR or the *Sentosa*® SA201 instruments

The *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24) is validated for viral total nucleic acid extraction from serum / EDTA plasma / urine samples for use with the *Sentosa*® SA ZIKV RT-PCR Test.

Important points before starting

- User must be familiar with operating the *Sentosa*® SX101 and ABI 7500 Fast Dx Real-Time PCR or *Sentosa*® SA201 instruments. Please refer to the respective user manuals supplied with the instruments for operating instructions.
- Before beginning the procedure, read the “Warnings and Precautions” section, page 8.
- The *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24)
    - Virus A1 tubes containing Proteinase K solution require gentle inversion followed by brief centrifugation (approx. 1,000 x g for 10 sec). Virus A2 tubes containing magnetic beads require thorough vortexing before use to ensure proper re-suspension.
    - Prior to use, Virus A3 (lyophilized carrier RNA) must be reconstituted with Virus A4 (carrier RNA buffer). Refer to detailed procedure step 2.8 for more information.
    - Mix the buffers in the bottles by gentle swirling, ensuring no foam or bubbles are present.
    - All components should be used within 30 minutes after removal of caps for extraction set-up.
- Thaw the *Sentosa*® SA ZIKV RT-PCR Test components at room temperature (approximately 15°C to 25°C). Do not exceed 30 minutes before placing the components onto the *Sentosa*® SX101 worktable.
- Ensure that the ABI 7500 Fast Dx Real-Time PCR instrument or *Sentosa*® SA201 is switched on at the beginning of the workflow (refer to the ABI 7500 Fast Dx Real-Time PCR Instrument or *Sentosa*® SA201 – Reference Guide for more details on how to operate the instrument).
- Do not discard the *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24) box as the user needs to scan the 2-D barcode on the box at step 2.13.

- For software, use current version or higher as follows:

Sentosa® SX101	Release 2.0 (Version 21.5.3.0)
ABI 7500 Fast Dx SDS Software	Version 1.4
Sentosa® SA201 Series Software	Version 1.0.1

- Screenshots are for illustration purposes only and may vary between installations.

Specimen collection, handling and storage

- Human EDTA plasma, serum and urine specimens may be used with *Sentosa*® SA ZIKV RT-PCR Test. These specimens should be collected and processed based on the standard operating procedures of respective laboratories or manufacturer's instructions for serum or plasma collection tubes.
- It is recommended to collect blood in gel separator tubes for serum or K2/K3 EDTA tubes for plasma.
- 250 µL of collected specimens should be transferred into fresh 1.5 mL *Sentosa*® SX Safe-Lock Tubes in a biosafety cabinet before the extraction procedure.

  NOTE: Remove visual precipitates, if any, from the samples by centrifuging the sample tubes (approx. 1,000 to 2,000 x g for 30 sec to 1 min) and transferring the supernatants into fresh 1.5 mL *Sentosa*® SX Safe-Lock Tubes.
- Specimens may be temporarily stored at 2°C − 8°C if they are to be extracted on the same day as collection. If not, it is recommended to freeze the specimens immediately at ⩽ −80°C. Repeated freeze-thaw of the specimens should be minimized.
- When transporting specimens ensure all application regulations for transport of potentially infectious biological specimens are met.
- Transport/ship specimens frozen on dry ice.

  NOTE: Inadequate specimen collection and / or inappropriate specimen processing, storage and transport may yield false negative results.

Procedure

The throughput of the *Sentosa*® SA ZIKV RT-PCR Test workflow, including nucleic acid extraction and RT-PCR, is 24 tests, including one positive control and one negative control. The total machine run time for the *Sentosa*® SX101 and the ABI 7500 Fast Dx Real-Time PCR or the *Sentosa*® SA201 is approximately 3 hours.

1. Sample preparation

1.1. Transfer 250 µL of each sample to a fresh 1.5 mL *Sentosa*® SX Safe-Lock Tubes in a biosafety cabinet.

NOTE:

- Remove visual precipitates, if any, from the samples by centrifuging the sample tubes (approx. 1,000 to 2,000 x g for 30 sec to 1 min) and transferring the supernatants into fresh 1.5 mL *Sentosa*® SX Safe-Lock Tubes.
- Sample processing volume by the *Sentosa*® SX101 is 200 µL.
- Elution volume is 100 µL.

2. *Automated nucleic acid extraction and PCR set-up on the Sentosa® SX101 instrument*

Figure 1 shows the positions of consumables / labware on the *Sentosa®* SX101 platform. Double line the *Sentosa®* SX Waste Bin with biohazard bags. Please refer to the layout as indicated by the *Sentosa®* SX101 instrument software or the appendix to load all items in the correct positions.

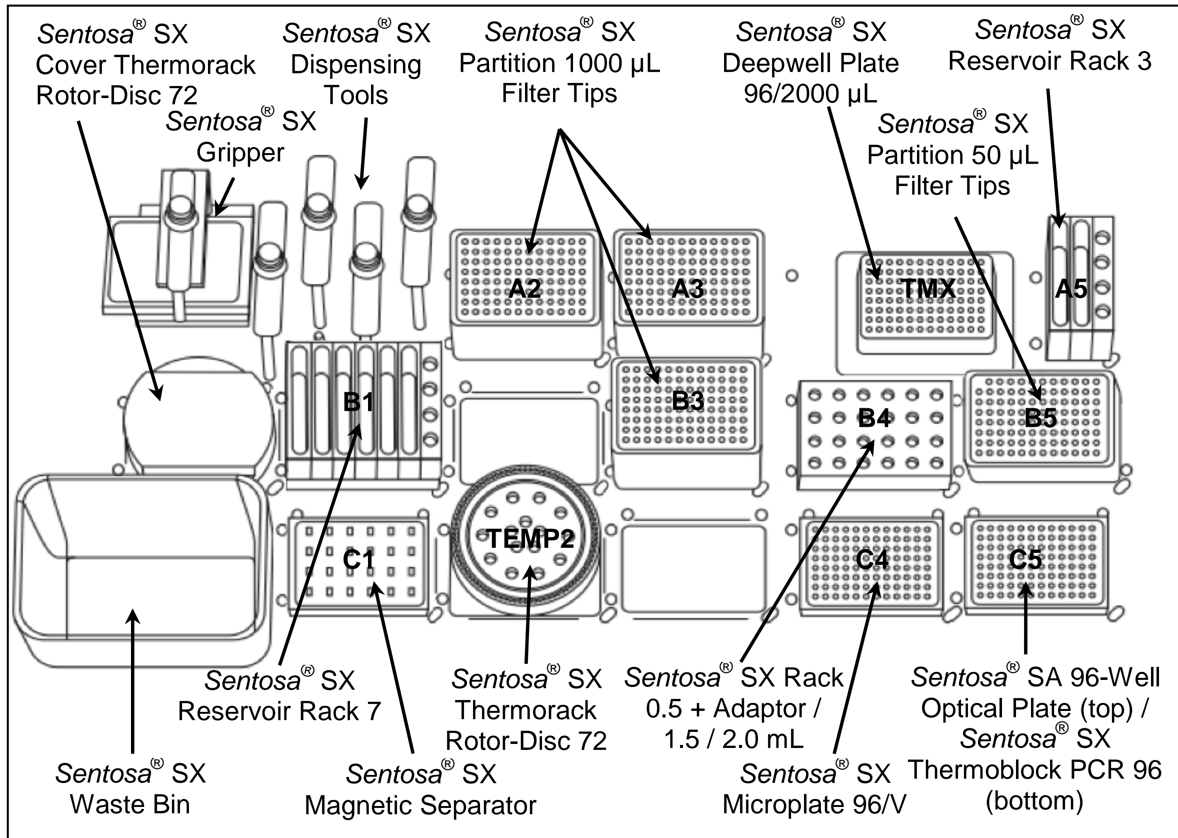
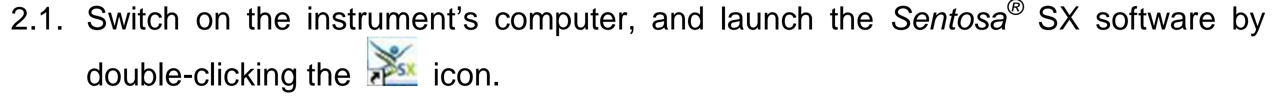


Figure 1. Layout of the *Sentosa®* SX101 platform for 24 tests

NOTE:

- Items shown are necessary for nucleic acid extraction and PCR set-up for 24 tests (application *"24-1 VirLys3 v2-1"*).
- Ensure that all consumables / labware are properly placed, aligned and secured into their respective positions. The biohazard bags must be properly placed in the *Sentosa®* SX Waste Bin before starting a protocol run. For more information, please refer to the *Sentosa®* SX101 instrument user manual.



2.1. Switch on the instrument's computer, and launch the *Sentosa*® SX software by double-clicking the icon.

The *"Login"* window opens.

Type the account name and password, and then click *"Login"*.

NOTE:

- Switch on the *Sentosa*® SX101 instrument after the *Sentosa*® SX software is launched.
- Switch on the ABI 7500 Fast Dx Real-Time PCR or the *Sentosa*® SA201 instrument prior to the start of the procedure.

2.2. Select *"Open / run applications"* from the *"Tasks"* menu.

2.3. Select the *"VelaDx"* account and choose the *"qPCR Tropical"* folder. Run the required application according to the following table.

Application (v2-1 or higher)	Number of tests	*Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24)	*Sentosa*® SA ZIKV RT-PCR Test
24-1 VirLys3	Up to 24 tests	One set of reagents (4x24)	One set of reagents (4x24)

2.4. Place the correct *Sentosa*® SX Reservoirs into positions 1, 2, 3, and 7 of the *Sentosa*® SX Reservoir Rack 7 (refer to **Figure 1**, B1) according to the following table. 100 mL Reservoir in position 7 is meant for liquid waste collection.

Application (v2-1 or higher)	*Sentosa*® SX Reservoir Rack 7 (B1)	
	Positions 1 to 3	**Position 7**
24-1 VirLys3	3 x 30 mL Reservoir	1 x 100 mL Reservoir

2.5. Place the correct *Sentosa*® SX Reservoirs into position A and B of the *Sentosa*® SX Reservoir Rack 3 (refer to **Figure 1**, A5) according to the following table.

Application (v2-1 or higher)	*Sentosa*® SX Reservoir Rack 3 (A5)	
	Position A	Position B
24-1 VirLys3	1 x 30 mL Reservoir	1 x 30 mL Reservoir

2.6. Transfer all reagents from the *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24) into their respective positions in *Sentosa*® SX Reservoir Rack 7 **and** *Sentosa*® SX Reservoir Rack 3 as indicated by the application protocol.

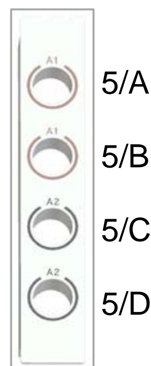
Application (v2-1 or higher)	*Sentosa*® SX Reservoir Rack 7 (B1)			
	Position 1	Position 2	Position 3	Position 7
	Virus B6	Virus B4	Virus B3	Waste Collection
24-1 VirLys3	1 x B6 (24)	1 x B4 (24)	1 x B3 (24)	N/A

Application (v2-1 or higher)	*Sentosa*® SX Reservoir Rack 3 (A5)	
	Position A	Position B
	Virus B1	Virus B2
24-1 VirLys3	1 x B1 (24)	1 x B2 (24)

NOTE:

- All reagents should be gently mixed, without foaming, before use.
- Ensure that all buffers in the bottles are transferred completely into the corresponding reservoir positions.

2.7. Place the *Sentosa*® SX RR Module A1/A2, illustrated in the figure below, in position 5 of the *Sentosa*® SX Reservoir Rack 7.



Sentosa® SX RR Module A1/A2

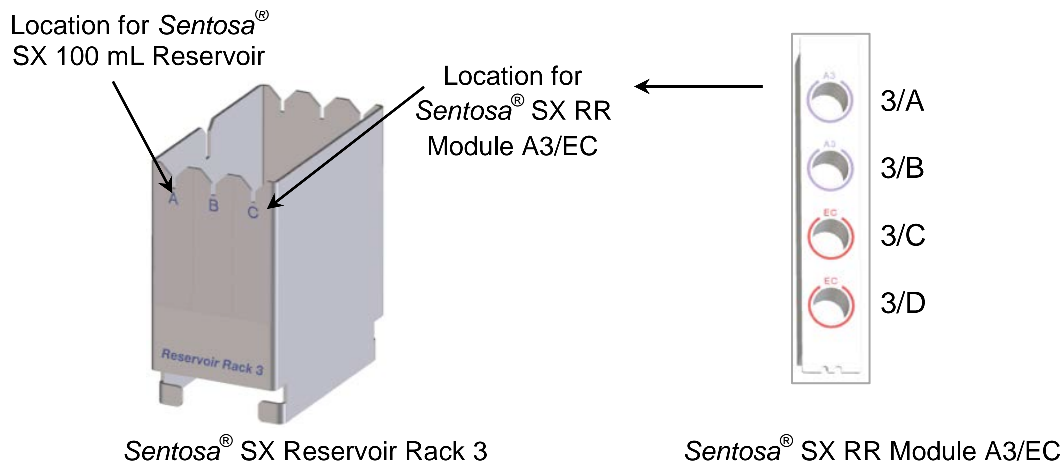
Load the Virus A1 tube and the Virus A2 tube from the *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24) on the *Sentosa*® SX RR Module A1/A2 according to the following table.

Application (v2-1 or higher)	*Sentosa*® SX RR Module A1/A2 (B1)			
	5/A	5/B	5/C	5/D
24-1 VirLys3	Virus A1 (24)	N/A	Virus A2 (24)	N/A

NOTE:

- The Virus A1 tube containing Proteinase K solution should be mixed by gentle inversion followed by brief centrifugation (approx. 1,000 x g for 10 sec).
- Ensure that the magnetic beads in Virus A2 tube from the *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24) are fully re-suspended. Vortex the tube for 1 minute and flick to collect the contents at the bottom of the tube. Ensure that no bubbles or multilayers are observed.
- Ensure that all tubes are uncapped.
- All reagents are designed for single-use only. Do not use the remaining reagents from previous runs.

2.8. Place the *Sentosa*® SX RR Module A3/EC in position C of the *Sentosa*® SX Reservoir Rack 3 as shown below, otherwise the instrument will detect an error.



Sentosa® SX Reservoir Rack 3

Sentosa® SX RR Module A3/EC

Load the Virus A3 (lyophilized carrier RNA) tube reconstituted with Virus A4 (carrier RNA buffer) from the *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24) and the EC1 (extraction control) tube from the *Sentosa*® SA ZIKV RT-PCR Test on the *Sentosa*® SX RR Module A3/EC according to the following table.

Application (v2-1 or higher)	*Sentosa*® SX RR Module A3/EC (A5)			
	3/A	3/B	3/C	3/D
24-1 VirLys3	Virus A3 (24)	N/A	EC1	N/A

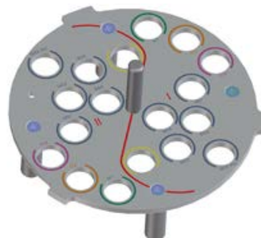
NOTE:

- The Virus A3 tube should be reconstituted with Virus A4 as described below.
  - Briefly spin Virus A3 and Virus A4 for 5 seconds.
  - For the *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24), add 125 µL of Virus A4 (24) to Virus A3 (24).
  - The reconstituted Virus A3 should be mixed by pulse vortexing for 30 seconds followed by brief centrifugation (approx. 1,000 x g for 10 sec).
- The EC1 tube should be mixed by pulse vortexing for 10 seconds followed by brief centrifugation (approx. 1,000 x g for 10 sec). Only one tube of EC1 is required.
- Ensure that all tubes are uncapped.
- All reagents are designed for single-use only. Do not use the remaining reagents from previous runs.

2.9. Assemble the *Sentosa*® SX Thermorack Rotor-Disc 72 with the *Sentosa*® SX Tube Holder Plate and *Sentosa*® SX Adaptor Rack and place it in position TEMP2 of the *Sentosa*® SX101 worktable (refer to **Figure 1**).

Sentosa® SX Adaptor Rack

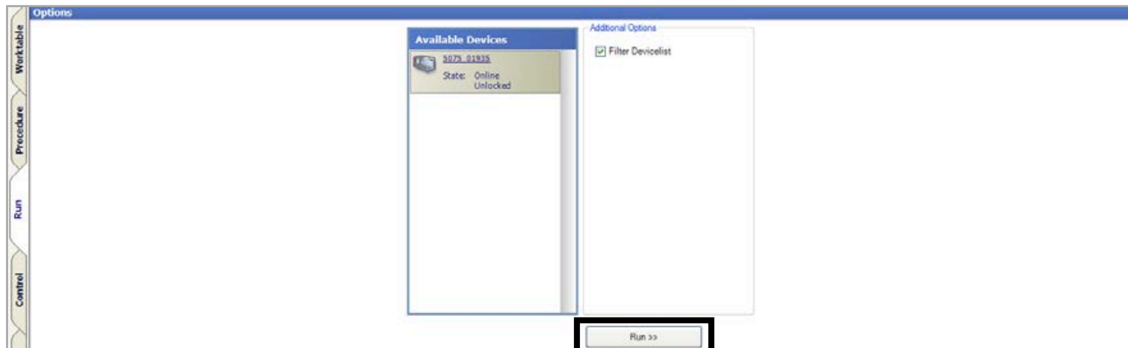
Sentosa® SX Thermorack Rotor-Disc 72



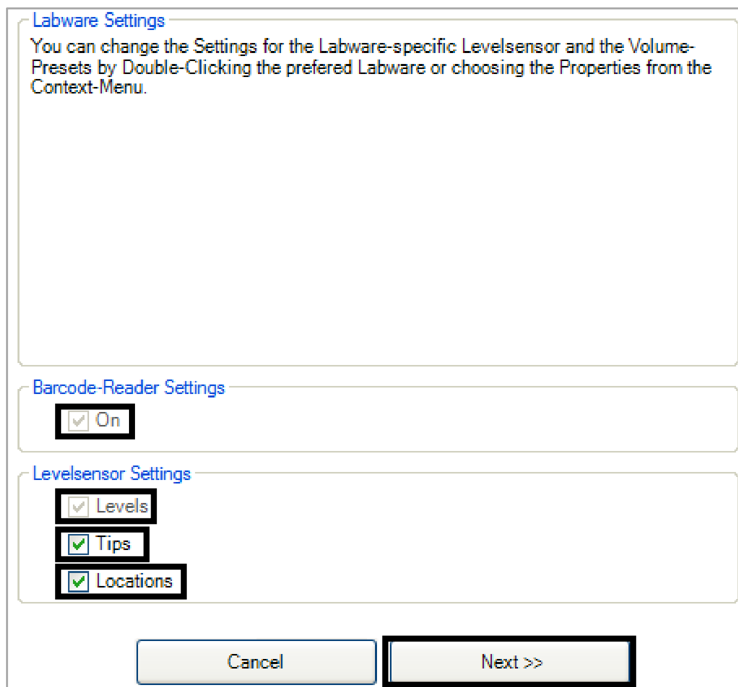
Sentosa® SX Tube Holder Plate For 2 Tests

2.10. Load the *Sentosa*® SX Partition 1000 µL Filter Tips, *Sentosa*® SX Partition 50 µL Filter Tips, *Sentosa*® SX Magnetic Separator, *Sentosa*® SX Deepwell Plate 96/2000 µL, *Sentosa*® SX Microplate 96/V, Sentosa® SX Thermoblock PCR 96 and Sentosa® SA 96-Well Optical Plates on the *Sentosa*® SX101 worktable.

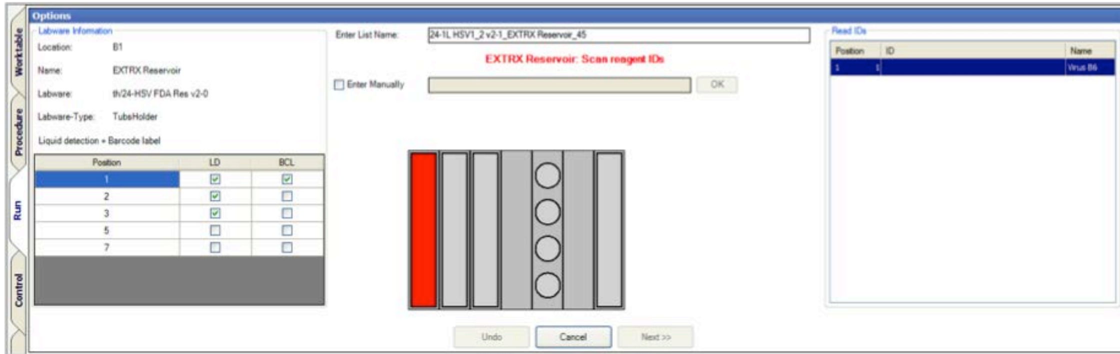
2.11. In the *"Run"* tab, select the *Sentosa*® SX101 instrument (5075 XXXXX, where XXXXX refers to the serial number) under *"Available devices"* and click *"Run"*.



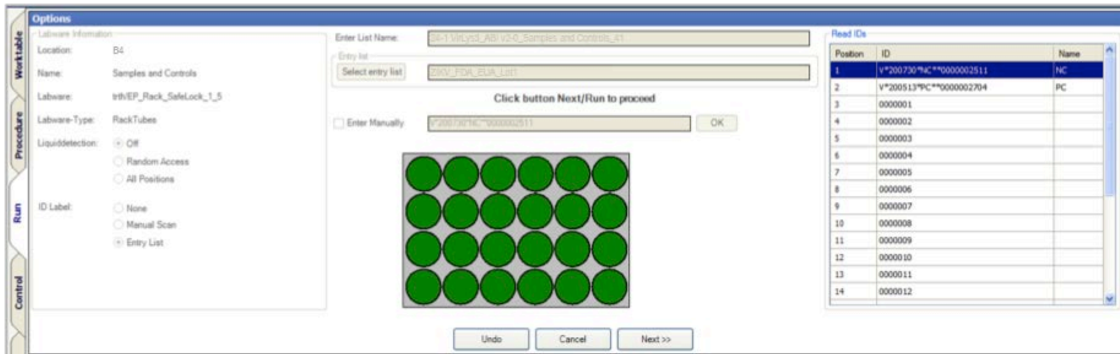
2.12. In *"Labware Settings"*, ensure that all checkboxes are activated, and then click *"Next"*.



2.13. Scan the 2-D barcode on the *Sentosa®* SX Virus Total Nucleic Acid Kit v2.0 (4x24) used in this run. Click *"OK"* and then click "*Next*".



2.14. Remove the NC1 and ZIKV PC from the *Sentosa®* SA ZIKV RT-PCR Test. Scan the NC1 and ZIKV PC barcode, and enter the sample IDs manually by activating the *"Enter manually"* checkbox.



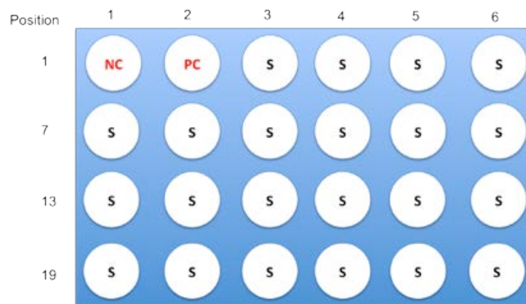
Once all samples are identified, click *"OK"* and then *"Next".*

2.15. Load NC1 and ZIKV PC to positions 1 and 2 of sample rack 1 (*Sentosa®* SX Rack 0.5 + Adaptor / 1.5 / 2.0 mL) after brief vortexing and spinning down.

Load sample tubes into the sample racks (*Sentosa®* SX Rack 0.5 + Adaptor / 1.5 / 2.0 mL) according to the following table.

Application (v2-1 or higher)	***Sentosa®* SX Rack 0.5 + Adaptor / 1.5 / 2.0 mL**
	Sample rack 1 (B4)
24-1 VirLys3	Positions 3 to 24

Layout of the samples for the *Sentosa*® SA ZIKV RT-PCR Test (4x24) is illustrated below.



NC – Negative control, PC – Positive control, S – Sample

Place the sample rack in position B4 of the *Sentosa*® SX101 platform (refer to Figure 1).

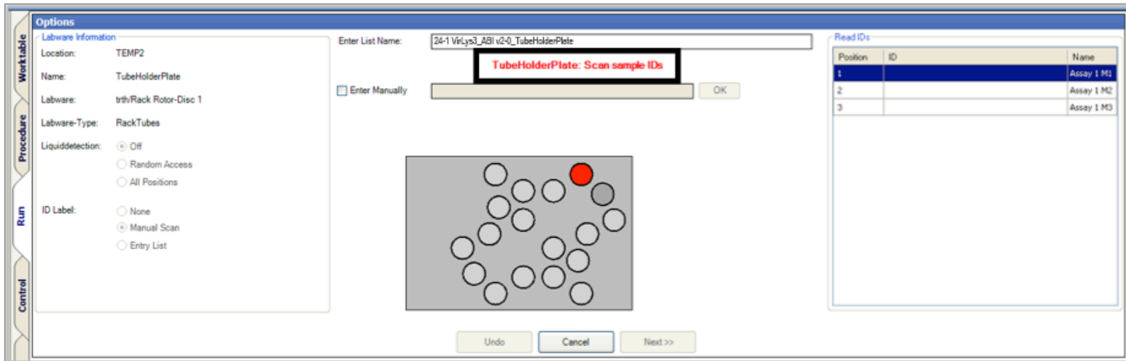
2.16. Prepare PCR reagents (ZIKV M1, RNA4 M2 and RNA4 M3) from the *Sentosa*® SA ZIKV RT-PCR Test. Pulse vortex ZIKV M1 for 10 seconds. Mix RNA4 M2 and RNA4 M3 by gentle inversion. Centrifuge ZIKV M1, RNA4 M2 and RNA4 M3 briefly (approx. 1,000 x g for 10 sec) to collect the contents at the bottom of the tubes.

Keep the RNA4 M3 tube on ice. Pipette 75 µL of RNA4 M3 to ZIKV M1 and gently invert the tube to mix the contents. Centrifuge ZIKV M1, containing RNA4 M3, briefly (approx. 1,000 x g for 10 sec) to collect the contents at the bottom of the tube, followed by barcode scanning.

Remove the caps of the ZIKV M1 and RNA4 M2 tubes and load both tubes into the *Sentosa*® SX Thermorack Rotor-Disc 72 in position TEMP2. The position of each tube is indicated on the *Sentosa*® SX Tube Holder Plate assembled on the *Sentosa*® SX Thermorack Rotor-Disc 72.

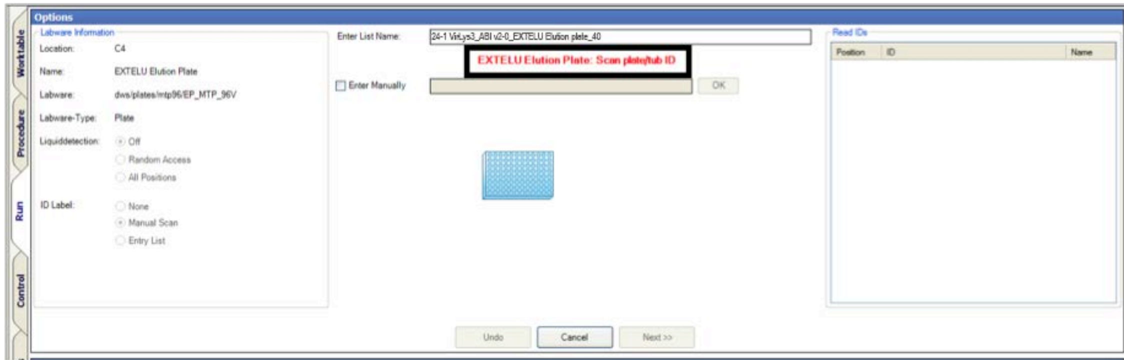
NOTE:

- RNA4 M3 is an enzyme in liquid state under storage conditions. Except RNA4 M3, all reagents should be thawed completely before use.
- RNA4 M3 is designed for maximum of 4 runs. It should be used directly out of the freezer or kept on ice during reagent preparation. Handle carefully to avoid contamination and store the remaining RNA4 M3 at −20°C immediately after use for subsequent runs.
- Do not mix components from kits with different lot numbers.

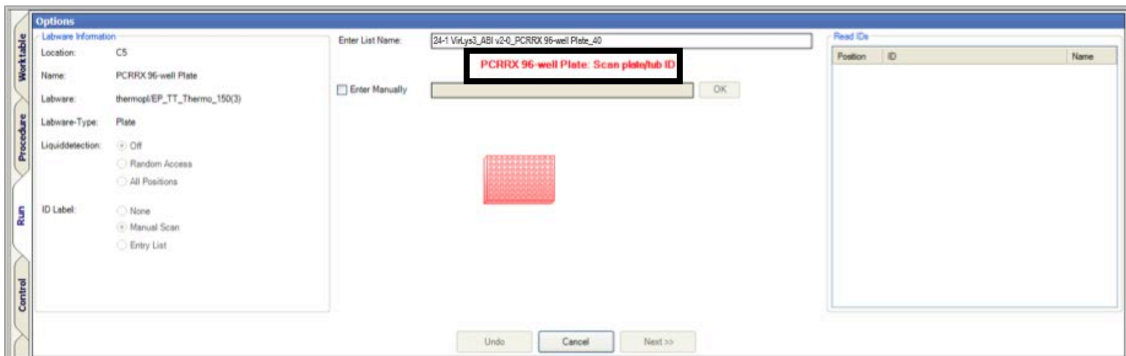


Once the last PCR reagent is loaded, click *“Next”*.

2.17. Scan the *Sentosa*® SX Microplate 96/V ID barcode, or enter the ID manually by checking the *“Enter manually”* check box. Click *“OK”* and then *“Next”*.



2.18. Identify the *Sentosa*® SA 96-Well Optical Plate by scanning the ID barcode, or enter the ID manually by checking the *“Enter manually”* box and key in a proper *Sentosa*® SA 96-Well Optical Plate ID (Recommended ID format is *“OP-96_YYYYMMDD_HHMM”*. e.g. OP-96_20121217_1534). Click *“OK”* and then *“Next”*.



2.19. The software conducts a volume check for each item; click *“Next”* for each *“volume check”* window:

2.19.1. *Sentosa*® SX Deepwell Plate 96/2000 µL

Labware Information

Location: TMX
Name: Separation Plate
Labware: plates/dwp96/EP_DWP_2000_BT800
Labware-Type: Plate
Liquiddetection: Off / Random Access / All Positions
ID Label: None / Manual Scan / Entry List

	Name	Minimum Volume [µL]	Volume [µL]
1		214	214

Cancel Next >>

2.19.2. *Sentosa*® SX Reservoir Rack 7

Labware Information

Location: B1
Name: EXTRX Reservoir
Labware: th/24-HSV FDA Res v2-0
Labware-Type: TubsHolder
Liquiddetection: Off / Random Access / Selected Positions
ID Label: None / Manual Scan / Entry List

	Name	Minimum Volume [µL]	Volume [µL]
1	Virus B6	2,822	6,000
2	Virus B4	15,505	17,000
3	Virus B3	15,505	17,000
5/A	A1	293	350
5/B		0	0
5/C	A2	579	600
5/D		0	0
7	Waste	0	10,000

Cancel Next >>

2.19.3. *Sentosa*® SX Rack 0.5 + Adaptor / 1.5 / 2.0 mL (sample rack)

Labware Information

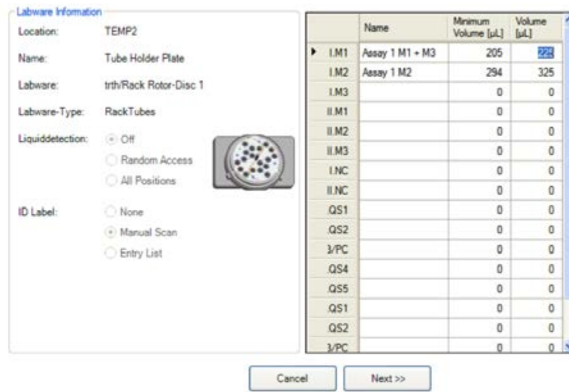
Location: B4
Name: Samples and Controls
Labware: trth/EP_Rack_SafeLock_1_5
Labware-Type: RackTubes
Liquiddetection: Off / Random Access / All Positions
ID Label: None / Manual Scan / Entry List

	Name	Minimum Volume [µL]	Volume [µL]
1	NC	214	300
2	PC	214	300
3	Sample 1	214	250
4	Sample 2	214	250
5	Sample 3	214	250
6	Sample 4	214	250
7	Sample 5	214	250
8	Sample 6	214	250
9	Sample 7	214	250
10	Sample 8	214	250
11	Sample 9	214	250
12	Sample 10	214	250
13	Sample 11	214	250
14	Sample 12	214	250
15	Sample 13	214	250
16	Sample 14	214	250

Cancel Next >>

2.19.4. PCR reagents

– Workflow –

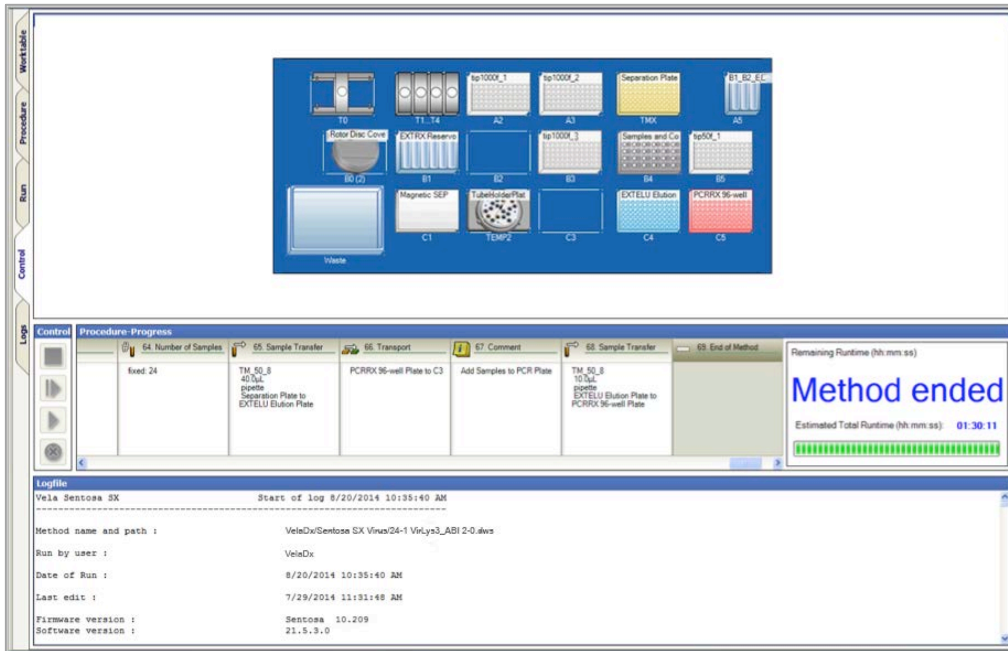


2.19.5. *Sentosa*® SX Microplate 96/V

2.19.6. *Sentosa*® SX Reservoir Rack 3

Click *“Run”* in the last window.

2.20. Nucleic acid extraction and PCR set-up steps are performed automatically. At the end of the protocol, the status of the run changes from *“running”* to *“completed”*.

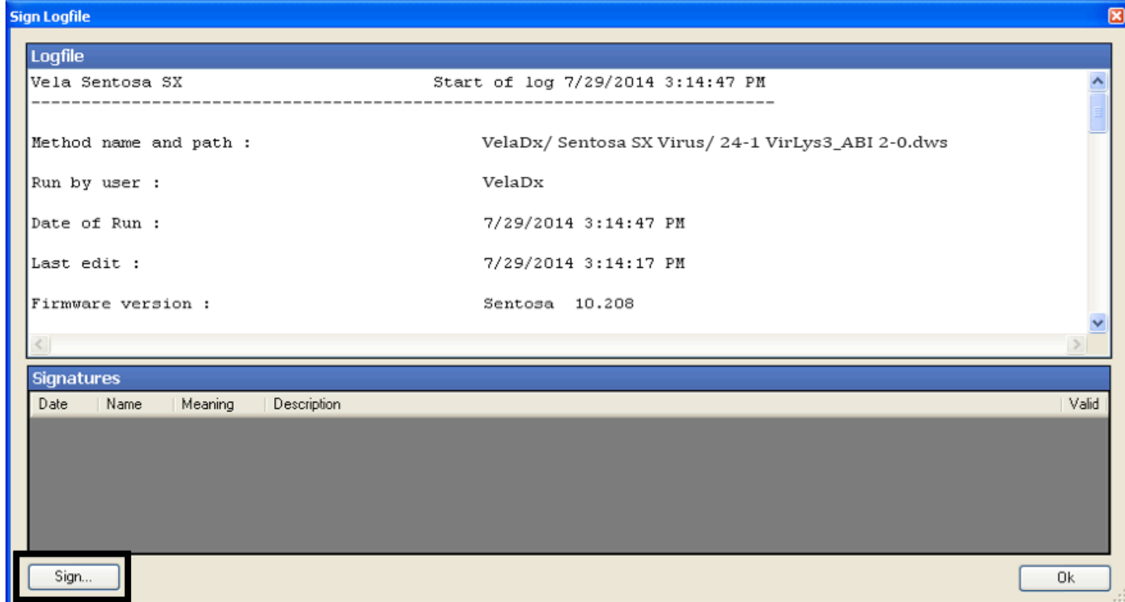


After the run is completed, carefully remove the MicroAmp® Fast Optical 96-Well Reaction Plate or the *Sentosa*® SA 96-Well Optical Plate. Apply a MicroAmp™ Optical Adhesive Film or a *Sentosa*® SA Optical Adhesive Seal on the *Sentosa*® SA 96-Well Optical Plate using the MicroAmp® Adhesive Film Applicator and seal the plate tightly to prevent contamination. Briefly spin down the PCR Plate and load it onto the ABI 7500 Fast Dx Real-Time PCR Instrument or *Sentosa*® SA201 for PCR.

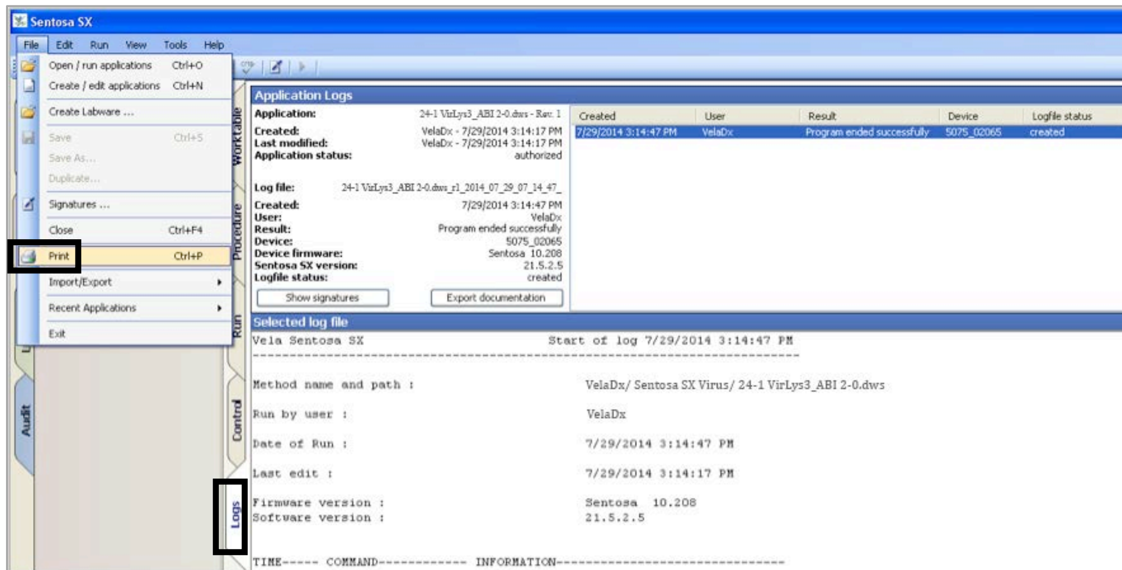
NOTE:

- MicroAmp™ Optical Adhesive Film / *Sentosa*® SA Optical Adhesive Seal should not be adjusted after placing it on the respective MicroAmp® Fast Optical 96-Well Reaction Plate / *Sentosa*® SA 96-Well Optical Plate.
- Ensure that there is no bubble present in the reaction well. If there is, gently flick the well and repeat the spinning down of the PCR plate.
- For nucleic acid storage, please refer to the “Storage of nucleic acids” section, on page 8.

2.21. Log file of the run is automatically generated which is then electronically signed by the operator by clicking *"Sign"*.



2.22. To print the log file, click on the *"Logs"* tab and select *"Print"* under the *"File-Print"* menu.



2.23. Clean and disinfect the *Sentosa*® SX101 instrument after each run. For instrument maintenance, please refer to the *Sentosa*® SX101 instrument user manual.

3. PCR on the ABI 7500 Fast Dx Real-Time PCR Instrument or Sentosa® SA201

3.1. Switch on the ABI 7500 Fast Dx Real-Time PCR Instrument or *Sentosa*® SA201 by pressing the power button on the instrument and wait for the initiation procedure to complete.

NOTE: Ensure the green indicator is lit and not flashing.

3.2. Launch and log in to the ABI 7500 Fast Dx SDS Software v1.4 or *Sentosa*® SA201 Series Software v1.0.1.

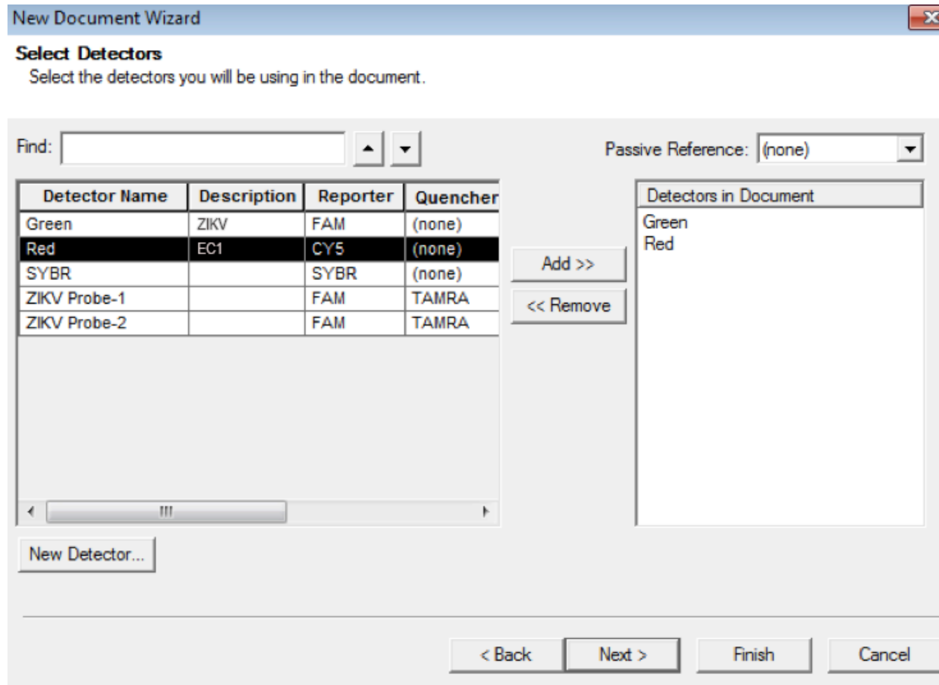
3.3. In the "*Quick Startup Document*" dialog box, select "*Create New Document*". Define the document by selecting from the drop-down list in each field in accordance with the figure below:

New Document Wizard
Define Document
Select the assay, container, and template for the document, and enter the operator name and comments.

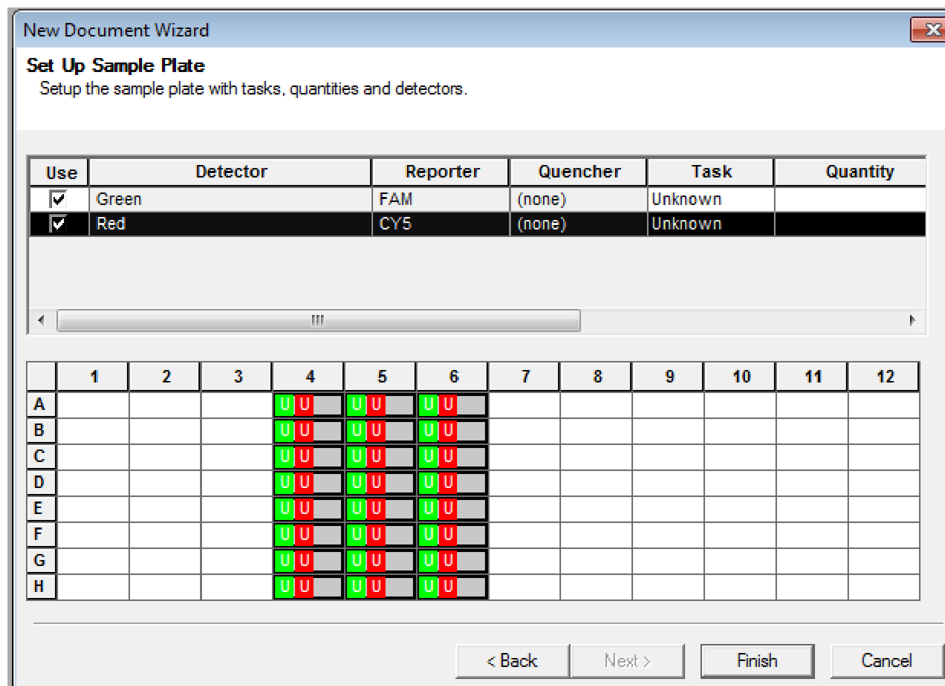
Assay: Standard Curve (Absolute Quantitation)
Container: 96-Well Clear
Template: Blank Document
Browse...
Run Mode: Fast 7500
Operator: INSTR-ADMIN
Comments:
Plate Name: Plate1
< Back | Next > | Finish | Cancel

3.4. Select and add "*Green*" detector / "*FAM*" reporter and "*Red*" detector / "*CY5*" reporter and choose "*(none)*" for "*Passive Reference*". Click "*Next*".

NOTE: "*Green*" detector detects ZIKV RNA and "*Red*" detector detects EC1.



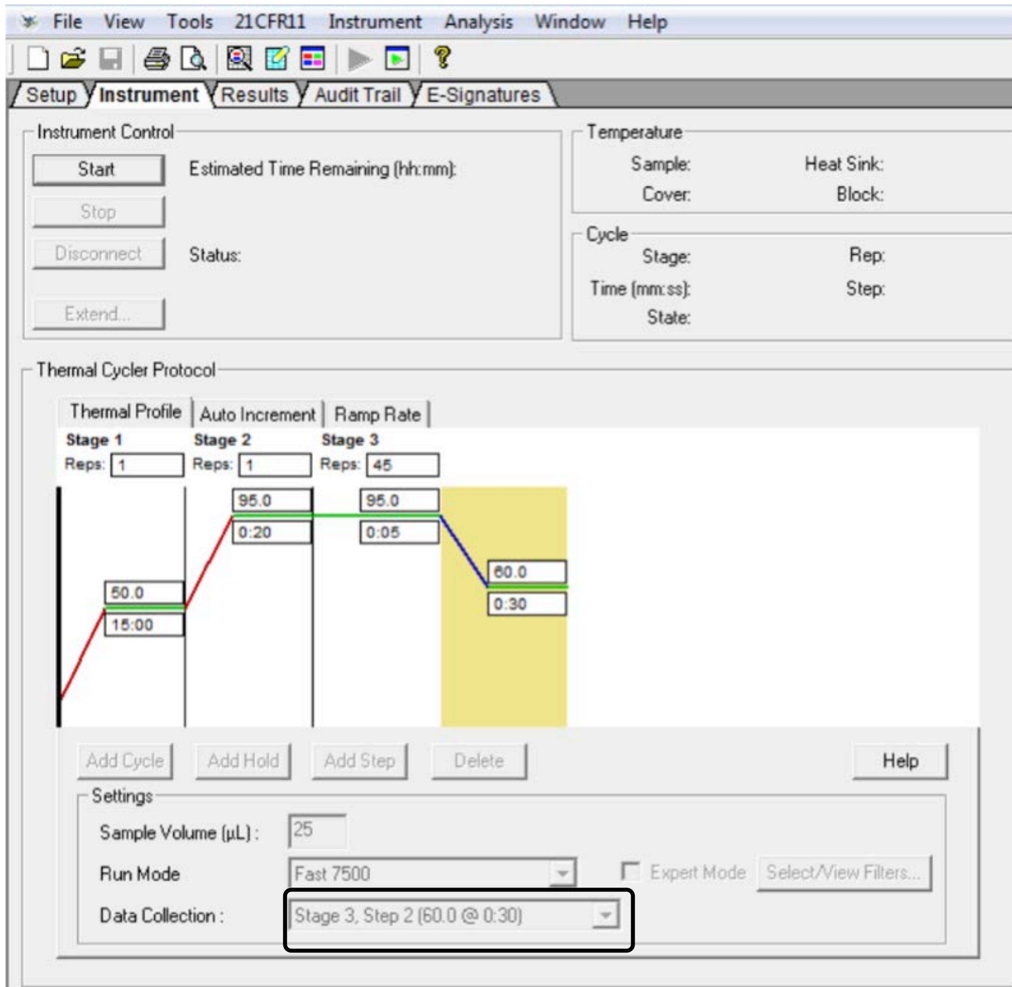
3.5. Set up the sample plate by selecting the wells and checking the checkboxes for detectors under the "*Use*" field as shown in the figure below. Click "*Finish*".



3.6. Program the cycling conditions as follows:

Stage	Reps	Temperature (°C)	Duration (min)
1	1	50	15:00
2	1	95	0:20
3	45	95	0:05
		60	0:30

Under Settings, enter “*25 µL*” as the sample volume. Ensure the run mode is set to *Fast 7500*. “*Stage 3, Step 2*” should be highlighted in yellow indicating data collection.

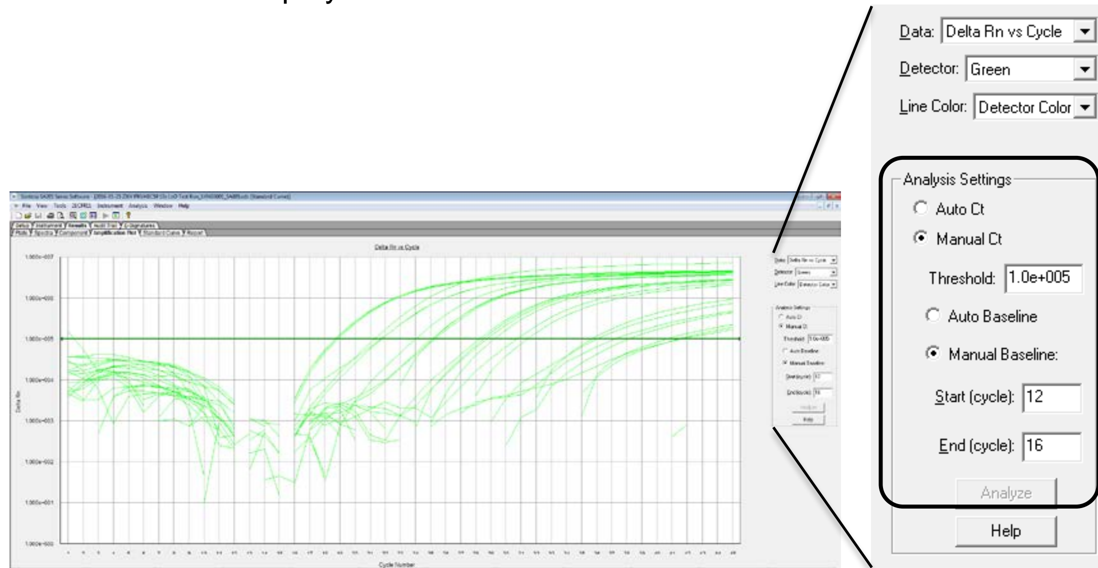


3.7. Select “*Save* as” and name the run file. Click “*Start*” to initiate the PCR run.

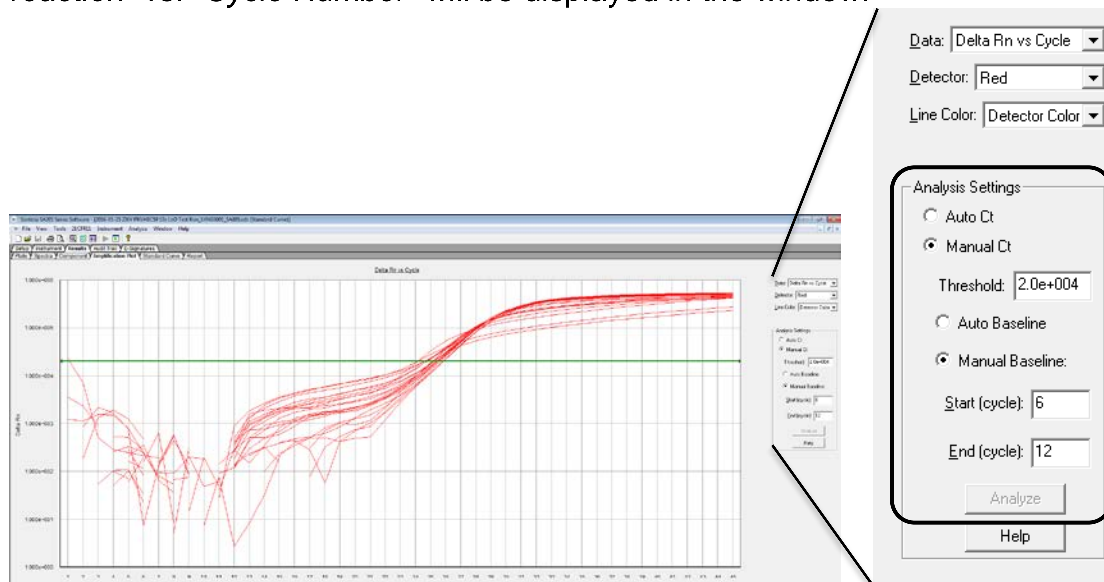
4. *Data analysis and export on the ABI 7500 Fast Dx Real-Time PCR Instrument or Sentosa® SA201.*

After completion of the run, save and analyze the data. Analyses should be performed separately for each target using a manual threshold and baseline settings as described from 4.1 to 4.2.

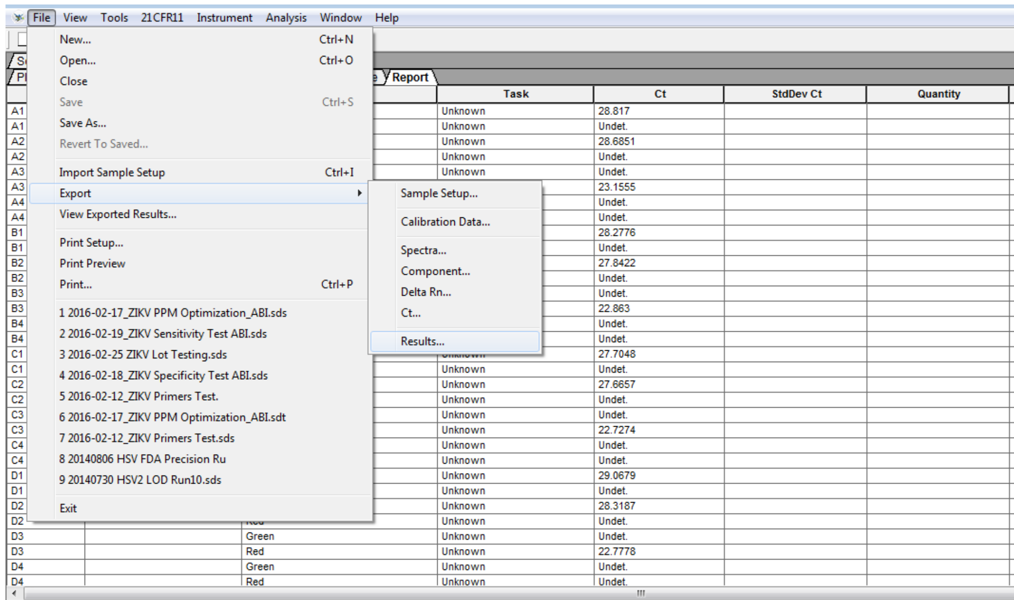
4.1. Under "*Amplification plot*" tab, in the "*Analysis* Settings", set the "*Manual* C_t" threshold and the "*Start (cycle)*" and "*End (cycle)*" of "*Manual Baseline*" for "*Green*" detector in accordance with the figure below. A graph of "*Delta reaction*" vs. "*Cycle Number*" will be displayed in the window.



4.2. Set the "*Manual* C_t" threshold and the "*Start (cycle)*" and "*End (cycle)*" of "*Manual Baseline*" for "*Red*" detector in accordance with the figure below. A graph of "*Delta reaction*" vs. "*Cycle Number*" will be displayed in the window.



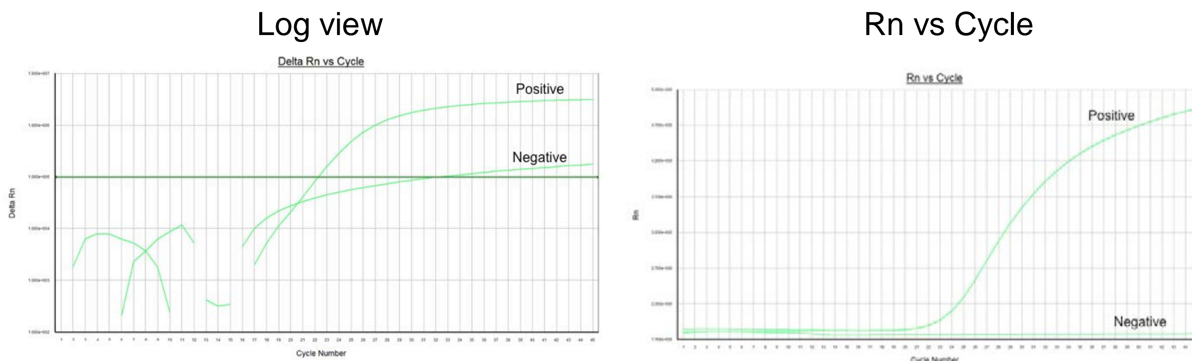
4.3. To export the results, click on “*File*” and mouse over “*Export*” in the drop-down list. Click on “*Results..*” in the secondary drop-down list. Export and save the .csv file. The report can also be printed in .pdf format by choosing the *“Print”* option under “*File*”.



4.4. The amplification plot and the background fluorescence view (Rn versus Cycle) / component data should be examined for positive samples. This is to confirm the C_t values reported and also to determine the presence of true amplification signal.

Example of a false positive curve:

The log view shows a curve with C_t value of approximately 32 although it is evident that the sample is negative based on the background fluorescence view and when compared to the positive sample.



Results

Negativity / positivity

The C_t range to define negativity / positivity for negative control, positive control and samples is listed in the following table. Fluorescence channel Cycling Green detects Zika virus and fluorescence channel Cycling Red detects the extraction control.

Fluorescence channel	C_t range for negativity (-) and positivity (+)					
	Negative control		Positive control		Samples	
	-	+	-	+	-	+
Green	≥ 40.0 or Undet	< 40.0	< 22.6, > 26.1 Or Undet	22.6 – 26.1	≥ 40.0 or Undet	< 40.0
Red	< 23.0, > 30.0 or Undet	23.0 – 30.0	< 23.0, > 30.0 or Undet	23.0 – 30.0	< 23.0, > 30.0 or Undet	23.0 – 30.0

Undet: Undetermined, indicating no C_t value reported

Result interpretation

Please refer to the tables below for result analysis. The Negative and Positive Control results must be evaluated first to determine run validity.

For **Negative control**

Negative control		Interpretation
Green	Red	
-	+	Run valid (proceed to Positive Control result interpretation)
+	+/-	Run invalid. All results are invalid and all samples must be retested.
-	-	Run invalid. All results are invalid and all samples must be retested.

For **Positive control**

Positive control		Interpretation
Green	Red	
+	+/-	Run valid (proceed to Sample result interpretation)
-	+/-	Run invalid. All results are invalid and all samples must be retested.

For **Samples**

Samples		Interpretation
Green	**Red**	
+	+/-	Zika virus RNA detected*. All Zika virus RNA detected results must be reported to the appropriate Public Health agency.
-	+	Zika virus RNA not detected.
-	-	Sample invalid. Repeat testing from the original sample or collect and test a new sample.

*For positive samples, the fluorescence channel Cycling Red can be negative due to competition with the target channels.

Run: Whole run on Applied Biosystems® 7500 Fast Dx or *Sentosa*® SA201.
Sample: Single sample in one well of the *Sentosa*® SA 96-Well Optical Plate or MicroAmp® Fast Optical 96-Well Reaction Plate.

Limitations

- A patient matched serum specimen is required for serological follow up testing of negative results, per the CDC testing algorithm. (Found at http://www.cdc.gov/zika/index.html.)
- The product is to be used by trained clinical laboratory personnel who have received specific training on the use of the *Sentosa*® SA ZIKV RT-PCR Test only.
- Strict compliance with the user manual is required for optimal PCR results.
- Do not use expired kit components. Expiration dates are printed on the box and labels of all components.
- Mutations that arise within the highly conserved regions of the viral genome covered by the kit's primers and / or probes may result in failure to detect the presence of the virus.
- Detection of viral RNA depends on the amount of virus present in the sample. This can be affected by sample collection methods, patient-related factors (e.g. age, symptoms), infection stage, and / or sample size.

Instrument maintenance

After every run, discard used sample tubes, plates, reagents and tips according to the local safety regulations. All samples and waste should be considered potentially infectious.

A reservoir collects liquid waste generated during the nucleic acid extraction procedure. Dispose of the liquid waste according to the local safety and environmental regulations. Dispose of the biohazard bags after each run.

Perform regular cleaning of the *Sentosa*® SX101 and Real-Time PCR instruments after each run. Please refer to the respective instrument user manuals for detailed procedures.

Ensure that maintenance is performed regularly to minimize the risk of error.

Always wear the appropriate personal protective equipment (PPE: laboratory coat, gloves, goggles) during cleaning / maintenance procedures.

Performance Characteristics

Analytical Sensitivity

The limit of detection (LoD) is defined as the lowest concentration of ZIKV RNA that can be consistently detected (in ⩾ 95% of samples tested under routine clinical laboratory conditions in a defined type of specimen). The LoD was determined for two strains of ZIKV, MR-766 and PRVABC59. The virus stocks of both strains were purchased from ATCC and the titers (provided by ATCC) of MR-766 and PRVABC59 were determined as 1.6×10^7 $TCID_{50}$/mL and 1.58×10^8 $TCID_{50}$/mL, respectively, by cytopathic effect (CPE).

Serial 10-fold dilutions of ZIKV strains MR-766 and PRVABC59 were prepared. Using the *Sentosa*® SA ZIKV RT-PCR Test and quantified in vitro transcribed RNA, the concentrations of the viral stocks were determined to be 4.85×10^{10} copies/mL and 1.44×10^9 copies/mL for MR-766 and PRVABC59, respectively.

Estimation of the Limit of Detection (LoD)

Based on the concentration of the viral stocks, 2-fold serial dilutions of each strain were prepared in pooled serum. For each concentration, 8 replicates were processed for RNA extraction and tested. For nucleic acid extraction, 200 µL of the samples were extracted using the *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24), followed by automated PCR set-up on the *Sentosa*® SX101. The PCR plate was then run on the ABI 7500 Fast Dx. The lowest concentration at which all 8 replicates were positive was treated as the initial LoD for subsequent confirmation. The results are noted in **Tables 2** and **3**.

Table 2. Limiting dilution for ZIKV, MR766 in serum matrix.

Target	Concentration ($\times 10^3$ copies/mL)	Call rate	Replicate 1 C_t	Replicate 2 C_t	Replicate 3 C_t	Replicate 4 C_t	Replicate 5 C_t	Replicate 6 C_t	Replicate 7 C_t	Replicate 8 C_t
ZIKV MR-766	12	8/8	33.63	33.94	33.33	32.92	33.99	34.08	33.25	33.16
	6	8/8	34.35	34.10	34.62	34.14	35.55	35.16	34.84	34.39
	3	8/8	35.81	36.02	38.51	35.64	35.67	34.27	33.86	35.11
	1.5	7/8	36.25	36.94	36.99	40.62	36.09	36.23	37.35	38.41
	0.75	3/8	42.06	39.39	42.46	40.01	Undet	39.29	42.43	39.10
	0.375	0/8	Undet	Undet	Undet	Undet	Undet	44.76	Undet	Undet

Undet: Undetermined, indicating no C_t value reported

Table 3. Limiting dilution for ZIKV, PRVABC59 in serum matrix.

Target	Concentration (x10^3 copies/mL)	Call rate	Replicate 1 C_t	Replicate 2 C_t	Replicate 3 C_t	Replicate 4 C_t	Replicate 5 C_t	Replicate 6 C_t	Replicate 7 C_t	Replicate 8 C_t
ZIKV PRVABC59	12	8/8	33.99	34.30	33.57	29.59	33.76	33.32	31.04	33.32
	6	8/8	34.76	35.79	35.30	36.78	34.51	35.69	35.82	35.27
	3	8/8	39.82	36.54	39.80	38.80	36.05	36.12	36.96	36.56
	1.5	8/8	37.22	29.28	38.28	39.91	38.05	36.48	37.88	37.86
	0.75	1/8	39.79	40.87	43.83	Undet	42.20	Undet	41.64	Undet
	0.375	0/8	Undet	42.52	Undet	44.40	Undet	43.02	Undet	42.78

Undet: Undetermined, indicating no C_t value reported

The *Sentosa*® SA ZIKV RT-PCR Test in conjunction with the *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24) and the ABI 7500 Fast Dx detected 8/8 replicates at a concentration of 3x10^3 copies/mL serum for ZIKV MR-766 and 1.5x10^3 copies/mL serum for ZIKV, PRVABC59.

Serial dilutions of ZIKV, PRVABC59 were prepared in pooled urine at 2-fold dilution above and below 1.5x10^3 copies/mL. For each concentration (including 1.5x10^3 copies/mL), 7 replicates were processed for RNA extraction and tested with *Sentosa*® SA ZIKV RT-PCR Test. The lowest concentration at which all 7 replicates were positive was treated as the initial LoD for subsequent confirmation in urine. The results are noted in **Table 4**.

Table 4. Limiting dilution for ZIKV, PRVABC59 in urine matrix.

Target	Concentration (x10^3 copies/mL)	Call rate	Replicate 1 C_t	Replicate 2 C_t	Replicate 3 C_t	Replicate 4 C_t	Replicate 5 C_t	Replicate C_t	Replicate 7 C_t
ZIKV PRVABC59	3	7/7	35.07	35.05	34.71	34.89	34.71	35.21	35.54
	1.5	7/7	36.21	36.77	37.06	35.74	38.27	37.31	36.04
	0.75	4/7	43.04	39.28	40.14	Undet	37.90	39.34	38.10

Undet: Undetermined, indicating no C_t value reported

Confirmation of the Limit of Detection (LoD)

The initial LoD ($3x10^3$ copies/mL) for ZIKV MR-766 in serum was extracted and tested in 22 replicates. The call rate was 14/22 (63.64%) and did not meet the ⩾ 95% positivity rate. The LoD was next confirmed for ZIKV strain MR-766 in serum at $6x10^3$ copies/mL, which is the next increment in 2-fold concentration. The call rate was 22/22 (100%). The LoD was then confirmed in EDTA plasma and urine at $6x10^3$ copies/mL, both achieving a call rate of 22/22 (100%). The final LoD for ZIKV MR-766 in serum, EDTA plasma and urine is determined as $6x10^3$ copies/mL. The data are summarized in **Table 5**.

Similarly, the initial LoD ($1.5x10^3$ copies/mL) for ZIKV PRVABC59 was tested in serum and urine with 22 replicates for each matrix. The call rate was 18/22 (81.82%) in serum and 16/22 (72.73%) in urine, and both matrices did not meet ⩾ the 95% positivity rate. The LoD was next confirmed for ZIKV, strain PRVABC59 in serum at $3x10^3$ copies/mL, the next increment in 2-fold concentration. The call rate was 22/22 (100%). The LoD was then confirmed in EDTA plasma and urine at $3x10^3$ copies/mL, both achieving a call rate of 22/22 (100%). The final LoD for ZIKV PRVABC59 in serum, EDTA plasma and urine was determined as $3x10^3$ copies/mL. The data are summarized in **Table 5**.

Table 5. Limit of detection: confirmation in serum, EDTA plasma and urine for ZIKV MR-766 and PRVABC59.

Target	Sample matrix	Concentration ($x10^3$ copies/mL)	Call Rate	Average C_t
ZIKV MR-766	Serum	3	14/22	39.06
	Serum	6	22/22	35.62
	EDTA Plasma	6	22/22	33.56
	Urine	6	22/22	34.30
ZIKV PRVABC59	Serum	1.5	18/22	37.97
	Urine	1.5	16/22	38.07
	Serum	3	22/22	35.68
	EDTA Plasma	3	22/22	36.91
	Urine	3	22/22	34.88

Analytical Sensitivity – FDA Reference Material

An analytical study was performed using FDA reference materials (S1 and S2) following a standard protocol provided by the FDA (**Table 6**). The protocol included range-finding and confirmatory LoD studies to evaluate the analytical sensitivity of the *Sentosa*® SA ZIKV RT-PCR Test.

Table 6. LoD confirmation of the *Sentosa*® SA ZIKV RT-PCR Test using the FDA reference materials.

Reference Materials	Specimen Type	Confirmed LoD* in RNA NAT Detectable Units/mL
S1	Serum	$3x10^4$
S1	Urine	$1x10^4$
S2	Serum	$1.5x10^4$
S2	Urine	$5x10^3$

*Study performed according to an FDA issued protocol

Reactivity

Reactivity of the Sentosa® SA ZIKV RT-PCR Test was evaluated for ZIKV MR-766 and PRVABC59, which are the same isolates used in the LoD study.

Sequences of both primers and probe were aligned to sequences of ZIKV strains from the National Center for Biotechnology Information (NCBI). The list of ZIKV is presented in the **Table 7**.

Forward primer (ZIK_F3) aligns to all 82 ZIKV sequences with 100% identity. Reverse primers, ZIK_R3 and ZIK_R5, only differ by a single nucleotide, and align to the same region of the Zika virus sequence. The combination of ZIK_R3 and ZIK_R5 align to all 82 Zika sequences with 100% identity. Probe sequence (ZIK_P3) aligns to all 82 Zika sequences with at least 95% (22/23 bases) identity.

Table 7. *In silico* analysis with 82 Zika sequences from the NCBI as at 24 May 2016.

Accession #	Description	ZIK_F3	ZIK_R3	ZIK_R5	ZIK_P3
AY632535.2	Zika virus strain MR 766, complete genome	100%	100%	94.70%	100%
DQ859059.1	Zika virus strain MR 766 polyprotein gene, complete cds	100%	100%	94.70%	100%
EU545988.1	Zika virus polyprotein gene, complete cds	100%	94.70%	100%	100%
KF268948.1	Zika virus isolate ARB13565 polyprotein gene, complete cds	100%	100%	94.70%	100%
KF268949.1	Zika virus isolate ARB15076 polyprotein gene, complete cds	100%	100%	94.70%	100%
KF268950.1	Zika virus isolate ARB7701 polyprotein gene, complete cds	100%	100%	94.70%	100%
KF383115.1	Zika virus strain ArB1362 polyprotein gene, complete cds	100%	100%	94.70%	100%
KF383116.1	Zika virus strain ArD7117 polyprotein gene, complete cds	100%	100%	94.70%	100%
KF383117.1	Zika virus strain ArD128000 polyprotein gene, complete cds	100%	100%	94.70%	100%
KF383118.1	Zika virus strain ArD157995 polyprotein gene, complete cds	100%	100%	94.70%	100%
KF383119.1	Zika virus strain ArD158084 polyprotein gene, complete cds	100%	100%	94.70%	100%
KJ776791.1	Zika virus strain H/PF/2013 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU312312.1	Zika virus isolate Z1106033 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU321639.1	Zika virus strain ZikaSPH2015, complete genome	100%	94.70%	100%	100%
KU365777.1	Zika virus strain BeH818995 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU365778.1	Zika virus strain BeH819015 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU365779.1	Zika virus strain BeH819966 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU365780.1	Zika virus strain BeH815744 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU497555.1	Zika virus isolate Brazil-ZKV2015, complete genome	100%	94.70%	100%	100%
KU501215.1	Zika virus strain PRVABC59, complete genome	100%	94.70%	100%	100%
KU501216.1	Zika virus strain 103344 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU501217.1	Zika virus strain 8375 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU509998.3	Zika virus strain Haiti/1225/2014, complete genome	100%	94.70%	100%	100%
KU527068.1	Zika virus strain Natal RGN, complete genome	100%	94.70%	100%	100%

Accession #	Description	ZIK_F3	ZIK_R3	ZIK_R5	ZIK_P3
KU647676.1	Zika virus strain MRS_OPY_Martinique_PaRi_2015 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU681081.3	Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127-14, complete genome	100%	94.70%	100%	95.70%
KU681082.3	Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, complete genome	100%	94.70%	100%	100%
KU707826.1	Zika virus isolate SSABR1, complete genome	100%	94.70%	100%	100%
KU720415.1	Zika virus strain MR 766 polyprotein gene, complete cds	100%	100%	94.70%	100%
KU729217.2	Zika virus isolate BeH823339 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU729218.1	Zika virus isolate BeH828305 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU740184.2	Zika virus isolate GD01 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU744693.1	Zika virus isolate VE_Ganxian, complete genome	100%	94.70%	100%	100%
KU761564.1	Zika virus isolate GDZ16001 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU820897.2	Zika virus isolate FLR polyprotein gene, complete cds	100%	94.70%	100%	100%
KU820898.1	Zika virus isolate GZ01 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU820899.2	Zika virus isolate ZJ03, complete genome	100%	94.70%	100%	100%
KU853012.1	Zika virus isolate Dominican Republic/2016/PD1, complete genome	100%	94.70%	100%	100%
KU853013.1	Zika virus isolate Dominican Republic/2016/PD2, complete genome	100%	94.70%	100%	100%
KU866423.1	Zika virus isolate Zika virus/SZ01/2016 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU870645.1	Zika virus isolate FB-GWUH-2016, complete genome	100%	94.70%	100%	100%
KU922923.1	Zika virus isolate MEX/InDRE/Lm/2016, complete genome	100%	94.70%	100%	100%
KU922960.1	Zika virus isolate MEX/InDRE/Sm/2016, complete genome	100%	94.70%	100%	100%
KU926309.1	Zika virus isolate Rio-U1, complete genome	100%	94.70%	100%	100%
KU926310.1	Zika virus isolate Rio-S1, complete genome	100%	94.70%	100%	100%
KU937936.1	Zika virus isolate ZIKVNL00013 polyprotein gene, complete cds	100%	94.70%	100%	95.70%
KU940224.1	Zika virus isolate Bahia09, partial genome	100%	94.70%	100%	100%
KU940227.1	Zika virus isolate Bahia08, partial genome	100%	94.70%	100%	100%

Accession #	Description	ZIK_F3	ZIK_R3	ZIK_R5	ZIK_P3
KU940228.1	Zika virus isolate Bahia07, partial genome	100%	94.70%	100%	100%
KU955589.1	Zika virus isolate Z16006 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU955590.1	Zika virus isolate Z16019 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU955591.1	Zika virus isolate Zika virus/A.africanus-tc/SEN/1984/41525-DAK, complete genome	100%	100%	94.70%	100%
KU955592.1	Zika virus isolate Zika virus/A.taylori-tc/SEN/1984/41662-DAK, complete genome	100%	100%	94.70%	100%
KU955593.1	Zika virus isolate Zika virus/H.sapiens-tc/KHM/2010/FSS13025, complete genome	100%	94.70%	100%	100%
KU955594.1	Zika virus isolate Zika virus/M.mulatta-tc/UGA/1947/MR-766, complete genome	100%	100%	94.70%	100%
KU955595.1	Zika virus isolate Zika virus /A.taylori-tc /SEN/1984/41671-DAK, complete genome	100%	100%	94.70%	100%
KU963573.1	Zika virus isolate ZIKV/Macaca mulatta/UGA/MR-766_SM150-V8/1947 polyprotein (GP1) gene, complete cds	100%	100%	94.70%	100%
KU963574.1	Zika virus isolate ZIKV/Homo sapiens/ NGA/IbH-30656_SM21V1-V3/1968 polyprotein (GP1) gene, complete cds	100%	100%	94.70%	100%
KU963796.1	Zika virus isolate SZ-WIV01 polyprotein gene, complete cds	100%	94.70%	100%	100%
KU991811.1	Zika virus isolate Brazil/2016/INMI1 polyprotein gene, complete cds	100%	94.70%	100%	100%
KX051563.1	Zika virus isolate Haiti/1/2016, complete genome	100%	94.70%	100%	100%
KX056898.1	Zika virus isolate Zika virus/GZ02/2016 polyprotein gene, complete cds	100%	94.70%	100%	100%
KX087101.2	Zika virus strain ZIKV/Homo sapiens/ PRI/PRVABC59/2015, complete genome	100%	94.70%	100%	100%
KX087102.1	Zika virus strain ZIKV/Homo sapiens/ COL/FLR/2015, complete genome	100%	94.70%	100%	100%
KX101060.1	Zika virus isolate Bahia02, partial genome	100%	94.70%	100%	100%
KX101063.1	Zika virus isolate Bahia05, partial genome	100%	94.70%	100%	100%
KX101064.1	Zika virus isolate Bahia11, partial genome	100%	94.70%	100%	100%
KX101065.1	Zika virus isolate Bahia15, partial genome	100%	94.70%	100%	100%
KX117076.1	Zika virus isolate Zhejiang04, complete genome	100%	94.70%	100%	100%
KX156774.1	Zika virus strain ZIKV/Homo sapiens/ PAN/CDC-259359_V1-V3/2015, complete genome	100%	94.70%	100%	100%

Accession #	Description	ZIK_F3	ZIK_R3	ZIK_R5	ZIK_P3
KX156775.1	Zika virus strain ZIKV/Homo sapiens/ PAN/CDC-259249_V1-V3/2015, complete genome	100%	94.70%	100%	100%
KX156776.1	Zika virus strain ZIKV/Homo sapiens /PAN/CDC-259364_V1-V2/2015, complete genome	100%	94.70%	100%	100%
KX185891.1	Zika virus isolate Zika virus/CN/SZ02/ 2016 polyprotein gene, complete cds	100%	94.70%	100%	100%
KX197192.1	Zika virus isolate ZIKV/H.sapiens/Brazil/PE243/2015, complete genome	100%	94.70%	100%	100%
KX198134.1	Zika virus strain ZIKV/Aedes africanus/ SEN/DAK-AR-41524_A1C1-V2/1984, complete genome	100%	100%	94.70%	100%
KX198135.1	Zika virus strain ZIKV/Homo sapiens/ PAN/BEI-259634_V4/2016, complete genome	100%	94.70%	100%	100%
KX247632.1	Zika virus isolate MEX_I_7 polyprotein gene, complete cds	100%	94.70%	100%	100%
KX247646.1	Zika virus isolate Zika virus/Homo sapiens/COL/UF-1/2016, complete genome	100%	94.70%	100%	100%
KX253996.1	Zika virus isolate ZKC2/2016, complete genome	100%	94.70%	100%	100%
KX262887.1	Zika virus isolate 103451, complete genome	100%	94.70%	100%	95.70%
LC002520.1	Zika virus genomic RNA, complete genome, strain: MR766-NIID	100%	100%	94.70%	100%
NC_012532.1	Zika virus, complete genome	100%	100%	94.70%	100%

Cross Reactivity

To evaluate the analytical specificity of the *Sentosa*® SA ZIKV RT-PCR Test for cross reactivity, viruses related to ZIKV, as well as other pathogens present in blood or urine, were tested. Each organism was spiked into the relevant sample matrix, extracted in triplicate and analyzed using the *Sentosa*® SA ZIKV RT-PCR Test workflow. Serum was used as a representative for blood-related sample matrices. For each organism, all three replicates were negative for ZIKV and positive for the extraction control. The results are summarized in **Table 8**.

Table 8. Cross Reactivity (wet testing): *Sentosa*® SA ZIKV RT-PCR Test.

Organism, Strain	Source	Test Concentration	Diluent	Green C_t (ZIKV)	Red C_t (EC1)
Dengue Type 1, strain D1/SG/05K847DK1/2005	Collaborator (Duke-NUS)	1x10^5 pfu/mL	Serum	Undet	25.78
				Undet	25.59
				Undet	25.98
Dengue Type 2, strain D2/SG/05K3295DK1/2005	Collaborator (Duke-NUS)	1x10^5 pfu/mL	Serum	Undet	26.18
				Undet	24.62
				Undet	25.51
Dengue Type 3, strain D3/SG/05K863DK1/2005	Collaborator (Duke-NUS)	1x10^5 pfu/mL	Serum	Undet	24.78
				Undet	25.25
				Undet	25.75
Dengue Type 4, strain D4/SG/06K2270DK1/2005	Collaborator (Duke-NUS)	1x10^5 pfu/mL	Serum	Undet	25.77
				Undet	26.07
				Undet	25.63
Chikungunya virus, strain Ross	Collaborator (Duke-NUS)	1x10^5 pfu/mL	Serum	Undet	25.48
				Undet	25.66
				Undet	25.39
Plasmodium falciparum	NIBSC	1x10^5 IU/mL	Serum	Undet	25.73
				Undet	26.02
				Undet	25.59
Parvovirus B19	NIBSC	1x10^5 IU/mL	Serum	Undet	25.71
				Undet	25.69
				Undet	25.64
Cytomegalovirus, Merlin	ATCC	1x10^5 IU/mL	Urine	Undet	26.24
				Undet	26.53
				Undet	26.33

Undet: Undetermined, indicating no C_t value reported

The *in silico* analysis was performed to determine the homology of the primer and probe sequences with the sequences of closely related viruses and other common organisms causing similar acute febrile illness. The BLAST command-line tool was utilized for this analysis. The command-line options used are: -task blastn-short -ungapped -evalue 1000. The percentage values were calculated by taking the ratio of number of identical bases in the top High-scoring segment pair (HSP) and the total number of query primer/probe bases. BLAST analysis output shows no significant combined homologies. Forward primers, reverse primers, or probe having alignments with the target sequence do not have nearby or correctly oriented primers or probe with alignment significant enough to produce a positive PCR reaction.

The causative agents presented in the *in silico* analysis are presented in **Tables 9 and 10**. The sequences were obtained from the NCBI GenBank public database.

Table 9. *In silico* analysis of viruses evaluated by wet testing.

Accession #	Description
KJ189368	Dengue virus serotype 1, Mexico 2012
KF887994	Dengue virus serotype 1, Thailand 2013
EU081227	Dengue virus serotype 1, Singapore 2005 (D1/SG/05K847DK1/2005)
KJ830750	Dengue virus serotype 2, Saudi Arabia 2014
KM279577	Dengue virus serotype 2, Singapore 2012
EU081177	Dengue virus serotype 2, Singapore 2005 (D2/SG/05K3295DK1/2005)
KJ622195	Dengue virus serotype 3, China 2013
KC762693	Dengue virus serotype 3, Indonesia 2010
EU081190	Dengue virus serotype 3, Singapore 2005 (D3/SG/05K863DK1/2005)
JN983813	Dengue virus serotype 4, Brazil 2010
GQ398256	Dengue virus serotype 4, Singapore 2005 (D4/SG/06K2270DK1/2005)
KR559473	Chikungunya virus strain WHCHK4
KJ579186	Chikungunya virus isolate BK63
NC_000883	Human parvovirus B19
GCF_000002 765	Plasmodium falciparum strain 3D7
NC_006273	Human herpesvirus 5 (cytomegalovirus)

No hit = no alignment found

Table 10. *In silico* analysis of closely related viruses, other common organisms causing acute febrile illness in humans, and human DNA.

Organism	Accession #	Description
Flavivirus	NC_004102	Hepatitis C virus genotype 1
	NC_009823	Hepatitis C virus genotype 2
	NC_009824	Hepatitis C virus genotype 3
	NC_009825	Hepatitis C virus genotype 4
	NC_009826	Hepatitis C virus genotype 5
	NC_009827	Hepatitis C virus genotype 6
	NC_007580	St. Louis encephalitis virus
	NC_001672	Tick-borne encephalitis virus
	NC_000943	Murray Valley encephalitis virus
	NC_001437	Japanese encephalitis virus
	NC_029055	Spondweni virus
	AY632542	Rocio virus strain SPH 34675
	NC_009028	Ilheus virus
	AY632538	Iguape virus strain SPAn 71686
	NC_002031	Yellow fever virus vaccine strain 17D

Organism	Accession #	Description
	DQ211652	West Nile virus strain NY99
Alphavirus	NC_003899	Eastern equine encephalitis virus (EEE)
	NC_003908	Western equine encephalomyelitis virus (WEE)
	NC_001544	Ross River virus
	NC_001786	Barmah Forest virus
	NC_001512	O'nyong-nyong virus
	NC_001547	Sindbis virus
	AF075254	Tonate virus
	HM147992	Una virus
	NC_003417	Mayaro virus
Other organisms	NC_001460	Human adenovirus A
	NC_011203	Human adenovirus B1
	NC_011202	Human adenovirus B2
	NC_001405	Human adenovirus C
	NC_010956	Human adenovirus D
	NC_003266	Human adenovirus E
	NC_001498	Measles virus
	NC_001545	Rubella virus
	NC_001612	Human enterovirus A
	NC_001472	Human enterovirus B
	NC_002058	Human enterovirus C
	NC_001430	Human enterovirus D
	NC_001859	Enterovirus E
	NC_021220	Enterovirus F strain BEV-261
	NC_010415	Enterovirus J strain 1631
	NC_013695	Enterovirus J strain N203
	NC_024073	Enterovirus sp. isolate CPML_8109/08
	NC_001489	Hepatitis A virus
	NC_003977	Hepatitis B virus (strain ayw)
	NC_001802	Human immunodeficiency virus 1
	NC_001722	Human immunodeficiency virus 2
	NC_001348	Human herpesvirus 3 (Varicella Zoster virus)
	NC_007605	Human herpesvirus 4 (EBV)
	NC_012633	Rickettsia africae ESF-5
	NC_009881	Rickettsia akari str. Hartford
	NC_017058	Rickettsia australis str. Cutlack, complete genome
	NC_007940	Rickettsia bellii RML369-C, complete genome
	NC_009879	Rickettsia canadensis str. McKiel, complete genome
	NC_003103	Rickettsia conorii str. Malish 7, complete genome
	NC_007109	Rickettsia felis URRWXCal2, complete genome
	NC_015866	Rickettsia heilongjiangensis 054, complete genome
	NZ_CM001467	Rickettsia helvetica C9P9 chromosome, whole genome shotgun sequence
	NC_016050	Rickettsia japonica YH DNA, complete genome

Organism	Accession #	Description
	NC_009900	Rickettsia massiliae MTU5, complete genome
	NC_016931	Rickettsia massiliae str. AZT80, complete genome
	NC_017044	Rickettsia parkeri str. Portsmouth, complete genome
	NC_016930	Rickettsia philipii str. 364D, complete genome
	NC_000963	Rickettsia prowazekii str. Madrid E chromosome, complete genome
	NC_017042	Rickettsia rhipicephali str. 3-7-female6-CWPP, complete genome
	NC_010263	Rickettsia rickettsii str. Iowa, complete genome
	NC_009882	Rickettsia rickettsii str. 'Sheila Smith', complete genome
	NZ_AABW01000 001	Rickettsia sibirica 246 chromosome, complete sequence, whole genome shotgun sequence
	NC_006142	Rickettsia typhi str. Wilmington, complete genome
	NC_001318	Borrelia burgdorferi B31
	NC_002737	Streptococcus pyogenes M1 GAS
	GCF_000013945	Leptospira borgpetersenii
	GCF_000092565	Leptospira interrogans
	GCF_000244515	Leptospira kirschneri
	GCF_000313175	Leptospira santarosai
	GCF_000002415	Plasmodium vivax
	GCF_000209065	Trypanosoma cruzi
	GCF_000699445	Schistosoma haematobium
	GCA_000151775	Schistosoma japonicum
	GCA_000237925	Schistosoma mansoni
	CP002099	Salmonella enterica subsp. enterica serovar Typhi str. Ty21a
	NC_000913	Escherichia coli str. K-12 substr. MG1655
	GCF_000001405	Homo sapiens (assembly GRCh38.p2)

No hit = no alignment found

Interference study

Interference studies were not performed for the *Sentosa*® SA ZIKV RT-PCR Test, since the test uses conventional real-time RT-PCR and an established extraction method using magnetic beads extraction for testing.

The *Sentosa*® SA ZIKV RT-PCR Test contains an extraction control (EC1), which is added to each specimen tested to show the nucleic acid extraction process proceeded. The extraction control (EC1) is amplified and detected in parallel to the ZIKV specific RNA, and ensures the integrity of ZIKV specific real-time RT-PCR results by indicating potential extraction issue and RT-PCR inhibition.

Co-infection/Competitive Interference Study

A microbial interference study was performed for the *Sentosa*® SA ZIKV RT-PCR Test, using dengue virus that is considered clinically relevant to co-infect with Zika virus. To assess the potential competitive interference due to co-infection, DENV-3 (strain D3/SG/05K863DK1/2005) and DENV-4 (strain D4/SG/06K2270DK1/2005) were chosen for the study. Based on the *in silico* analysis, both strains of DENV-3 and DENV-4 have high homology (≥ 70%) to the assay's reverse primers.

To determine if competitive interference occurs with the assay, co-infection studies were performed using ZIKV, strain PRVABC59 spiked into two different matrices, serum and urine, at 3xLoD. The co-infecting dengue targets (DENV-3 or DENV-4) were spiked at different concentrations, including the highest possible concentration to determine potential interference. Each sample was tested in triplicates.

As shown the Table 11, 3xLoD ZIKV spiked with DENV-3 or DENV 4 at $1x10^5$, $1x10^4$ and $1x10^3$ pfu/mL in serum and urine are detectable with similar Ct values as the control (ZIKV, 3xLoD). Co-infection of Zika virus and dengue virus did not result in competitive interference.

Table 11. Co-infection/Competitive interference with DENV-3 and DENV-4

Matrix	**Serum**			**Urine**		
Channel	**Green C_t**	**Average Green**	**Red C_t**	**Green C_t**	**Average Green**	**Red C_t**
ZIKV, 3x LOD	32.64	33.08	25.33	33.71	33.63	26.43
	34.19		25.42	33.56		26.42
	32.42		25.52	33.60		26.35
ZIKV, 3x LOD + 1x10^5 pfu/mL DENV-3	31.18	32.13	25.54	32.79	32.96	26.24
	31.45		25.55	33.42		26.05
	33.77		25.44	32.68		25.80
ZIKV, 3x LOD + 1x10^4 pfu/mL DENV-3	32.99	32.06	24.89	33.48	33.60	25.86
	31.06		25.36	33.43		26.08
	32.14		25.25	33.89		26.26
ZIKV, 3x LOD + 1x10^3 pfu/mL DENV-3	32.61	32.52	25.53	33.27	33.55	26.21
	32.15		25.61	33.50		26.38
	32.80		25.49	33.87		25.98

ZIKV, 3x LOD + 1x10^5 pfu/mL DENV-4	31.62	31.61	25.16	33.40	33.49	25.95
	32.97		25.35	33.47		25.92
	30.23		25.72	33.61		25.86
ZIKV, 3x LOD + 1x10^4 pfu/mL DENV-4	33.47	32.98	24.94	33.66	33.33	26.01
	33.13		25.39	33.15		26.02
	32.34		25.42	33.17		25.85
ZIKV, 3x LOD + 1x10^3 pfu/mL DENV-4	32.18	32.28	25.47	33.23	32.72	26.11
	33.19		25.50	31.93		26.04
	31.48		25.32	33.00		25.78

Clinical Evaluation

EDTA Plasma

Thirty-four (34) EDTA plasma clinical samples obtained from outside sources were determined by the suppliers to be positive for ZIKV. All 34 samples were re-tested using the RealStar Zika Virus RT-PCR Kit 1.0 as the reference device, and 28 out of 34 plasma clinical samples were confirmed positive. The six plasma clinical samples that were negative are considered negative for the reference result in the analyses below. The *Sentosa*® SA ZIKV RT-PCR Test was positive for 24 out of the 28 reference positive plasma clinical samples. **Table 12** shows the results for the 28 reference positive samples.

Table 12. *Sentosa*® SA ZIKV RT-PCR Test Results for 28 ZIKV Positive Clinical Samples (EDTA Plasma)

Sample ID	Days between symptom onset and collection	Vendor C_t	Reference Device C_t	*Sentosa* C_t	*Sentosa* Results
1043-TDS-0052	3	30.44	30.56	30.55	Positive
1043-TDS-0055	5	38.72	37.60	Undet	Negative
1043-TDS-0056	7	36.24	38.00	Undet	Negative
1043-TDS-0058	4	36.89	35.50	35.66	Positive
1043-TDS-0059	3	33.39	34.21	33.18	Positive
1043-TDS-0060	2	34.75	39.14	38.65	Positive
1043-TDS-0062	3	38.23	37.95	37.41	Positive
1043-TDS-0064	3	35.19	35.37	35.96	Positive
1043-TDS-0067	3	34.61	34.55	33.15	Positive
1043-TDS-0068	4	37.23	37.46	36.97	Positive

Sample ID	Days between symptom onset	Vendor C_t	Reference Device C_t	*Sentosa* C_t	*Sentosa* Results
1043-TDS-0069	5	38.48	36.23	35.66	Positive
1043-TDS-0071	3	32.16	32.38	31.46	Positive
1043-TDS-0076	5	33.79	32.17	31.12	Positive
1043-TDS-0079	7	34.4	34.66	33.74	Positive
1043-TDS-0080	3	41.53	37.33	Undet	Negative
1043-TDS-0085	2	29.47	30.10	28.56	Positive
1043-TDS-0086	4	40.78	36.64	38.43	Positive
1043-TDS-0087	5	41.59	38.09	39.40	Positive
1043-TDS-0088	5	36.74	35.22	35.73	Positive
1043-TDS-0089	4	39.66	37.07	39.53	Positive
1043-TDS-0090	4	35.31	34.04	32.29	Positive
1043-TDS-0091	3	32.45	31.31	30.75	Positive
1043-TDS-0096	4	34.79	33.41	31.11	Positive
1043-TDS-0099	5	41.94	37.82	38.23	Positive
BUH01 (Plasma)	7	32.88	33.37	34.02	Positive
BUH02 (Plasma)	6	30.05	36.04	37.85	Positive
BUH08 (Plasma)	5	31.85	33.14	33.74	Positive
BUH09 (Plasma)	3	33.14	33.67	41.15	Negative

Undet: Undetermined, indicating no C_t value reported

A total of 50 contrived plasma samples were prepared by spiking ZIKV strain PRVABC59 into individual plasma samples to 1.5xLoD (4.5×10^3 copies/mL, n=25) and 5xLoD (15×10^3copies/mL, n=25). A total of 56 individual plasma samples, including six plasma clinical samples, that were negative by the reference device were used as Zika RNA negative samples. The 50 contrived plasma samples were positive and the 56 negative plasma samples were negative with the *Sentosa*® SA ZIKV RT-PCR Test. The clinical evaluation results for plasma samples are summarized in **Table 13**.

Table 13. Clinical Evaluation Results for the *Sentosa*® SA ZIKV RT-PCR Test (EDTA Plasma).

Specimen Category	*Sentosa* SA ZIKV RT-PCR Test		
	Number Tested	Positive	Negative
Natural Zika Positive Samples	28	24	4
Contrived Zika Positive Samples (1.5x LoD)	25	25	0
Contrived Zika Positive Samples (5x LoD)	25	25	0
Expected Zika Negative samples	56	0	56
Positive % Agreement	94.9% (74/78) 95% CI[b]: 87.5% to 98.0%		
Negative % Agreement	100% (56/56)[a]		

	95% CI[b]: 93.6% to 100%

CI = Confidence Interval
[a] Includes six plasma clinical samples that were tested negative by the reference device.
[b] Wilson (Score) 95% Confidence Interval.

Serum

Twenty-eight (28) serum clinical samples were obtained from an outside source. The 28 serum clinical samples were determined by the supplier to be positive for ZIKV. The samples were re-tested using the reference device and 19 out of 28 serum clinical samples were confirmed positive. The nine serum clinical samples that were negative are considered negative for the reference result in the analyses below. The *Sentosa*® SA ZIKV RT-PCR Test was positive for 15 out of the 19 reference positive serum clinical samples. **Table 14** shows the results for the 19 reference positive samples.

Table 14. *Sentosa*® SA ZIKV RT-PCR Test Results for 19 ZIKV Positive Clinical Samples (Serum).

Sample ID	Days between symptom onset and collection	7. Vendor C_t	Reference Device C_t	*Sentosa* C_t	*Sentosa* Results
1043-TDS-0162	4	33.95	32.90	32.66	Positive
1043-TDS-0164	3	36.37	35.41	35.19	Positive
1043-TDS-0169	5	36.33	37.23	37.49	Positive
1043-TDS-0176	4	37.98	38.83	Undet	Negative
1043-TDS-0183	4	29.97	32.16	30.98	Positive
1043-TDS-0184	4	30.99	34.25	33.75	Positive
1043-TDS-0190	4	35.04	33.56	32.87	Positive
1043-TDS-0191	4	41.14	36.85	40.42	Negative
1043-TDS-0193	3	40.99	40.10	Undet	Negative
1043-TDS-0195	5	30.44	32.28	31.88	Positive
1043-TDS-0196	5	33.22	35.71	35.60	Positive
1043-TDS-0197	3	34.67	35.21	34.89	Positive
1043-TDS-0201	3	34.78	36.55	37.71	Positive
1043-TDS-0208	2	25.67	26.47	26.04	Positive
1043-TDS-0211	4	32.54	33.96	34.29	Positive
1043-TDS-0214	2	36.75	38.41	38.54	Positive
1043-TDS-0216	3	28.26	31.81	30.36	Positive
1043-TDS-0218	3	37.11	37.02	Undet	Negative
1043-TDS-0223	2	32.5	33.46	33.14	Positive

Undet: Undetermined, indicating no C_t value reported

A total of 50 contrived serum samples were prepared by spiking ZIKV strain PRVABC59 into individual serum samples to 1.5xLoD ($4.5x10^3$ copies/mL, n=25) and 5xLoD ($15x10^3$copies/mL, n=25). A total of 59 individual serum samples, including 9 clinical serum samples, that were negative by the reference device were used as Zika RNA negative samples. The 50 contrived serum samples were positive and the 59 negative serum samples were negative with the *Sentosa*® SA ZIKV RT-PCR Test. The clinical evaluation results for serum samples are summarized in **Table 15**.

Table 15. Clinical Evaluation Results for the *Sentosa*® SA ZIKV RT-PCR Test (Serum).

Specimen Category	*Sentosa* SA ZIKV RT-PCR Test		
	Number Tested	Positive	Negative
Natural Zika Positive Samples	19	15	4
Contrived Zika Positive Samples (1.5x LoD)	25	25	0
Contrived Zika Positive Samples (5x LoD)	25	25	0
Expected Zika Negative samples	59	0	59
Positive % Agreement	94.2% (65/69) 95% CI[b]: 86.0% to 97.7%		
Negative % Agreement	100% (59/59)[a] 95% CI[b]: 93.9% to 100%		

CI = Confidence Interval

[a] Includes nine serum clinical samples that were tested negative by the reference device.

[b] Wilson (Score) 95% Confidence Interval.

Urine

Fourteen (14) urine clinical samples were obtained from outside sources. Each urine clinical sample was collected alongside a patient-matched EDTA plasma or serum sample and tested with the reference device and the *Sentosa*® SA ZIKV RT-PCR Test. All 14 urine clinical samples were confirmed positive by the reference device. The *Sentosa*® SA ZIKV RT-PCR Test was positive for twelve (12) urine clinical samples as two (2) of the samples were invalid after re-testing. **Table 16** shows the results for the fourteen (14) reference positive samples.

Table 16. *Sentosa*® SA ZIKV RT-PCR Test Results for 14 ZIKV Positive Clinical Samples (Urine)

Patient ID	Days between symptom onset and collection	Sample Type	Reference C_t	Reference Results	*Sentosa* C_t	*Sentosa* Results
1043-TDS-0057	4	EDTA	Undet	Negative	Undet	Negative

Patient ID	Days between	Sample Type	Reference C_t	Reference Results	*Sentosa* C_t	*Sentosa* Results
		plasma				
		Urine	33.58	Positive	36.05	Positive
1043-TDS-0058	4	EDTA plasma	35.50	Positive	35.66	Positive
		Urine	33.40	Positive	Undet	Sample Invalid
1043-TDS-0059	3	EDTA plasma	34.21	Positive	33.18	Positive
		Urine	34.07	Positive	35.46	Positive
1043-TDS-0060	2	EDTA plasma	39.14	Positive	38.65	Positive
		Urine	27.45	Positive	32.02	Positive
1043-TDS-0064	3	EDTA plasma	35.37	Positive	35.96	Positive
		Urine	36.23	Positive	38.02	Positive
1043-TDS-0076	5	EDTA plasma	32.17	Positive	31.12	Positive
		Urine	32.31	Positive	36.29	Positive
1043-TDS-0202	3	Serum	Undet	Negative	Undet	Negative
		Urine	34.16	Positive	Undet	Sample Invalid
1043-TDS-0206	6	Serum	Undet	Negative	Undet	Negative
		Urine	29.50	Positive	36.29	Positive
BUH01	7	EDTA plasma	33.37	Positive	34.02	Positive
		Urine	26.31	Positive	27.41	Positive
BUH02	6	EDTA plasma	36.04	Positive	37.85	Positive
		Urine	31.27	Positive	28.36	Positive
BUH03	6	EDTA plasma	Undet	Negative	Undet	Negative
		Urine	28.44	Positive	29.54	Positive
BUH06	4	EDTA plasma	Undet	Negative	Undet	Negative
		Urine	30.34	Positive	31.59	Positive
BUH08	5	EDTA plasma	33.14	Positive	33.74	Positive
		Urine	36.40	Positive	39.64	Positive
BUH09	3	EDTA plasma	33.67	Positive	41.15	Negative
		Urine	32.83	Positive	31.79	Positive

Undet: Undetermined, indicating no C_t value reported

A total of 52 contrived urine samples were prepared by spiking ZIKV, strain PRVABC59 into individual urine samples to 1.5xLoD (4.5×10^3 copies/mL, n=27) and 5xLoD (15×10^3 copies/mL, n=25). A total of 52 individual urine samples were used as Zika RNA negative

samples. The contrived and negative samples were only tested with *Sentosa*® SA ZIKV RT-PCR. The urine samples collected from three individuals resulted in invalid samples in both negative and contrived samples and are excluded from the number of samples tested. The clinical evaluation results for urine samples are summarized in **Table 17**.

Table 17. Clinical Evaluation Results for the *Sentosa*® SA ZIKV RT-PCR Test (Urine)

Specimen Category	***Sentosa* SA ZIKV RT-PCR Test**		
	Number Tested	**Positive**	**Negative**
Natural Zika Positive Samples	12[a]	12	0
Contrived Zika Positive Samples (1.5x LoD)	24[b]	24	0
Contrived Zika Positive Samples (5x LoD)	25	25	0
Expected Zika Negative samples	49[b]	0	49
Positive % Agreement	100% (61/61) 95% CI[c]: 94.1% to 100%		
Negative % Agreement	100% (49/49) 95% CI[c]: 92.7% to 100%		

CI = Confidence Interval

[a] Two invalid clinical samples by *Sentosa*® SA ZIKV RT-PCR Test were excluded.

[b] Three individual urine samples resulted in invalid samples in both negative (unspiked) and contrived (spiked) samples were excluded. Two of the three individual urine samples were re-tested and remain invalid.

[c] Wilson (Score) 95% Confidence Interval (CI)

Troubleshooting guide

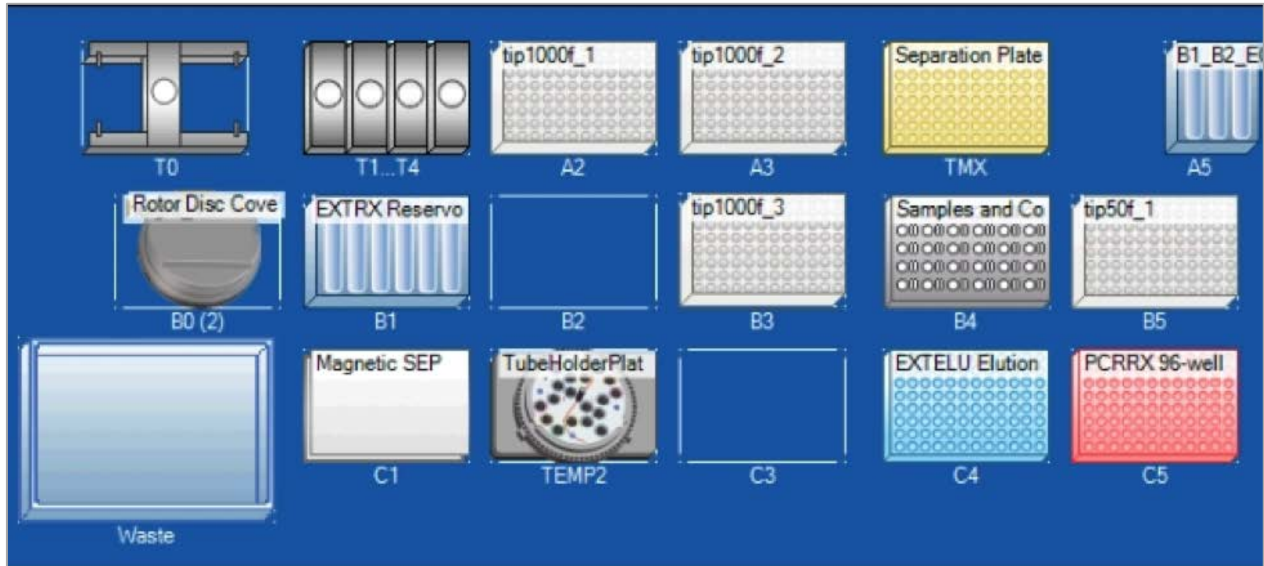
The troubleshooting guide may be helpful in solving any problems that may arise. For more information, please contact the authorized Vela Diagnostics representative. Vela Diagnostics Service and Support is always ready to answer any questions about the information and protocols in this user manual or sample and assay technologies (for contact information, refer to the back cover or visit www.veladx.com).

	Comments and recommended actions
1. Possible installation error	
a) Software initialization failed.	Please ensure base software has been successfully installed and default settings of the folder / files remain unchanged. For further assistance, please contact Vela Diagnostics Service and Support.
2. General handling	
a) Software display error.	Logout or re-start the software.
b) ABI 7500 Fast Dx Real-Time PCR Instrument or *Sentosa*® SA201 does not respond.	Re-start the machine for the run.
c) Error message displayed on the screen.	When an error message is displayed during a protocol run, please refer to the instrument / software user manuals.
3. Precipitates in the reagents of *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24)	
a) Storage of reagents.	Reagents might precipitate upon storage. If required, incubate the reagents in a water bath at 37°C for 30 minutes and shake occasionally to dissolve the precipitates.
4. Consistent late C_t values observed for samples	
a) Magnetic beads were not completely re-suspended.	Virus A2 (magnetic beads) requires thorough vortexing before use to ensure proper re-suspension.
b) Frozen samples were not mixed properly after thawing.	Thaw frozen samples with mild agitation to ensure thorough mixing.

		Comments and recommended actions
c)	Degraded nucleic acids.	Ensure that samples are stored correctly and not subjected to multiple freeze-thaw cycles. Repeat the extraction procedure with new samples.
d)	Incomplete sample lysis.	Ensure that Virus B1 (lysis buffer) does not contain precipitates. If required, incubate it in a water bath at 37°C for 30 minutes and shake occasionally to dissolve the precipitates.
e)	Clogging of pipette tip due to insoluble material in the samples.	Insoluble material was not removed from the sample prior to starting the extraction procedure on the *Sentosa*® SX101 instrument. To remove insoluble material, centrifuge the diluted sample suspension at approx. 1,000 to 2,000 x g for 30 sec to 1 min, and transfer the supernatant to a fresh sample tube.
5.	**No signal for positive control (ZIKV PC) in the fluorescence channel Green**	
a)	Storage conditions for one or more components did not comply with the instructions given in the “Storage” section.	Check the storage conditions (refer to the kit label) of the reagents and use a new kit, if necessary.
b)	Extraction / assay kit has expired.	Check the expiration date (refer to the kit label) of the reagents and use a new kit, if necessary.
6.	**Weak or no signal of the extraction control (EC) in the fluorescence channel Red subjected to extraction using the *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24)**	
a)	PCR inhibition.	Collect new sample from the same patient and repeat the whole *Sentosa*® workflow.

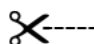
		Comments and recommended actions
b)	EC is not added into the sample.	Ensure the correct SX 101 application is used (refer to the "Protocol: Automated nucleic acid extraction, PCR set-up and detection on *Sentosa*® SX101 and *Sentosa*® ABI 7500 Fast Dx Real-Time PCR Instrument" section on page 9 and follow the instructions closely).
c)	Loss of nucleic acid during extraction.	No signal of the extraction control may indicate the loss of nucleic acid during the extraction. Ensure the correct SX 101 application is used (refer to the "Protocol: Automated nucleic acid extraction, PCR set-up and detection on *Sentosa*® SX101 and ABI 7500 Fast Dx Real-Time PCR Instrument" section on page 9 and follow the instructions closely).
d)	Storage conditions for one or more kit components did not comply with the instructions given in the "Storage" section.	Check the storage conditions (refer to the kit label) of the reagents and use a new kit if necessary.
e)	Extraction / assay kit has expired.	Check the expiration date (refer to the kit label) of the reagents and use a new kit if necessary.
7.	**Signals with the negative control in the fluorescence channel Green or Orange of the analytical PCR**	
a)	Contamination occurred during extraction / PCR set-up.	Ensure that the workspace and instruments are decontaminated as recommended. Repeat extraction and PCR protocols with new reagents.

Appendix A: *Sentosa*® SX101 layout for 24-1 VirLys3 v2-1 protocol (for 24 tests)



Position	**Labware**
T0	*Sentosa*® SX Gripper
T1 to T4	*Sentosa*® SX Dispensing Tools
A2	*Sentosa*® SX Partition 1000 µL Filter Tips
A3	*Sentosa*® SX Partition 1000 µL Filter Tips
TMX	*Sentosa*® SX Deepwell Plate 96/2000 µL
A5	*Sentosa*® SX Reservoir Rack 3
B0 (top)	Sentosa® SX Cover Thermorack Rotor-Disc 72
B0 (bottom)	*Sentosa*® SX Cover Thermorack Rotor-Disc 72 Adaptor
B1	*Sentosa*® SX Reservoir Rack 7
B2	Empty
B3	*Sentosa*® SX Partition 1000 µL Filter Tips
B4	*Sentosa*® SX Rack 0.5 + Adaptor / 1.5 / 2.0 mL
B5	*Sentosa*® SX Partition 50 µL Filter Tips
C1	*Sentosa*® SX Magnetic Separator
TEMP2 (top)	*Sentosa*® SX Thermorack Rotor-Disc 72
TEMP2 (bottom)	*Sentosa*® SX Thermorack Rotor-Disc 72 Adaptor
C3	Empty
C4	*Sentosa*® SX Microplate 96/V
C5 (top)	*Sentosa*® SA 96-Well Optical Plate / MicroAMP® Fast Optical 96-Well Reaction Plate
C5 (bottom)	*Sentosa*® SX Thermoblock PCR 96

Symbols

Symbol	Meaning
	Contains sufficient for <*n*> tests
	Use-by date
	Do not reuse
	Catalog number
	Component
	Number
	Content
	Lot number
	Control
	Negative control
	Positive control
	Document / label identification number
	Temperature limit
	Manufacturer
	Consult instructions for use
	Cut here
	In Vitro Diagnostic
	Caution: Federal law restricts this device to sale by or on the order of a licensed healthcare practitioner

References

1) CDC 2016. Centers for Disease Control and Prevention, Zika Virus. [ONLINE] Available at: http://www.cdc.gov/zika/about/. [Accessed 18 April 2016].

2) WHO 2016. World Health Organization, Zika Virus. [ONLINE] Available at: http://www.who.int/mediacentre/factsheets/zika/en/. [Accessed 18 April 2016].

3) Mackay, I.M. (2004). Real-time PCR in the microbiology laboratory. *Clin Microbiol Infect.* 10(3), 190–212.

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Limited License Agreement

Use of this product signifies the agreement of any purchaser or user of the *Sentosa*® SA ZIKV RT-PCR Test (4x24) to the following terms:

1. The *Sentosa*® SA ZIKV RT-PCR Test (4x24) may be used solely in accordance with the *Sentosa*® SA ZIKV RT-PCR Test (4x24) user manual and for use with components contained in the test only.
2. Vela Diagnostics grants no license under any of its intellectual property to use or incorporate the enclosed components of this test with any components not included within this test except as described in the *Sentosa*® SA ZIKV RT-PCR Test (4x24) user manual.
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Visit our website: www.veladx.com

For use under an Emergency Use Authorization (EUA) only.

Refer to the *Sentosa*® SA ZIKV RT-PCR Test complete Instructions for Use available at www.veladx.com.

Intended use

The *Sentosa*® SA ZIKV RT-PCR Test is a real-time RT-PCR test intended for the qualitative detection of RNA from the Zika virus in serum, EDTA plasma or urine (collected alongside a patient-matched serum or plasma specimen) from individuals meeting Centers for Disease Control and Prevention (CDC) Zika virus clinical criteria (e.g., clinical signs and symptoms associated with Zika virus infection) and / or CDC Zika virus epidemiological criteria (e.g., history of residence in or travel to a geographic region with active Zika transmission at the time of travel, or other epidemiological criteria for which Zika virus testing may be indicated). Testing is limited to U.S. laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests, and similarly qualified non-U.S. laboratories.

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

Negative results do not preclude Zika virus infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

The *Sentosa*® SA ZIKV RT-PCR Test is intended for use by trained clinical laboratory personnel who have received specific training on the use of the *Sentosa*® SA ZIKV RT-PCR Test. The test is only for use under the Food and Drug Administration's Emergency Use Authorization.

The *Sentosa*® SA ZIKV RT-PCR Test is configured for automated workflow using the *Sentosa*® SX101 instrument, in conjunction with the Applied Biosystems® 7500 Fast Dx Real-Time PCR instrument (ABI 7500 Fast Dx) or the *Sentosa*® SA201. The *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24) is used for extraction.

Assay Principle

The *Sentosa*® SA ZIKV RT-PCR Test comprises a ready-to-use kit for the detection of Zika virus (ZIKV) RNA by PCR on the ABI 7500 Fast Dx Real-Time PCR instrument or *Sentosa*® SA201, with nucleic acid extraction and PCR assay set-up using the *Sentosa*® SX101 instrument. The ZIKV master mix contains reagents and enzymes for reverse transcription and specific amplification of a 103 base pair (bp) fragment of the NS4A gene within the open reading frame (ORF) of the ZIKV. The master mix also contains specific primers/probe for the direct detection of ZIKV amplicons in the fluorescence channels Cycling Green of the ABI 7500 or *Sentosa*® SA201.

Detection of the targets occurs in two channels: green and red on the ABI 7500 Fast Dx Real-Time PCR instrument or *Sentosa*® SA201. Output is recorded as the increase of fluorescence over time in comparison to background signal. Monitoring the fluorescence intensities during the PCR run allows the detection of the accumulating product without having to re-open the reaction tubes after the PCR run.

In addition, the *Sentosa*® SA ZIKV RT-PCR Test contains a second set of primers / probes designed to detect an extraction control (EC1) target in the fluorescence channel Cycling Red. This extraction control is used as a control for the nucleic acid extraction procedure and as a PCR inhibition control. The EC1 amplification does not compromise the detection limit of the analytical ZIKV PCR.

The test also contains a negative control (NC1) and a positive control (ZIKV PC) that allow the user to assess whether the PCR reaction has been performed properly.

Pathogen detection by PCR is based on the amplification of specific regions of the pathogen genome. In real-time PCR, the amplified products are detected via fluorescent dyes linked to oligonucleotide probes that bind specifically to the target sequences. Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Real-time monitoring of the fluorescence intensities during the PCR run allows the detection of the accumulating products[1].

Kit contents

Item	Cap color	Description	Quantity (tube)	Volume / tube
ZIKV M1 (24)	Green	Primer and probe mix	4	150 µL
RNA4 M2 (24)	Orange	Amplification mix	4	325 µL
RNA4 M3 (96)	Pink	Reverse transcriptase	1	330 µL
NC1	Yellow	Negative control (NC)	4	300 µL
ZIKV PC	Blue	Positive control (PC)	4	300 µL
EC1	Red	Extraction control (EC)	4	200 µL

Storage

The components of the *Sentosa*® SA ZIKV RT-PCR Test are stable for 12 months (until the expiration date stated on the label) when stored at −20°C. They should not be subjected to repeated freeze-thaw cycles as this may compromise assay performance. Each set of reagents (ZIKV M1, RNA4 M2, NC1, ZIKV PC and EC1) is meant for single-use and not recommended for reuse. RNA4 M3 is designed for a maximum of four runs.

Read before use

- The product is to be used by trained clinical laboratory personnel who have received specific training on the use of the *Sentosa*® SA ZIKV RT-PCR Test only.
- Strict compliance with the protocol is required for optimal PCR results.
- RNA4 M3 is an enzyme in liquid state under storage conditions. Except RNA4 M3, all reagents should be thawed completely before use.
- All reagents, except RNA4 M2 and RNA4 M3, require thorough mixing by quick vortex. Mix RNA4 M2 and RNA4 M3 by gentle inversion. Centrifuge all tubes briefly (approx. 1,000 x g for 10 sec) to collect the contents at the bottom of the tubes. Avoid foaming of the reagents.
- **RNA4 M3 is designed for a maximum of 4 runs. It should be used directly out of the freezer or kept on ice during reagent preparation. Handle carefully to avoid contamination and store the remaining RNA4 M3 at −20°C immediately after use for subsequent runs.**
- All reagents, except RNA4 M3, are designed for single-use only. Do not use the remaining reagents from previous runs.

Warnings and precautions

- For *In Vitro* Diagnostic Use.
- A patient matched serum specimen is required for serological follow up testing of negative results, per the CDC testing algorithm. (Found at http://www.cdc.gov/zika/index.html.)
- Use sterile pipette tips with filters.
- During manual steps, ensure that the tubes are closed when

possible to avoid contamination.

- **Do not mix components from kits with different lot numbers**
- Proceed continuously from one part of the workflow to the next. Do not exceed 30 minutes of transfer time between the *Sentosa*® SX101 and the ABI 7500 Fast Dx Real-time PCR instrument or *Sentosa*® SA201.
- For additional information, please refer to the *Sentosa*® SA ZIKV RT-PCR Test complete Instructions for Use and the material safety data sheet (MSDS).

Procedure

Extraction reagents preparation

Prepare reagents from the *Sentosa*® SX Virus Total Nucleic Acid Kit v2.0 (4x24) (item no.: 300353) as follows:

- Reconstitute Virus A3 (lyophilized carrier RNA) with Virus A4 (carrier RNA buffer) by adding 125 µL of Virus A4 to Virus A3. Pulse vortex for 30 seconds followed by brief centrifugation (approx. 1,000 x g for 10 sec).
- Invert Virus A1 (Proteinase K solution) gently followed by a brief centrifugation (approx. 1,000 x g for 10 sec). Vortex Virus A2 (magnetic beads) vigorously for 1 minute and flick to collect the contents at the bottom of the tube.

Assay reagents preparation

- Transfer 75 µL of RNA4 M3 to ZIKV M1 and gently invert the tube to mix the content. Centrifuge ZIKV M1 tube, containing RNA4 M3, briefly (approx. 1,000 x g for 10 sec) to collect the contents at the bottom of the tube. Store the remaining RNA4 M3 at -20°C for subsequent runs.

Sample preparation

- Transfer 250 µL of sample into a fresh 1.5 mL *Sentosa*® SX Safe-Lock Tube or Sarstedt tube in a biosafety cabinet.

  NOTE: Remove visual precipitates, if any, from the samples by briefly spinning the sample tubes (approx. 1,000 to 2,000 x g for 30 sec to 1 min) and transferring the supernatants into fresh 1.5 mL *Sentosa*® SX Safe-Lock Tube(s) or Sarstedt tube(s).
- Load the sample tubes directly into the *Sentosa*® SX Rack(s) in the *Sentosa*® SX101 instrument and start the required application immediately.

Workflow

Nucleic acid extraction and PCR set-up procedures are automated using *Sentosa*® SX101 instrument.

Log in to the *Sentosa*® SX software and run the application. Successive windows will assist in the loading of samples, reagents and consumables on the worktable.

On the final window, click *"Run".*

After the run is completed, seal the MicroAmp® Fast Optical 96-Well Reaction Plate or *Sentosa*® SA 96-Well Optical Plate with Barcode using the respective *Sentosa*® SA Optical Adhesive Seal or the MicroAmp® Optical Adhesive Film.

PCR

Briefly spin down the PCR Plate and load it onto the Applied Biosystems® 7500 Fast Dx Real Time PCR System or the *Sentosa*® SA201.

Applied Biosystems® 7500 Fast Dx / Sentosa® SA201:

- Turn on the Applied Biosystems® 7500 Fast Dx Real-Time Instrument or the *Sentosa*® SA201 and wait for the initiation procedure to complete.
- Launch and log in to the Applied Biosystems® 7500 Fast System SDS Software or the *Sentosa*® SA201 Series Software.
- Run Reverse Transcription PCR:

  In the "*Quick Startup document*" dialog box, select "*Create New Document*". In the "*Assay*" drop-down list, select "*Standard Curve (Absolute Quantitation)*". The default setting for "*Container*" should be "*96-Well Clear*". The default setting for "*Template*" should be "*Blank Document*". In the "*Run Mode*" drop-down list, select "*Fast 7500*".
- Define the fluorescent detectors:

Detection	Detector Name	Reporter Dye	Quencher Dye
Zika virus RNA	GREEN	FAM	(None)
EC1	RED	CY5	(None)

- Select "*None*" for Passive Reference.
- Set up the sample plate by selecting the wells and checking both the green and red detectors for use.

Set the cycling conditions as follows:

Stage	Reps	Temperature (°C)	Duration (min)
1	1	50	15:00
2	1	95	0:20
3	45	95	0:05
		60	0:30

- Save the run file and start the run.
- Perform data analysis after the run is completed.

Data analysis

Input the parameters for analysis settings as follows:

Channel	Threshold	Manual Baseline	
Green	1.0e+005	Start (cycle) 12	End (cycle) 16
Red	2.0e+004	Start (cycle) 6	End (cycle) 12

Ensure that analysis is performed for the green and red fluorescence channels using the ABI 7500 Fast Dx SDS Software or the *Sentosa*® SA201 Series Software.

Export the results and save the .csv file.

The amplification plot and the background fluorescence view (Rn versus Cycle) / component data should be examined for positive samples. This is to confirm the C_t value(s) reported and also to determine if there is a true amplification signal.

Data interpretation

Negativity / positivity of samples

The table below displays the C_t range to define negativity (-) and positivity (+) for negative control, positive control and sample(s). Fluorescence channel Cycling Green detects the Zika virus and fluorescence channel Cycling Red detects the extraction control.

Fluorescence channel	C_t range for negativity (-) and positivity (+)					
	Negative control		Positive control		Sample	
	-	+	-	+	-	+
Green	≥ 40.0 or Undet	< 40.0	< 22.6, > 26.1 or Undet	22.6 – 26.1	≥ 40.0 or Undet	< 40.0
Red	< 23.0, > 30.0 or Undet	23.0 - 30.0	< 23.0, > 30.0 or Undet	23.0 – 30.0	< 23.0, > 30.0 or Undet	23.0 – 30.0

Undet: Undetermined, indicating no C_t value reported

The following tables summarize results and their corresponding interpretations. The Negative and Positive Control results must be evaluated first to determine run validity.

For **Negative control**

Negative control		Interpretation
Green	Red	
-	+	Run valid (proceed to Positive Control result interpretation)
+	+/-	Run invalid. All results are invalid and all samples must be retested.
-	-	Run invalid. All results are invalid and all samples must be retested.

For **Positive control**

Positive control		Interpretation
Green	Red	
+	+/-	Run valid (proceed to Sample result interpretation)
-	+/-	Run invalid. All results are invalid and all samples must be retested.

For **Samples**

Sample		Interpretation
Green	Red	
+	+/-	Zika virus RNA detected*. All Zika virus RNA detected results must be reported to the appropriate Public Health agency.
-	+	Zika virus RNA not detected.
-	-	Sample invalid. Repeat testing from the original sample or collect and test a new sample.

*For positive samples, the fluorescence channel Cycling Red can be negative due to competition with the target channel.

Run: Whole run on Applied Biosystems® 7500 Fast Dx or *Sentosa*® SA201.
Sample: Single sample in one well of the MicroAmp® Fast Optical 96-Well Reaction Plate or the *Sentosa*® SA 96-Well Optical Plate.

Instrument maintenance

After every run, discard used sample tubes, plates, reagents and tips according to the local safety regulations. All samples and waste should be considered potentially infectious.

A reservoir collects liquid waste generated during the nucleic acid purification procedure. Dispose of the liquid waste according to the local safety and environmental regulations. Dispose of the biohazard bags after each run.

Perform regular cleaning of the *Sentosa*® SX101 instrument and the Applied Biosystems® 7500 Fast Dx Real-Time PCR instrument or *Sentosa*® SA201 after each run. Refer to the respective instrument user manuals for detailed procedures.

Ensure that maintenance is performed regularly to minimize the risk of error.

Always wear the appropriate personal protective equipment (PPE: laboratory coat, gloves, goggles) during cleaning / maintenance procedures.

Regulatory status

For use under an Emergency Use Authorization (EUA) only.

References

1) Mackay, I.M. (2004). Real-time PCR in the microbiology laboratory. *Clin Microbiol Infect.* 10(3), 190–212.

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