For use under the Emergency Use Authorization (EUA) only

For *in vitro* diagnostic use

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Instructions for Use

R_x Only

LightMix® Zika rRT-PCR Test

Cat. No. 53-0702-96 Roche SAP n°07987897001

Kit with lyophilized reagents for 96 PCR reactions (20 µl) for detection of Zika Virus RNA

1. Intended Use

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

Results are for the identification of Zika virus RNA. Zika virus RNA is generally detectable in serum during the acute phase of infection (approximately 7 days following onset of symptoms, if present). Positive results are indicative of current infection. Laboratories are required to report all positive results to the appropriate public health authorities. Within the United States and its territories positive results must be reported to appropriate state/local 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 ***LightMix® Zika rRT-PCR Test*** is intended for use by trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures. The ***Test*** is only for use under the Food and Drug Administration's Emergency Use Authorization.

2. Test Principle

The ***LightMix® Zika rRT-PCR Test*** is a real-time reverse transcription polymerase chain reaction (rRT-PCR) test. The Zika virus primers and probe set is designed to detect RNA from the Zika virus in serum or EDTA plasma from patients presenting signs and symptoms of the Zika virus infection in conjunction with epidemiological risk factors.

Nucleic acid is isolated and purified from serum or EDTA plasma specimens using the Roche MagNA Pure 96 System together with the Roche MagNA Pure 96 DNA and Viral NA Large Volume reagent kit. Alternatively the nucleic acid is isolated and purified using the MagNA Pure Compact Instrument and MagNA Pure Compact Nucleic Acid Isolation Kit I - Large Volume.

The purified nucleic acid is reverse transcribed into cDNA and then subsequently amplified using the Roche LightCycler® Multiplex RNA Virus Master reagents in the Roche LightCycler® 480 Instrument II, or alternatively in the Roche **cobas z** 480 Analyzer (open channel). In the process, the probes anneal to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5′ nuclease activity of Taq DNA polymerase degrades the probes, causing the reporter dyes to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity and emission wavelengths are monitored at each PCR cycle by the Roche LightCycler® 480 Instrument II or alternatively in the Roche **cobas z** 480 Analyzer (open channel).

3. Kit Contents

- 1 Vial **LightMix® Zika rRT-PCR Test PSR** (**yellow** cap, 96 reactions)
  Contains pathogen-specific reagent (PSR), i.e., lyophilized primers and FAM-labeled probe that specifically detect Zika virus.
- 1 Vial **LightMix® Zika rRT-PCR Test ivRNA Positive Control** (**black** cap, 32 reactions)
  Contains lyophilized synthetic RNA, designed to react with the **LightMix® Zika rRT-PCR Test PSR** to indicate whether the ***Test*** has been successful.

4. Storage and Shelf Life

- Store all kit components at 4-24℃ in the dark upon receipt. **Do <u>not</u> freeze** the lyophilized reagents.
- Do not use any kit component past the labeled expiration date.
- Reconstituted **LightMix® Zika rRT-PCR Test PSR** reagent is stable for up to 2 weeks if stored at 4℃ protected from light.
- Reconstituted **LightMix® Zika rRT-PCR Test ivRNA Positive Control** reagent should be stored frozen until the labeled expiration date. Avoid multiple freeze-thaw cycles.

5. Materials Required But Not Supplied

Note: All Catalog Numbers listed in this section are from Roche Diagnostics GmbH (Mannheim, Germany), unless otherwise specified.

<u>General</u>
- Clean pipettes/tips reserved for RNA work only
- RNase, DNase free-disposable plasticware
- Centrifuge capable of spinning microwell plates
- Microcentrifuge
- Vortex mixer

<u>Nucleic acid extraction, Automated</u>
- MagNA Pure 96 Instrument (Cat.-No. 06 541 089 001)
- MagNA Pure 96 DNA and Viral NA Large Volume Kit (Cat.-No. 06 374 891 001)
- MagNA Pure Tip 1000 µL (Cat.-No. 06 241 620 001)
- MagNA Pure 96 Processing Cartridges (Cat.-No. 06 241 603 001)
- MagNA Pure 96 Output Plate (Cat.-No. 06 241 611 001)
- MagNA Pure Sealing Foil (Cat.-No. 06 241 638 001)
- MagNA Pure 96 Internal Control Tube, IVD (Cat.-No. 06 374 905 001)
- MagNA Pure 96 Waste Cover (Cat.-No. 06 541 275 001)
- MagNA Pure 96 System Fluid (Internal) (Cat.-No. 06 430 112 001) *or* (External) (Cat.-No. 06 640 729 001)

<u>Nucleic acid extraction, Automated (Alternative)</u>
- MagNA Pure Compact Instrument (Cat.-No. 03 731 146 001)
- MagNA Pure Compact Nucleic Acid Isolation Kit I - Large Volume (Cat.-No. 03 730 972 001)
- Internal Control Tube, 2.0 ml Sarstedt Tubes (without cap: Sarstedt #72.608; with cap: Sarstedt #72.693)

<u>Controls</u>
- RNA Process Control Kit LSR (Cat. No. 07 958 293 001)

<u>Amplification and Detection</u>
- LightCycler® 480 Instrument II (Cat.-No. 05 015 278 001) *or* **cobas z** 480 Analyzer (Cat.-No. 05 200 881 001)
- LightCycler® Software Version 1.5 or higher *or* **cobas z** 480 Software Version 1.5 or higher with UDF Version 1.0 or higher
- LightCycler® 480 Multiwell Plate 96 white, with seals (Cat.-No. 04 729 692 001) *or* c4800 MWP (Cat.-No. 05 232 724 001)
- LightCycler® Multiplex RNA Virus Master (Cat.-No. 06 754 155 001).

6. Precautions, Warning, and Material Safety Data (MSDS)

- For In Vitro Diagnostic Use under Emergency Use Authorization only
- Use of this product is limited to specified laboratories and clinical laboratory personnel who have been trained in the techniques of real-time PCR and *in vitro* diagnostic procedures on authorized instruments.
- Laboratory biosafety guidance for working with Zika virus specimens is provided at http://www.cdc.gov/zika/state-labs/index.html. It is recommended that laboratories perform a risk assessment when conducting new tests and safety precautions should be based on the laboratory's risk assessment. The Zika virus is considered a pathogen that can be safely worked with in a biosafety level 2 (BSL-2) laboratory.
- Use appropriate laboratory and personal protective equipment when using this kit.
- Treat all specimens and waste as potentially infectious.
- The laboratory work-flow must conform to standard practices. Due to the risk of contamination of PCR with previously generated PCR amplicons, PCR set-up and PCR amplification must be performed in physically separated areas.
- Lyophilized PCR positive control should be reconstituted and handled in a location designated for the handling of high-copy nucleic acid (e.g., designated dead air box) apart from the normal PCR setup area.
- Avoid microbial or nuclease contamination of the reagents while pipetting the aliquots. The use of disposable sterile tips is essential.
- Do not mix reagents from different lots.
- Do not use the reagents after the expiration date.
- Performance of this assay has only been established with the specimen types listed in the Intended Use. Performance with other specimen types has not been evaluated.
- Proper sample collection, storage, and transport are essential for correct results.
- Refer to the respective Operator's Manuals for all the operative and safety instructions of equipment used in the assay.
- Handle all reagents following the respective manufacturer's instructions for use.
- Dispose of the unused reagents and waste materials according to the applicable laws and regulations.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Due to the relatively fast molecular evolution of RNA viruses, there is an inherent risk for any RT-PCR based test system that accumulation of mutations over time may lead to false negative results.

Material Safety Data (MSDS)
According to U.S. OSHA 29 CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the EU Directives 67/548/EC and 1999/45/EC any products which do not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

7. Sample Collection

- The ***LightMix®* Zika rRT-PCR Test** is intended for detection of RNA from the Zika virus in serum and EDTA plasma.
- Specimens should be collected according to the manufacturer's instructions for the specimen collection device.
- Shipping should be performed according to the policies of the shipping performer, customs regulations, and the requirements of the receiving laboratory.
- Follow the recommended infection control precautions for Zika or other viruses in handling all specimens.

8. Testing Procedure

The ***LightMix®* Zika rRT-PCR Test** requires the use of the following controls for quality control purposes:

1. A "no template" negative control (Negative Process Control) is needed to document specificity of the ***Test*** and absence of cross-contamination. PCR-grade water is used in place of clinical samples from the beginning of the sample extraction process. The Negative Process Control is run in parallel with unknown samples in each sample extraction run.
2. A second "no template" negative control (Negative RT-PCR Control) is needed as an rRT-PCR control. PCR-grade water is used at the rRT-PCR step to test for absence of cross-contamination.

3. A Zika positive template control (Positive Control) is needed to document that the ***Test*** can detect Zika nucleic acid if present. The Positive Control is the **LightMix® Zika rRT-PCR Test ivRNA Positive Control,** a synthetic RNA transcript containing the virus region targeted by the ***Test***. The Positive Control is used starting at the rRT-PCR step, then run in parallel with unknown samples and the negative controls in each assay run.
4. A positive extraction control (Extraction Control) is needed to document that the nucleic acid extraction method for the ***Test*** sufficiently preserves the target nucleic acid. The Extraction Control is the Roche RNA Process Control LSR (RPC). The Extraction Control is run together with every serum or EDTA plasma sample from the beginning of the extraction process and detected by rRT-PCR using a primer pair and a Cy5-labeled probe in a duplex reaction with the Zika virus specific primer pair and probe.

8.1 Nucleic Acid Extraction

Materials and equipment:
Refer to Section 5 for all materials and equipment required for nucleic acid extraction, but not provided with this kit.

Sample materials and process controls:
- Appropriate specimens for nucleic acid extractions are serum or EDTA plasma.
- For each extraction batch, always include at least one Negative Process Control by replacing specimen with PCR-grade water. The Negative Process Control will serve as an external processing control for both extraction and amplification.
- For the RNA Process Control **(RPC 1:4)**:
  - Dilute as per the RPC Instructions for Use.
  - Take 1.0 ml of the RPC Working Solution and place into the MP96 Internal Control Tube.
  - Add 3.0 ml of the RPC Diluent into the Internal Control tube and mix briefly by vortexing. This is the **RPC 1:4.**

Extraction using MagNA Pure 96 System
Refer to the following document for details:
- MagNA Pure 96 System Operator's Manual
- MagNA Pure 96 DNA and Viral NA Large Volume Kit (Instructions for Use)

Perform the MagNA Pure 96 System run following the steps below:
- For each sample, place 500 µl of the sample into one well of a MagNA Pure 96 processing cartridge. This cartridge is hereafter referred to as the "source plate".
- Create a work order in the MagNA Pure 96 software with the following specifications:
  - **Order Type:** Purification
  - **MagNA Pure Kit Name:** DNA/Viral NA LV 2.0
  - **Protocol:** Pathogen Universal 500 3.0 or higher
  - **Volume, Sample:** 500 µl
  - **Volume, Elution:** 100 µl
  - **Internal Control:** RPC 1:4 (User defined)
  - **Target Plate:** MP96 Output Plate
  - **Sample names:** (*Enter as appropriate in the Sample Table).*
- Save the work order by selecting the SAVE button; enter a file name for the order and click *Save*.
- Verify that the deck waste tip holders are free of tips and that there is a waste cover in place.
- Load the appropriate reagents and consumables onto the MagNA Pure 96 instrument deck.
- Load the source plate onto the instrument.
- Close the load flap and start the run.
- When the run is finished, open the load flap. Unload the MagNA Pure 96; carefully remove the output plate containing the eluates.

Extraction using MagNA Pure Compact Instrument
Refer to the following document for details:
- MagNA Pure Compact Instrument Operator's Manual
- MagNA Pure Compact Nucleic Acid Isolation Kit I - Large Volume (Instructions for Use)

Perform the MagNA Pure Compact Instrument run following the steps below:
- For each sample, place 500 µl of the sample into a Sample Tube.
- For each sample, create an Internal Control tube. Place 20 µl of the **RPC 1:4** into an IC tube.

- Follow the MagNA Pure Compact Nucleic Acid Isolation Kit I - Large Volume, IFU section, **Protocol for the Isolation of Nucleic Acids** to setup the MagNA Pure Compact run
- Create a work order in the MagNA Pure Compact software with the following specifications:
  - **Protocol:** Total_NA_Plasma_500
  - **Elution Volume:** 100 µl
  - **Internal Control:** Yes
  - **Sample Materials:** Serum or EDTA Plasma
  - **Sample names:** (*Enter as appropriate)*
- Close the load flap and start the run.
- When the run is finished, open the load flap. Unload the MagNA Pure Compact; carefully remove the elution tubes containing the eluates and cover with screw-caps.

Handling of specimen extracts:
Keep the specimen extracts at 2-8°C until PCR amplification. If not used immediately, freeze the extracts at -80ºC. Do not freeze or thaw extracts more than once prior to PCR amplification.

8.2 PCR Amplification

Materials and equipment:
PCR instrument: Amplification and detection can be accomplished by following the instructions below using either the LightCycler® 480 Instrument II *or* the **cobas z** 480 Instrument.

Refer to the following documents for details:
- LightCycler® 480 Instrument Operator's Manual *or* **cobas z** 480 Operator Manual
- LightCycler® Multiplex RNA Virus Master Instructions for Use

Refer to Section 5 for all other materials and equipment required for PCR amplification, but not provided with this kit.

Programming
Refer to the instrument operator's manual for details. Start programming before preparing solutions. The protocol consists of four program steps listed in Table 1 below, including:
1. Reverse Transcription of the viral RNA
2. Denaturation: Sample denaturation and enzyme activation
3. Cycling: PCR-amplification
4. Cooling: cooling of the thermal block

Set the Detection Format as follows:
Detection Format 530 Channel Set Quant Factor **10** and Max Integration Time **2 sec**
LightCycler® 480 Instrument II 465-510
cobas z 480 Analyzer (open channel) 465-510
Detection Format 670 Channel Set Quant Factor **10** and Max Integration Time **2 sec**
LightCycler® 480 Instrument II 618-660
cobas z 480 Analyzer (open channel) 610-670

Table 1 PCR Amplification Programming Parameters

Program Step:	***RT Step***	***Denaturation***	***Cycling***			***Cooling***
Parameter						
Analysis Mode	None	None	Quantification mode			None
Cycles	*1*	1	45			1
Target [℃]	55	95	95	60	72	40
Hold [hh:mm:ss]	00:05:00	00:05:00	00:00:05	00:00:15	00:00:15	00:00:30
Ramp Rate [℃/s]	4.4	4.4	4.4	2.2	4.4	1.5
Acquisition Mode	None	None	None	**Single**	None	None

Preparation of LightMix® Zika rRT-PCR Test Assay-Specific Reagents
- **LightMix® Zika rRT-PCR Test PSR** (**yellow** cap):
  - **Add 50 µl** PCR grade water to each vial. Mix the solution (vortex) and spin down.
  - **Use 0.5 µl** reconstituted reagent for a 20 µl PCR reaction.
  - Refer to Section 4 for proper storage of the reconstituted reagent.

- **LightMix® Zika rRT-PCR Test ivRNA Positive Control** (**black** cap):
  - **Add 320 µl** PCR grade water to the vial. Mix by pipetting the solution up and down 10 times.
  - **Use 10 µl** reconstituted reagent for a 20 µl PCR reaction.
  - Refer to Section 4 for proper storage of the reconstituted reagent.

Preparation of the reaction mix

- In a cooled reaction tube, prepare the reaction mix for single reactions (left) or one 96-well plate (right) (Table 2).

Table 2 Preparation of LightMix® Zika rRT-PCR Reaction Mix

For use with the Roche LightCycler® Multiplex RNA Virus Master		
One reaction	**Component**	**100 reactions**
4.4 µl	**Water**, PCR grade (Colorless Cap)	440 µl
0.5 µl	Reconstituted **LightMix® Zika rRT-PCR Test PSR** (**Yellow** Cap)	50 µl
1.0 µl	**RNA Process Control Detection Assay** (**Yellow** Cap)	100 µl
4.0 µl	**RT-qPCR Reaction Mix, 5x concentration** (**Red** Cap)	400 µl
0.1 µl	**RT-Enzyme Solution, 200x Concentration** (**Blue** Cap)	10 µl
10.0 µl	**Volume of Reaction Mix**	**1000µl**

- Mix gently and spin down
- **Transfer 10 µl** of the **LightMix® Zika rRT-PCR Reaction Mix** per well.
- **Add 10 µl** of sample or control to each well for a final reaction volume of 20 µl. Seal the plate and centrifuge.
- Load the plate and start the run.

9. Interpretation of the Results

Upon completion of the PCR run, perform data analysis using the instrument software as described in the operator's manual.

- Click the **Analysis** box on the left side of the software.
- Choose the **Abs Quant/2nd Derivative Max** under the **Create New Analysis** window. Make sure the defaults settings are:
  - **Analysis Type:** Abs Quant/2nd Derivative Max
  - **Subset:** All Samples
  - **Program:** Amplification
  - **Name:** Abs Quant/2nd Derivative Max for All Samples
    Click on the √ to complete the analysis.
- Click the **Calculate** button.

The amplification results are reported as Cp values. The instrument software also tentatively reports an *assay result* as positive (a Red curve in the amplification plot from the software), negative (a Green curve in the amplification plot from the software), or uncertain (a Blue curve in the amplification plot from the software).

Table 3 summarizes the steps for the interpretation of the ***Test*** results. The results from the Controls should be examined prior to interpretation of any clinical specimen result. If these Controls are not valid, the clinical specimen results are not valid and should not be interpreted.

Table 3 Summary of Result Interpretation

(*Note: Follow evaluation in this table in the order as listed.*)

Step	Controls / Specimens	Zika Assay	RPC Assay	Interpretation
1	Negative Process Control	-	N/A[a]	Proceed to evaluate Negative RT-PCR Control results.
		+	N/A[a]	Invalidate the run.[b]
2	Negative RT-PCR Control	-	-	Proceed to evaluate Positive Control results.
		+	-	Invalidate the run.[b]
		-	+	
		+	+	
3	Positive Control	+	N/A[a]	Proceed to evaluate Clinical Specimen results.
		-	-	Invalidate the run.[c]
		-	+	
4	Clinical Specimen[d]	+ (FIR ≥ 10%)	N/A[a]	Report as **Positive** for Zika Virus
		+ (FIR < 10%)	N/A[a]	**Indeterminate** specimen result[e]
		-	+	Report as **Negative** for Zika Virus.[f]
		-	-	**Indeterminate** specimen result[e]

a. N/A: Result of the RPC Assay is not required and should not be assessed. The result of the Zika Assay determines the interpretation.

b. If either of the Negative Controls fails, re-test the extracted specimen and controls. If either of the Negative Controls fails again, re-test with all specimens re-extracted.

c. If the Positive Control fails for the Zika Virus assay, the entire run is invalid and potential sources of the PCR process failure should be identified and corrected. Re-test the extracted specimen and controls, and re-analyze. If the Positive Control reaction fails again, re-test with a freshly reconstituted **LightMix® Zika rRT-PCR Test ivRNA Positive Control,** fresh aliquots of extracted specimens or all specimens re-extracted.

d. If a clinical specimen is reported as Zika Assay Positive by the instrument software, the maximum fluorescence intensity of the specimen should be compared to the maximum fluorescence intensity of the Positive Control from the same run to derive a Fluorescence Intensity Ratio (FIR).

   1. The maximum fluorescence intensity is defined as the RFI value of the final cycle of amplification.
   2. To obtain the fluorescence data, follow the steps outlined in **Figure 1**. For more information, consult the operator manual of the respective instrument.
   3. To calculate the FIR value in %, divide the RFI value for a Zika Assay Positive specimen by the corresponding RFI value of the Positive Control, then multiply by 100.

      FIR (%) = (RFI-specimen / RFI-Positive Control) x 100
   4. For a specimen with FIR ≥ 10%, the result of the Zika Assay is considered valid. The interpretation of the specimen is “Positive for Zika Virus”.
   5. For a specimen with FIR < 10%, the result of the Zika Assay is considered invalid. The interpretation of the specimen is “Indeterminate”. To facilitate the interpretation, refer to **Figure 2** below which provides: a) the maximum fluorescence intensity (defined as FIR = 100%), as determined by the Positive Control; b) an example of an Indeterminate sample with FIR < 10%; and c) close-up of an Indeterminate sample and the Negative RT-PCR Control.

e. An **Indeterminate** specimen result requires the following interpretation:

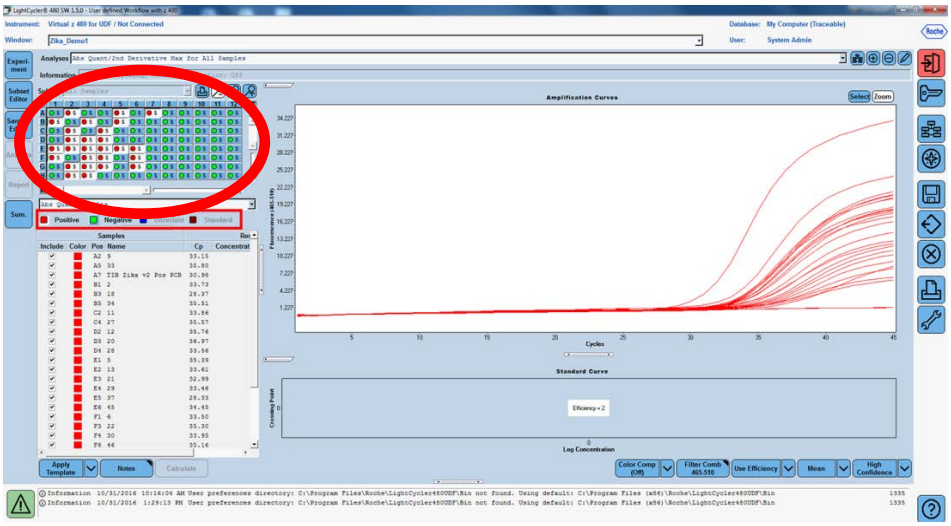
   1. If the Zika Virus Assay is reported as uncertain or invalid, re-test the extracted specimen and controls and re-analyze.
      - If the uncertain or invalid assay result is repeated, re-test with the specimens re-extracted or request a new sample.
      - If the uncertain or invalid assay result is repeated once again, the final interpretation of the specimen is “Indeterminate”. Please refer to note f below.

2. If the Zika Virus Assay is reported as negative AND the RPC Assay is reported as negative, re-test the extracted specimen: 1) undiluted and 2) at a 1:10 dilution (using nuclease-free PCR-grade water).
    - Valid positive or negative re-test results from the undiluted specimen will be taken as the final result.
    - Valid positive Zika Virus Assay results from the 1:10 dilution will also be considered the final result.
    - Any negative RPC Assay result from the 1:10 dilution requires the original clinical specimen to be re-extracted and re-tested.

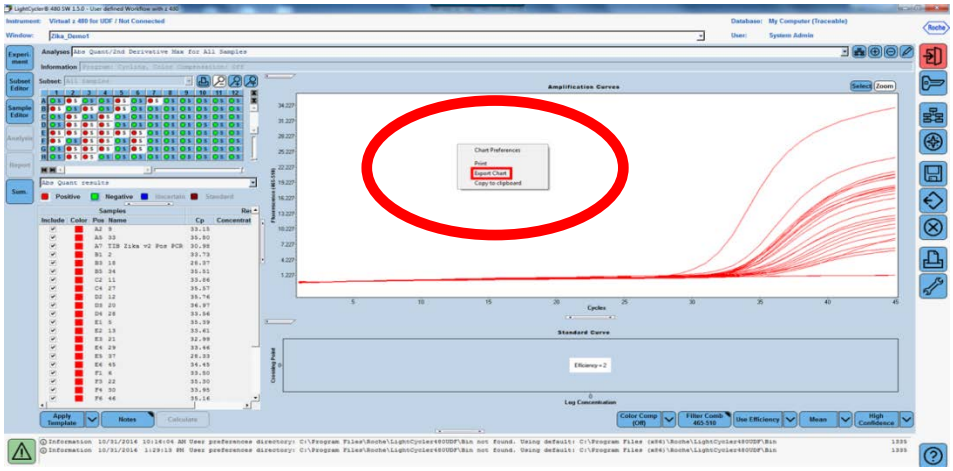
f. A **Negative for Zika Virus** result requires the following action: a patient matched serum specimen is required for serological follow up testing of negative results, per the CDC testing algorithm. (Found at http://www.cdc.gov/zika/index.html). It is recommended that any specimen with an Indeterminate final result is also followed with the same serological testing.

Figure 1 Obtaining Fluorescence Data

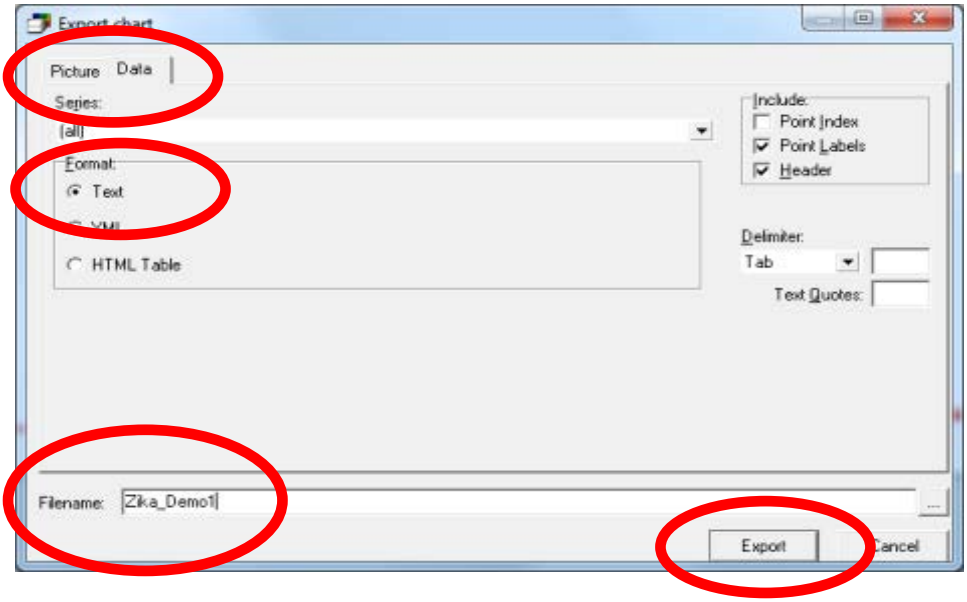
a) After calculating the run results using the 2nd Derivative Max algorithm, click the colored boxes on the left side of the software screen on LightCycler® 480 or **cobas z** 480 so that only the red Positive box is selected, as shown below. This will select the Positive Control reaction, as well as all samples called positive.



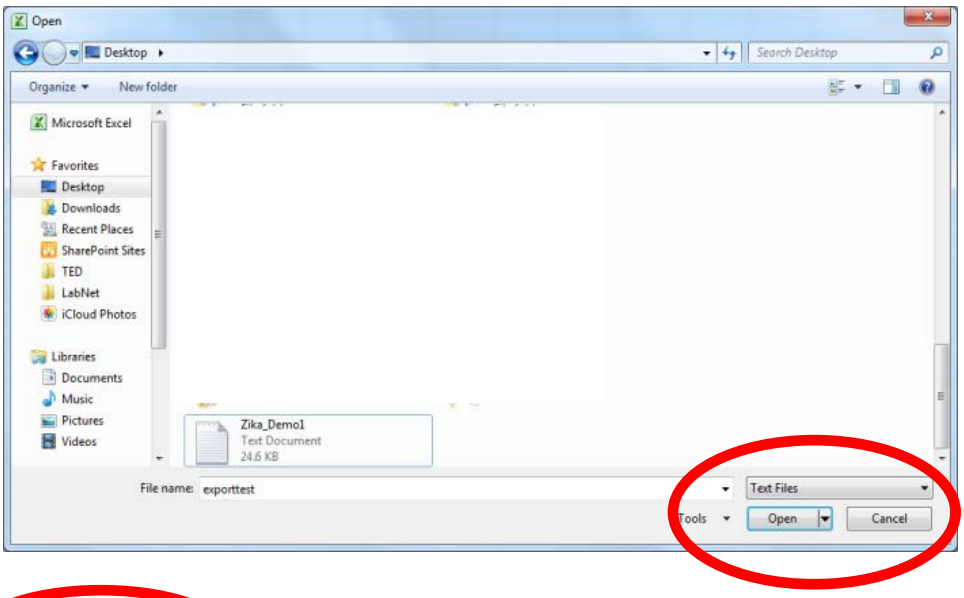
b) Right-click on the Amplification Curves window and select Export Chart:

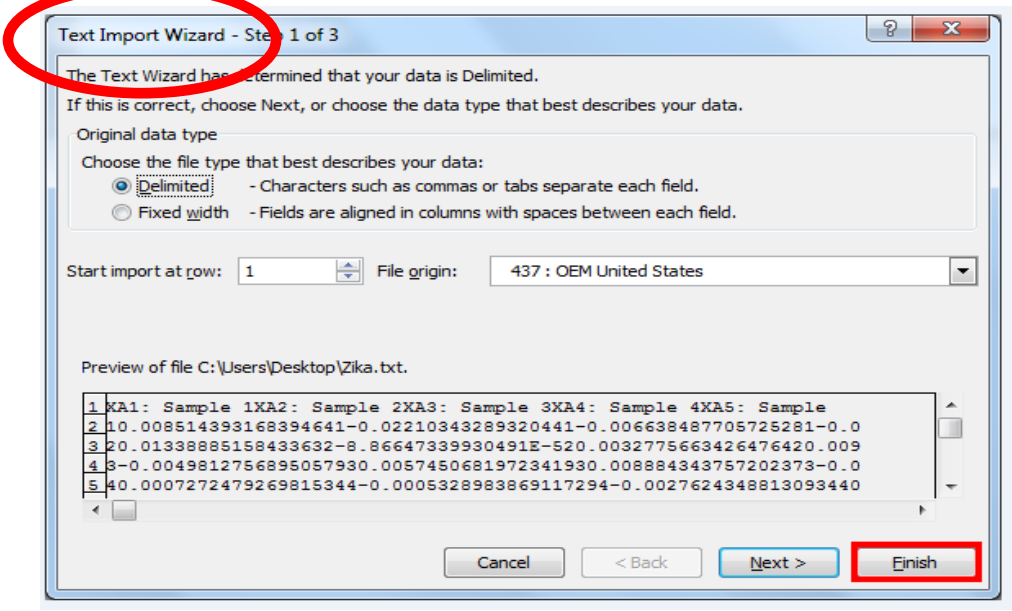


c) Choose the Data tab and the Text radio button. At the bottom of the window, choose a file path, enter a file name and press Export to save the run data as a tab-delimited text file

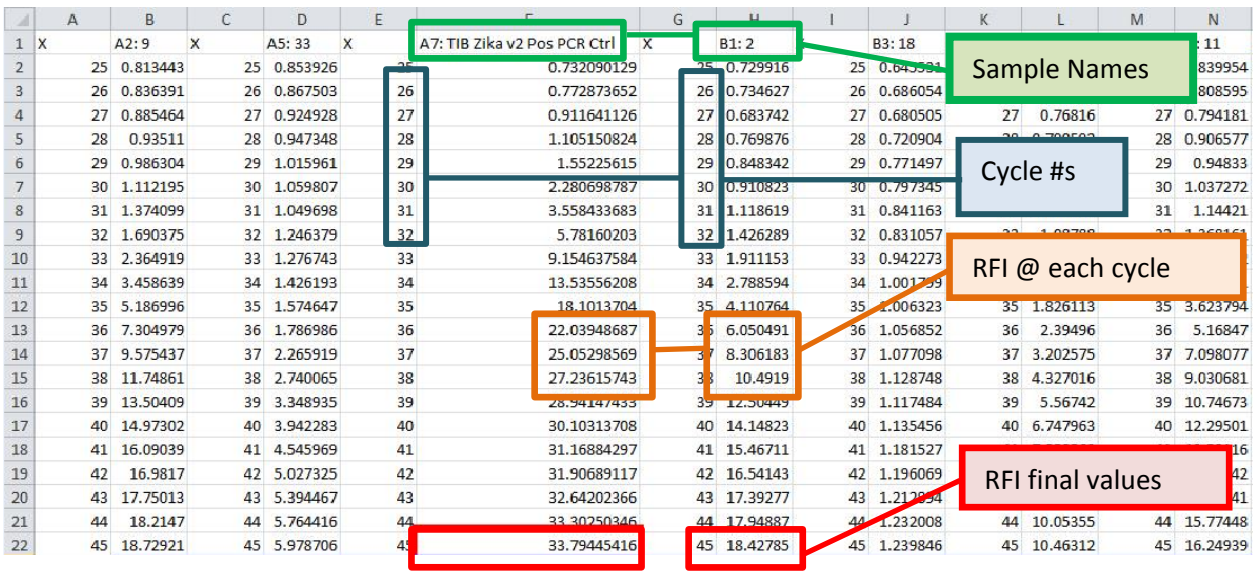


d) Open the new text file with appropriate software (e.g., Microsoft Excel). If using Excel, be sure to select 'Text Files' from the file type dropbox. Click 'Open'; the Text Import Window will open in Excel. Click 'Finish' to proceed.





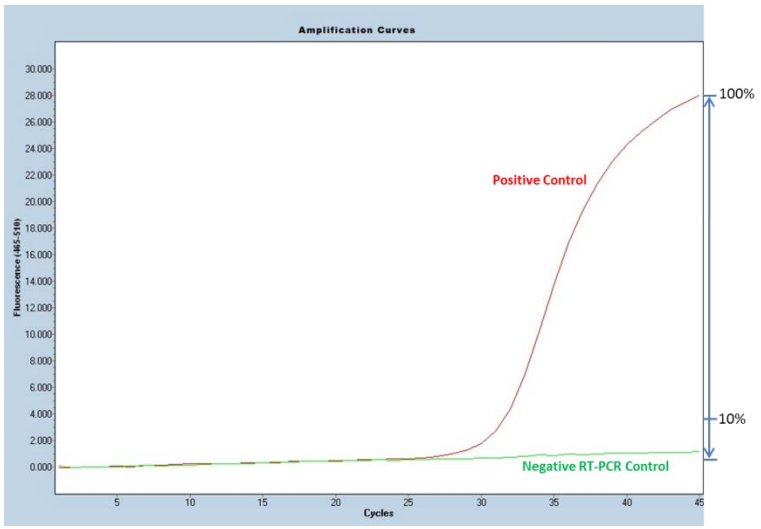
e) A multi-column spreadsheet will be displayed. The sample name is in Row 1 and the numerical values underneath correspond to the RFI at each cycle of the run. The value at the final cycle is the RFI for the FIR calculation.



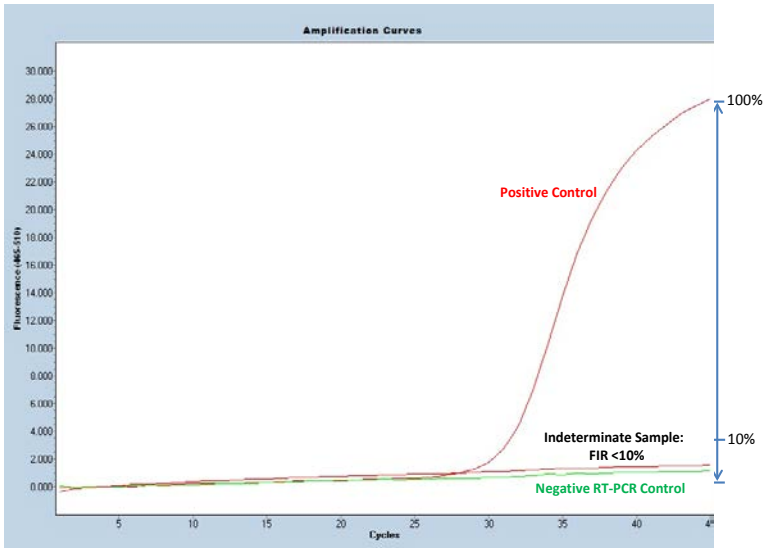
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	X	A2: 9	X	A5: 33	X	A7: TIB Zika v2 Pos PCR Ctrl	X	B1: 2		B3: 18				11
2	25	0.813443	25	0.853926	25	0.732090129	25	0.729916	25	0.645331				839954
3	26	0.836391	26	0.867503	26	0.772873652	26	0.734627	26	0.686054				808595
4	27	0.885464	27	0.924928	27	0.911641126	27	0.683742	27	0.680505	27	0.76816	27	0.794181
5	28	0.93511	28	0.947348	28	1.105150824	28	0.769876	28	0.720904			28	0.906577
6	29	0.986304	29	1.015961	29	1.55225615	29	0.848342	29	0.771497			29	0.94833
7	30	1.112195	30	1.059807	30	2.280698787	30	0.910823	30	0.797345			30	1.037272
8	31	1.374099	31	1.049698	31	3.558433683	31	1.118619	31	0.841163			31	1.14421
9	32	1.690375	32	1.246379	32	5.78160203	32	1.426289	32	0.831057				
10	33	2.364919	33	1.276743	33	9.154637584	33	1.911153	33	0.942273				
11	34	3.458639	34	1.426193	34	13.53556208	34	2.788594	34	1.001759				
12	35	5.186996	35	1.574647	35	18.1013704	35	4.110764	35	1.006323	35	1.826113	35	3.623794
13	36	7.304979	36	1.786986	36	22.03948687	36	6.050491	36	1.056852	36	2.39496	36	5.16847
14	37	9.575437	37	2.265919	37	25.05298569	37	8.306183	37	1.077098	37	3.202575	37	7.098077
15	38	11.74861	38	2.740065	38	27.23615743	38	10.4919	38	1.128748	38	4.327016	38	9.030681
16	39	13.50409	39	3.348935	39	28.94147433	39	12.50445	39	1.117484	39	5.56742	39	10.74673
17	40	14.97302	40	3.942283	40	30.10313708	40	14.14823	40	1.135456	40	6.747963	40	12.29501
18	41	16.09039	41	4.545969	41	31.16884297	41	15.46711	41	1.181527				16
19	42	16.9817	42	5.027325	42	31.90689117	42	16.54143	42	1.196069				42
20	43	17.75013	43	5.394467	43	32.64202366	43	17.39277	43	1.212994				41
21	44	18.2147	44	5.764416	44	33.30250346	44	17.94887	44	1.232008	44	10.05355	44	15.77448
22	45	18.72921	45	5.978706	45	33.79445416	45	18.42785	45	1.239846	45	10.46312	45	16.24939

Figure 2 Example of Indeterminate Sample Determination

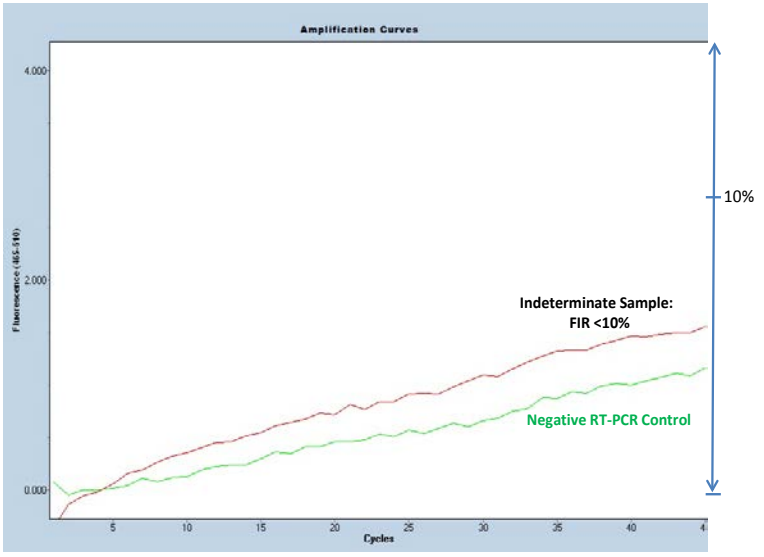
a) Maximum fluorescence intensity (FIR = 100%) as determined by the Positive Control



b) Example of an Indeterminate sample with FIR < 10%



c) Close-up of the Indeterminate sample and the Negative RT-PCR Control



10. Assay Limitations

- All results should be interpreted by a trained professional in conjunction with the patient's history and clinical signs and symptoms, and epidemiological risk factors.
- Interpretation of results from the ***LightMix® Zika rRT-PCR Test*** must account for the possibility of false-negative and false-positive results.
- Negative results do not preclude infection with Zika virus and should not be the sole basis of a patient treatment/management or public health decision.
- **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).**
- False positive results may occur from cross-contamination by target organisms, their nucleic acids or amplified product.
- Failure to follow the assay procedures may lead to false negative results.
- Improper collection, storage, or transport of specimens may lead to false negative results.
- Specimen collection conducted prior to symptom onset may lead to false negative results.
- Specimen collection after nucleic acid can no longer be found in the patient (approximately 7 days post-onset of symptoms for sera) may lead to false negative results.
- Inhibitors present in the samples may lead to false negative results.
- The test is not validated as a quantitative test for treatment monitoring.
- Performance of this assay has only been established for serum and EDTA plasma. Performance with other specimen types has not been evaluated.
- Potential mutations within the target regions of the virus genome covered by the primer and/or probes of the test may result in failure to detect the presence of the pathogen.

11. Performance Characteristics

Analytical Sensitivity/Limit of Detection (LoD)

The analytical sensitivity of the ***LightMix® Zika rRT-PCR Test*** was assessed by determination of limit of detection (LoD) through two steps: first an initial range finding study, followed by a final confirmation. The viral material used in the study (Zika virus, strain H/PF/2013) was obtained from the European Virus Archive (EVA, Ref-SKU: 001v-EVA1545). No viral inactivation step was performed.

Initial range finding study: The initial range finding study to determine a tentative LoD was performed only on the primary workflow. Virus strain from EVA was spiked into EDTA plasma at the highest node. The virus strain was then serially diluted in EDTA plasma in a total of 11 dilution nodes. Sample preparation was performed with the MagNAPure 96 DNA and Viral NA Large Volume Kit on the MagNA Pure 96 Instrument. Two extractions were performed per dilution node. Each isolation of the 11 dilution nodes was tested in triplicate RT-PCR amplifications using the LightCycler® 480 Instrument II, yielding 6 results per dilution node. The results of the initial testing are shown in Table 4 below. The lowest concentration at which all six replicates (6/6) samples were positive was treated as the tentative LoD. The tentative LoD was determined to be 1.81 $TCID_{50}$/mL.

Table 4 Limit of Detection, Initial Estimation - MagNA Pure 96, LightCycler 480 Workflow

Dilution Factor from Stock	Concentration ($TCID_{50}$/mL)*	GC/mL***	GC /RXN**	Zika Positive Result	Average Cp	Cp-SD	Hit Rate
5.00E+05	28.9	5780	145	6/6	31.8	0.11	100%
1.00E+06	14.5	2890	72	6/6	32.7	0.10	100%
2.00E+06	7.23	1450	36	6/6	33.3	0.34	100%
4.00E+06	3.61	723	18	6/6	33.9	0.23	100%
8.00E+06	**1.81**	**361**	**9**	**6/6**	**34.6**	0.50	**100%**
1.60E+07	0.90	181	4.5	4/6	35.5	0.18	67%
3.20E+07	0.45	90	2.3	4/6	35.5	0.28	67%
6.40E+07	0.23	45	1.1	2/6	35.9	0.14	33%
1.28E+08	0.11	23	0.6	1/6#	35.5	N/A	17%
2.56E+08	0.06	11	0.3	0/6	N/A	N/A	0%
5.12E+08	0.03	6	0.1	0/6	N/A	N/A	0%

** The Concentration at each dilution node is calculated by dividing the EVA reported $TCID_{50}$/ml by the dilution factor. 50% Tissue Culture Infective Dose ($TCID_{50}$) is the measure of infectious virus titer. European Virus Archive, Zika Virus Strain H/PF/2013: $TCID_{50}$/ml=10^7.16.*

*** 1 $TCID_{50}$/mL is estimated to represent 5 Genome Copies (GC) per RT-PCR reaction, determined by comparison of the Cp values of pre-determined quantities of the Positive Control and the Zika Virus Strain H/PF/2013 EVA in the LoD study.*

**** 1 $TCID_{50}$/mL = 200 GC/mL, determined from the facts that 1 $TCID_{50}$/mL = 5 GC/RXN and that the **Test** workflow uses: 1) Initial specimen volume prepared is 0.5mL of 1mL (factor of 2), 2) 50% sample preparation isolation efficiency is assumed (factor of 2), and 3) 10 % of the sample preparation eluate is utilized in the RT-PCR (factor of 10). Therefore, in the **Test** workflow, 1 TCID50/mL = 5 GC/RXN x 2 x 2 x 10 = 200 GC/mL.*

 # One reaction resulted in an Indeterminate call per FIR evaluation

LoD confirmation study: 20 individual replicates were tested with 6 concentrations around the tentative LoD. The final LoD was determined to be the lowest concentration resulting in positive detection of at least 95% (19/20) of true positive replicates.

The LoD confirmation studies were performed first on the workflow MagNA Pure 96 + LightCycler 480 (Table 5) to establish the claimed Limit of Detection of the ***Test***. Then, this claimed LoD level was confirmed on the alternative workflows: MagNA Pure 96 + **cobas z** 480 (Table 6), and MagNA Pure Compact + LightCycler 480 (Table 7).

Table 5 Limit of Detection, Confirmation - MagNA Pure 96, LightCycler 480 Workflow

Concentration ($TCID_{50}$/mL)	GC/mL	GC /RXN	Zika Positive Result	Average Cp	Cp-SD	Hit Rate
7.23	1450	36	20/20	35.3	0.45	100%
3.61	723	18	20/20	36.8	0.82	100%
1.81	361	9	20/20	37.0	0.58	100%
0.9	**181**	**4.5**	**19/20**	**37.5**	**0.47**	**95%**
0.45	90	2.3	7/20	37.3	0.40	35%
0.23	45	1.1	9/20	38.2	0.49	45%

Table 6 Limit of Detection, Confirmation - MagNA Pure 96, cobas z 480 Workflow

Concentration ($TCID_{50}$/mL)	GC/mL	GC /RXN	Zika Positive Result	Average Cp	Cp-SD	Hit Rate
7.23	1450	36	20/20	34.5	0.45	100%
3.61	723	18	20/20	35.7	0.43	100%
1.81	**361**	**9**	**20/20**	**36.2**	**0.44**	**100%**
0.9	181	4.5	16/20	36.4	0.40	80%
0.45	90	2.3	11/20	38.0	0.70	55%
0.23	45	1.1	5/20	38.0	0.20	25%

Table 7 Limit of Detection, Confirmation - MagNA Pure Compact, LightCycler 480 Workflow

Concentration ($TCID_{50}$/mL)	GC/mL	GC /RXN	Zika Positive Result	Average Cp	Cp-SD	Hit Rate
1.81	361	9	19/20	34.9*	0.44	95%
0.9	**181**	**4.5**	**19/20**	**38.0**	**0.94**	**95%**
0.45	90	2.3	16/20	35.9*	0.43	80%

** These reactions were conducted in a separate run from the 0.9 $TCID_{50}$/mL reactions. The two runs utilized separate dilutions of the Zika virus and different manufacturing lots of the MagNA Pure Compact Kits. All controls for both runs were valid.*

The claimed Limit of Detection of the Test is 0.9 $TCID_{50}$/mL or 1.81 GC/mL, equivalent in all 3 workflows. LoDs are considered equivalent within a 1-3xLoD range.

Analytical Sensitivity – FDA Reference Materials

An analytical study was performed using FDA reference materials (S1 and S2) following a standard protocol provided by the FDA, which includes a LoD range finding study and a confirmatory LoD study, to evaluate the analytical sensitivity of the ***Test*** using the workflow: MagNA Pure 96 + LightCycler 480. The results are presented in Table 8 below:

Table 8 Summary of LoD Confirmation Results Using the FDA Reference Materials

Reference Material	Specimen Type	Confirmed LOD* in RNA NAAT Detectable Units/mL
S1	EDTA plasma	1×10^4
S2	EDTA plasma	5×10^3

**Study performed according to an FDA issued protocol*

Analytical Specificity

a) Analytical Reactivity

Reactivity of the ***LightMix® Zika rRT-PCR Test*** with additional isolates of Zika virus was assessed by conducting *in silico* analysis on the Zika primers and probes from the ***Test*** for such reactivity.

Additional Zika virus sequences from the virus database have been aligned to the ***Test*** primer and probe sequences to demonstrate coverage of the ***Test*** to a broad range of known Zika virus strains. Results are summarized in Table 9. The ***Test*** primers include no known mismatches to database sequences when primer wobble positions are considered, and the ***Test*** probe shows a maximum of one mismatch (in 3 cases) to the additional virus sequences analyzed. It was demonstrated using the EVA reference strain that a single mismatch under the probe does not negatively influence the sensitivity of the ***Test***.

Table 9 Reactivity of Zika Strains

GenBank Acct#	Strain Location	Forward Primer	Probe	Reverse Primer
AY632535.2	Uganda	100%	100%	100%
NC_012532.1	Uganda	100%	100%	100%
HQ234498.1	Uganda	100%	100%	100%
KF383121.1	(Unknown)	100%	100%	100%
KF383119.1	(Unknown)	100%	100%	100%
KF383118.1	(Unknown)	100%	100%	100%
LC002520.1	Uganda	100%	100%	100%
KU720415.1	Uganda	100%	100%	100%
DQ859059.1	Uganda	100%	100%	100%
KF383117.1	(Unknown)	100%	100%	100%
KU681082.3	Philippines	100%	100%	100%
EU545988.1	Micronesia	100%	100%	100%
JN860885.1	Cambodia	100%	100%	100%
HQ234501.1	Senegal	100%	100%	100%
KF268949.1	Central African Republic	100%	100%	100%
KF383120.1	(Unknown)	100%	100%	100%
KF383116.1	(Unknown)	100%	100%	100%
KF993678.1	Canada	100%	100%	100%
KU321639.1	Brazil	100%	100%	100%
KU312312.1	Suriname	100%	100%	100%
KU365780.1	Brazil	100%	100%	100%
KU365779.1	Brazil	100%	100%	100%
KU365778.1	Brazil	100%	100%	100%
KU365777.1	Brazil	100%	100%	100%
KU501217.1	Guatemala	100%	100%	100%
KU501216.1	Guatemala	100%	100%	100%

GenBank Acct#	Strain Location	Forward Primer	Probe	Reverse Primer
KU501215.1	Puerto Rico	100%	100%	100%
KU647676.1	Martinique	100%	100%	100%
KU509998.1	Haiti	100%	100%	100%
KU681081.3	Thailand	100%	100%	100%
KU527068.1	Brazil: Rio Grande do Norte, Natal	100%	100%	100%
KU707826.1	Brazil	100%	100%	100%
KU497555.1	Brazil	100%	100%	100%
KU740184.1	China	100%	100%	100%
KU744693.1	China	100%	100%	100%
KU761564.1	China	100%	100%	100%
KU729218.1	Brazil	100%	100%	100%
KU729217.2	Brazil	100%	100%	100%
KU820899.1	China	100%	100%	100%
KU820897.1	Colombia: Barranquilla	100%	100%	100%
HQ234500.1	Nigeria	100%	96%	100%
HQ234499.1	Malaysia	100%	96%	100%
KF268950.1	Central African Republic	100%	100%	100%
KF268948.1	Central African Republic	100%	100%	100%
KF383115.1	(Unknown)	100%	100%	100%
KJ776791.1	French Polynesia	100%	96%	100%

Additionally the following Zika virus isolates were wet-tested with the ***Test*** as part of the FDA Analytical Sensitivity evaluation:

PRVABC59: Puerto Rico, 2015 provided by CDC. GenBank #KU501215
FSS13025: Cambodia, 2010 provided by UTMB. GenBank #JN860885

b) Cross Reactivity

Cross-reactivity of the ***LightMix® Zika rRT-PCR Test*** has been evaluated by testing additional organisms that (1) produce symptoms similar to Zika virus; or (2) have a significant likelihood to result in cross-reactivity due to genetic similarity with Zika virus; and are likely to be observed in the currently affected area (South America, including Brazil; Central America and the Caribbean).

High concentration extracts containing genomic RNA or DNA from various organisms were analyzed using the ***Test*** with the LightCycler® 480 Instrument II. All tested organisms showed negative reactivity with the ***Test***. Results are summarized below in Table 10.

Table 10 Cross-Reactivity (Wet Tested)

Organism	Strain	Source / Sample Type	GC*/ml	Reference Cp Value	Zika rRT-PCR Cp Value
Dengue virus 1	VR344 (Thai 1958 strain)	RNA	3.30E+08	20.92	Neg
Dengue virus 2	VR345 (TH-36 strain)	RNA	1.10E+08	22.54	Neg
Dengue virus 3	VR216 (H87 strain)	RNA	3.67E+08	20.76	Neg
Dengue virus 4	VR217 (H241 strain)	RNA	4.53E+08	20.45	Neg
Yellow fever Brazil strain	17D RKI #142/94/1	RNA	2.49E+08	21.33	Neg
Yellow fever 17D vaccine strain	Trinidad 79A 788379	RNA	7.47E+07	23.12	Neg
West Nile virus lineage 1	Lineage 1 NY99	RNA	9.19E+07	22.81	Neg
West Nile virus lineage 2	Lineage 2 goshawk-Hungary/04	RNA	2.54E+09	17.89	Neg
Saint Louise Encephalitis virus	ATCC VR 1265	RNA	1.93E+08	21.71	Neg
Usutu virus	ArB1803/ IPD Dakar	RNA	1.66E+09	18.52	Neg
Sindbis virus	Edgar 339 /ENIVD EQA	RNA	7.50E+06	N.A.	Neg

Organism	Strain	Source / Sample Type	GC*/ml	Reference Cp Value	Zika rRT-PCR Cp Value
ChikV African isolate	S27-African prototype	RNA	6.57E+09	16.89	Neg
ChikV LR2006	LR 2006	RNA	3.16E+09	17.95	Neg
ChikV St Martin	H20235 Saint Martin/2013	RNA	1.42E+09	19.10	Neg
Mayaro virus	Institut Pasteur Paris	RNA	1.0E+06**	N.A.	Neg
Parvovirus (B19)	Instand EQA sample	RNA	2.68E+05	30.46	Neg
Plasmodium falciparum	Clinical sample	DNA	1.50E+09	15.00	Neg

** GC = genome copies, as determined by correlation to the Positive Control, quantitated by OD 260 measurement.*
***Plaque Forming Unit/ml (PFU/ml)*
N.A. : Not Available
Neg : Negative

In silico analysis: The organisms wet-tested above have been analyzed *in silico* (BLAST analysis of the Forward and Reverse Primers versus Zika Virus Strain H/PF/2013 sequence). All analyzed organism sequences showed negative reactivity with the ***Test***, allowing no amplicons to be produced. The results are summarized below in Table 11.

Table 11 *In silico* Analysis of Cross Reactivity (Wet Tested Organisms)

Organism	Strain	Taxonomy ID	GenBank ID	Forward Primer	Probe	Reverse Primer
Dengue virus 1	VR344 (Thai 1958 strain)	11053	X69395.1, X69396.1, D10513.1, X70952.1	33%	34%	45%
Dengue virus 2	VR345 (TH-36 strain)	11060	D10514.1, X69191.1, X72849.1,	45%	34%	40%
Dengue virus 3	VR216 (H87 strain)	11069	KU050695.1	41%	43%	45%
Dengue virus 4	VR217 (H241 strain)	11070	KR011349.2	41%	43%	40%
Yellow fever Brazil strain	17D RKI #142/94/1	40005	JN628279.1	37%	39%	40%
Yellow fever 17D vaccine strain	Trinidad 79A 788379	40005	X03700.1	37%	39%	40%
West Nile virus lineage 1	Lineage 1 NY99	11082	DQ211652.1	37%	43%	45%
West Nile virus lineage 2	Lineage 2 goshawk-Hungary/04	11082	DQ116961.1	63%	57%	59%
Saint Louise Encephalitis virus	ATCC VR 1265	11080	EF158070.1	66%	60%	68%
Usutu virus	ArB1803/ IPD Dakar	64286	KC754958.1	66%	58%	72%
Sindbis virus	Edgar 339 /ENIVD EQA	11034	Specific strain not in Genbank	0%	47%	0%
ChikV African isolate	S27-African prototype	37124	NC_004162	66%	56%	63%
ChikV LR2006	LR 2006	37124	KT449801.1	66%	60%	63%
ChikV St Martin	H20235 Saint Martin/2013	37124	[a] Sequence not in Genbank	37%	39%	45%
Mayaro virus	Institut Pasteur Paris	59301	Specific strain not in Genbank	54%	47%	0%
Parvovirus (B19)	Instand EQA sample	10798	Specific strain not in Genbank	0%	0%	0%
Plasmodium falciparum	Clinical sample	5833	Specific strain not in Genbank	0%	0%	72%

[a] *Sequence not in Genbank: http://www.european-virus-archive.com/viruses/chikungunya-virus-strain-h20235stmartin2013*

Additionally the following organisms have been analyzed *in silico*. The sequences of the ***Test*** oligonucleotides were blasted against the organisms. No reactivity was identified that could lead to undesired amplification of potentially cross-reacting target sequences. The results are summarized below in Table 12.

Table 12 *In silico* Analysis of Cross Reactivity Against All Available Sequences in the Database

Organism	Taxonomy ID	Overall Homology		
		Forward Primer	Probe	Reverse Primer
Dengue virus 1	11053	0%	0%	59%
Dengue virus 2	11060	0%	0%	59%
Dengue virus 3	11069	54%	0%	54%
Dengue virus 4	11070	50%	0%	54%
Chikungunya virus (Asian, East/Central/South African (ECSA) and West African)	37124	0%	0%	0%
West Nile virus	11082	0%	52%	54%
Yellow fever virus	11089	45%	52%	54%
Japanese encephalitis virus	11072	0%	52%	54%
St. Louis encephalitis virus	11080	45%	43%	50%
Murray Valley encephalitis virus	11079	45%	43%	54%
Ebola Virus (SEBOV Gulu, ZEBOV Mayinga)	1570291	0%	56%	41%
Marburg virus (MARV Musoke)	11269	45%	47%	59%
Usutu virus (Seq homology with the target region for with Altona Zika test)	64286	0%	0%	50%
Plasmodium (vivax, falciparum,ovale)	5820	0%	0%	72%
Hepatitis A Virus (HAV)	208726	0%	43%	0%
Hepatitis B Virus (HBV)	10407	0%	0%	59%
Hepatitis C Virus (HCV)	11103	62%	60%	0%
Human Immunodeficiency Virus (HIV-1 Group M)	388795	58%	0%	50%
Influenza virus A	41857	41%	43%	45%
Rubella virus	11041	0%	0%	0%
Toxoplasma gondii	5811	70%	69%	77%
Leptospira	171	0%	0%	77%
Treponema pallidum	160	0%	56%	63%
Rickettsia (conorii, prowazekii, typhi)	780	62%	0%	72%
Trypanosoma cruzi	5693	83%	65%	68%
Leishmania (donovani/infatum/chagasi)	38568	66%	65%	0%
Salmonella typhi	90370	0%	82%	0%
Cytomegalovirus (CMV)	10358	54%	56%	72%
Adenovirus C5	28285	0%	0%	45%
Epstein-Barr Virus (EBV)	10376	0%	0%	0%
Varicella Zoster Virus (VZV)	10335	0%	0%	59%
Herpes Simplex Virus Type I (HSV-1)	10298	0%	0%	59%
Herpes Simplex Virus Type II (HSV-2)	10310	0%	0%	59%
Human T-cell Lymphotropic Virus Type I (HTLV I)	11908	45%	47%	50%
Human T-cell Lymphotropic Virus Type II (HTLV II)	11909	0%	43%	0%
Hepatitis E Virus (HEV)	12461	0%	56%	54%
Human Herpes Virus 6B (HHV-6)	32604	45%	0%	59%
Hepatitis G Virus (GBV_C)	54290	50%	52%	50%
Human Immunodeficiency Virus (HIV-2)	11709	0%	52%	54%
Candida albicans	5476	62%	0%	72%
Staphylococcus aureus	1280	66%	0%	77%
Staphylococcus epidermis	1282	70%	0%	72%
Staphylococcus haemolyticus	1283	70%	0%	72%
Streptococcus viridans	78535	41%	39%	50%

Organism	Taxonomy ID	Overall Homology		
		Forward Primer	Probe	Reverse Primer
Propionibacterium acnes	1747	0%	0%	0%
Escherichia coli	562	0%	82%	86%
Spondweni virus	64318	58%	39%	40%
Eastern Equine Encephalitis Virus (EEE)	11021	0%	0%	54%
Western Equine Encephalitis Virus (WEE)	11039	45%	0%	54%
Ross River virus	11029	0%	43%	45%
Barmah Forest virus	11020	41%	39%	45%
O'nyong-nyong virus	11027	37%	47%	45%
Tonate virus	60877	33%	30%	40%
Una virus	59304	45%	39%	40%
Measles virus	11234	0%	0%	59%
Enterovirus all serotypes	12059	54%	0%	72%
Adenovirus–all serotypes (members of the family *Adenoviridae*)	10508	54%	47%	54%
Borrelia burgdorferi	139	0%	0%	63%
Schistosomiasis	6181	83%	69%	81%
Group A *Streptococcus*	36470	33%	34%	45%

Potential cross-reactivity of the ***LightMix®* *Zika rRT-PCR Test*** has been further re-assessed by evaluating additional sequence alignments of a selected group of organisms against the ***Test*** oligos in an additional BLAST analysis, querying a smaller database which contains only the top hit sequences of the particular organism, and the E-value thus significantly decreased as compared to the values in Tables 11 and 12. (The lower the E-value, the more significant the score and the alignment.) Predicted for each oligo set is the smallest possible predicted amplicon size that could be generated in PCR amplification. Any oligonucleotide less than 40% homologous to the target sequence is not expected to bind. Results are provided in Table 13.

Table 13 Additional *in silico* Analysis of Cross Reactivity

Organism	Taxonomy ID	Overall Homology			Smallest Possible Amplicon Predicted
		Forward Primer	Probe	Reverse Primer	
Schistosomiasis	6181	83%	69%	81%	**155730 bp**
Enterovirus all serotypes	12059	54%	56%	72%	**3119 bp**
Cytomegalovirus (CMV)	10358	83%	56%	72%	**NONE**
Rickettsia sp.	780	62%	52%	72%	**690012 bp**
Leptospirosis	171	66%	43%	77%	**NONE**
Plasmodium sp.Plasmodium vivax	5820	45%	47%	72%	**NONE**
Trypanosoma cruzi (Chagas)	5693	83%	65%	68%	**NONE**
Toxoplasma gondii	5811	79%	69%	77%	**5905443 bp**
Candida albicans	5476	62%	65%	72%	**75499 bp**
Staphylococcus aureus	1280	75%	60%	77%	**31647 bp**
Staphylococcus epidermis	1282	70%	52%	72%	**10957 bp**
Staphylococcus haemolyticus	1283	70%	56%	72%	**NONE**
Escherichia coli	562	66%	82%	86%	**NONE**

c) Microbial Interference Studies

Due to high homology after the cross-activity re-analysis, the potential effects of interfering organisms on the performance of the ***Test*** were analyzed. The conclusion from the investigation was that oligonucleotides are provided in significant excess in the ***Test*** RT-PCR relative to the clinically relevant levels of the organisms. The presence of cross-reacting organism nucleic acid would not affect the ability of the ***Test*** oligonucleotides to be available to bind with Zika virus nucleic acid.

d) Interference Substances Studies

The nucleic acid extraction and rRT-PCR methods provided in the ***Test*** utilize well-established technologies. Therefore no interference testing was performed.

Sample Matrix Equivalence Study

The equivalency of using serum and EDTA plasma clinical matrices in the ***LightMix® Zika rRT-PCR Test*** has been demonstrated.

A total of 40 paired serum/plasma samples, including 10 negative, 20 low positive (1.8x LoD), and 10 moderate positive (7x LoD) were prepared by spiking individual clinical serum and EDTA plasma isolates (collected from non-Zika endemic regions) with cultured Zika Virus Strain H/PF/2013 (EVA). The paired serum/plasma samples were analyzed using the ***Test*** with the LightCycler® 480 Instrument II. All positive samples for serum and plasma, except one moderate individual plasma sample which dropped out, generated positive results. Similar Cp results were achieved between the serum and plasma matrices at each dilution level, showing equivalency of the clinical sample matrices for the ***Test***. The results are summarized below in Table 14.

Table 14 Sample Matrix Equivalency

	Plasma			Serum		
Name	**Cp Mean**	**Cp SD**	**Hits**	**Cp Mean**	**Cp SD**	**Hits**
Moderate/7x LOD	35.9	0.4	9/10	36.2	0.5	10/10
Low/1.8x LOD	37.9	1.3	20/20	37.4	1.0	20/20
Negative	-	-	0/10	-	-	0/10

Clinical Evaluation

The performance characteristics of the ***LightMix® Zika rRT-PCR Test*** have been established using a clinical study. Two separate sample panels were prepared for the study. The first panel included all natural specimens and the second panel included contrived samples along with natural negative specimens.

Panel 1 (Natural specimens): A total of 161 natural (undiluted) clinical EDTA plasma specimens were included in the study (samples under the groups A and B below were collected from febrile patients in the Zika virus endemic regions of Colombia and El Salvador):

A. 88 clinical samples which were “reactive” by the Roche in-house screening assay (the NS5 assay) and confirmed positive by the **cobas®** Zika Test (under an IND);
B. 23 clinical samples which were “reactive” by the NS5 assay and negative by the **cobas®** Zika Test;
C. 50 clinical samples collected from non-Zika virus endemic regions, presumed to be true Zika negatives.

Panel 2 (Contrived samples): A total of 122 contrived samples and natural specimens were included in the study:

- 72 contrived Zika positive specimens: 18 replicates of 4 levels (1.5x, 2x, 3x, and 5x LoD), each set prepared by spiking Zika Virus Strain H/PF/2013 EVA into one negative plasma specimen and preparing the dilutions. A total of 18 sets of the 4-level contrived samples were prepared using 18 individual negative plasma specimens;
- 50 clinical samples collected from non-Zika virus endemic regions, presumed to be true Zika negatives, non-overlapping with the specimens used in Panel 1 but collected in the same batch as negatives above.

A total of 283 samples from the two sample panels were analyzed retrospectively in a blinded fashion. Samples from both panels have been evaluated with the ***Test*** method according to the Instructions for Use using the primary workflow. Samples from Panel 1 were additionally analyzed with the Comparator method (Lanciotti *et al*, Emerg Infect Dis 2008;14(8):1232-1239) as described in further details below. For the ***Test***, any sample that generated a Cp with a legitimate amplification curve with a FIR ≥ 10% was considered positive; for the Comparator, any sample that generated a Cp with a legitimate amplification curve was considered positive. No cut-off value was used for either method.

The results of the clinical performance evaluation are summarized in Table 15. The results from the Comparator method are reported as the expected results for the Panel 1 samples. For the Panel 2 samples, all the contrived samples are expected positive and all the natural negative specimens are expected negative. In summary, the ***LightMix® Zika rRT-PCR Test*** demonstrated a positive percent agreement (PPA) of 88.0% (95% CI: 81.7% - 92.4%), and a negative percent agreement (NPA) of 90.8% (95% CI: 84.9% - 94.5%) with the expected results.

Table 15 Summary of Clinical Evaluation Results

Specimen Category	Number Tested	LightMix® Zika rRT-PCR Test		
		Zika Positive	Indeterminate	Zika Negative
Natural Zika Positive Clinical Specimens as Determined by the Zika comparator from Lanciotti	70*	56	0	14**
Contrived Zika Positive Clinical Specimens (1.5X LoD)	18	16	1	1
Contrived Zika Positive Clinical Specimens (2X LoD)	18	18	0	0
Contrived Zika Positive Clinical Specimens (3X LoD)	18	17	1	0
Contrived Zika Positive Clinical Specimens (5X LoD)	18	18	0	0
Zika negative Plasma Endemic Specimens as determined by the comparator from Lanciotti	43*	11***	2	30
Zika negative Plasma Non-endemic Specimens as determined by the comparator from Lanciotti	48****	0	0	48
Expected Zika Negative Non-endemic Clinical Specimens	50*****	0	0	50
Positive Percent Agreement	**88.0% (125/142), 95% CI: 81.7% - 92.4%**			
Negative Percent Agreement	**90.8% (128/141), 95% CI: 84.9% - 94.5%**			

**111/113 specimens were collected in Q4 2015 and Q1 2016 from symptomatic, febrile patients in Colombia and El Salvador. The specimens were confirmed to be Chikungunya and Dengue virus negative, and of unknown Zika status. 2/113 specimens were presumed negatives as they were collected in non-endemic areas in the US but turned out positive when tested by Lanciotti's assay.*
***11/14 of the "False Negative" samples were tested positive and 2/14 of the "False Negative" samples were tested negative by a commercial blood screening **cobas**® Zika Test under an IND from the FDA.*
****8/11 of the "False Positives" samples were tested positive and 3/11 of the "False Positive" samples were tested negative by a commercial blood screening **cobas**® Zika Test under an IND from the FDA.*
*****48/50 presumed negative samples (collected in the US with unknown febrile indication and the following specifications: healthy donors (HxV negative), and geographic restrictions (cannot reside in Florida at the time of collection) were tested negative by Lanciotti's assay.*
****** Presumed negative samples (collected in the US with unknown febrile indication and the following specifications: healthy donors (HxV negative), and geographic restrictions (cannot reside in Florida at the time of collection).*

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13. Version History

V160823	Release Version
V161122	Amendment Update

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