IVD For *in Vitro* Diagnostic Use

CE

Zika Virus Real-TM Handbook

Real-Time PCR test for qualitative detection of Zika Virus

Σ **50**

REF **V73-50FRT**

NAME
Zika Virus Real-TM

INTRODUCTION
Zika virus (ZIKV) is a member of the virus family Flaviviridae and the genus Flavivirus. It is spread by daytime-active Aedes mosquitoes, such as A. aegypti and A. albopictus. Its name comes from the Zika Forest of Uganda, where the virus was first isolated in 1947. It was subsequently identified in humans in 1952 in Uganda and the United Republic of Tanzania. Outbreaks of Zika virus disease have been recorded in Africa, the Americas, Asia and the Pacific.
The incubation period of Zika virus disease is not clear, but is likely to be a few days. The symptoms are similar to other arbovirus infections such as dengue, and include fever, skin rashes, conjunctivitis, muscle and joint pain, malaise, and headache. These symptoms are usually mild and last for 2-7 days.
During large outbreaks in French Polynesia and Brazil in 2013 and 2015 respectively, national health authorities reported potential neurological and auto-immune complications of Zika virus disease. Recently in Brazil, local health authorities have observed an increase in Guillain-Barré syndrome which coincided with Zika virus infections in the general public, as well as an increase in babies born with microcephaly in northeast Brazil. Agencies investigating the Zika outbreaks are finding an increasing body of evidence about the link between Zika virus and microcephaly.
Zika virus is transmitted to people through the bite of an infected mosquito from the Aedes genus, mainly Aedes aegypti in tropical regions. This is the same mosquito that transmits dengue, chikungunya and yellow fever. However, sexual transmission of Zika virus has been described in 2 cases, and the presence of the Zika virus in semen in 1 additional case.
There is no specific treatment or vaccine currently available. The best form of prevention is protection against mosquito bites.
Infection with Zika virus may be suspected based on symptoms and recent history (e.g. residence or travel to an area where Zika virus is known to be present). Zika virus diagnosis can only be confirmed by laboratory testing for the presence of Zika virus RNA in the blood or other body fluids, such as urine or saliva.

INTENDED USE
Zika Virus Real-TM is a Real-Time test for the qualitative detection of Zika Virus RNA in the blood, plasma, urine, saliva, tissue, amniotic liquid. ZIKA RNA is extracted from specimens, amplified using RT-amplification and detected using fluorescent reporter dye probes specific for Zika or Zika IC.

PRINCIPLE OF ASSAY
Zika virus detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region by using specific primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product. The real-time monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening of the reaction tubes after the PCR run. **Zika Virus Real-TM** PCR kit is a qualitative test that contains the Internal Control (IC), which must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition. **Zika Virus Real-TM** PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase by using a chemically modified polymerase (TaqF), which is activated by heating at 95 °C for 15 min.

MATERIALS PROVIDED

Reagent	*Description*	*Volume, ml*	*Quantity*
RT-G-mix-2	colorless clear liquid	0.015	1 tube
RT-PCR-mix-1-FRT *ZIKV*	colorless clear liquid	0.6	1 tube
RT-PCR-mix-2	colorless clear liquid	0.3	1 tube
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
TM-Revertase (MMlv)	colorless clear liquid	0.015	1 tube
Pos Control cDNA/IC ZIKV	colorless clear liquid	0.2	1 tube
RNA-buffer	colorless clear liquid	0.2	1 tube
Pos Control ZIKV-rec	colorless clear liquid	0.1	1 tube
Negative Control (C–)*	straw-colored clear liquid	1.2	1 tube
IC ZIKV-rec**	colorless clear liquid	0.5	1 tube

** must be used in the isolation procedure as Negative Control of Extraction.*

*** add 10 µl of Internal Control (IC) to each sample/control during the RNA purification procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

- RNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers.
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Personal Real Time PCR thermocycler.
- Disposable polypropylene microtubes for PCR or PCR-plate**.**
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**
For *In Vitro* Diagnostic Use Only

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Do not pipette by mouth.
3. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
4. Do not use a kit after its expiration date.
5. Dispose of all specimens and unused reagents in accordance with local regulations.
6. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant.
7. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
8. Material Safety Data Sheets (MSDS) are available on request.
9. Use of this product should be limited to personnel trained in the techniques of DNA amplification.
10. PCR reactions are sensitive to contamination. Measures to reduce the risk of contamination in the laboratory include physically separating the activities involved in performing PCR in compliance with good laboratory practice.
11. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where you performed previous step.

⚠ Sampling of biological materials for PCR-analysis, transportation, and storage are described in details in the handbook of the manufacturer. It is recommended that this handbook is read before beginning of the work.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the in Vitro diagnostic procedures (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

STORAGE INSTRUCTIONS

All components of the **Zika Virus Real-TM** PCR kit (except for RT-G-mix-2, RT-PCR-mix-1-FRT ZIKV, RT-PCR-mix-2, polymerase (TaqF), and TM-Revertase (MMlv)) are to be stored at 2–8 ºC. All components of the **Zika Virus Real-TM** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

RT-G-mix-2, RT-PCR-mix-1-FRT *Zika virus,* RT-PCR-mix-2, polymerase (TaqF), and TM-Revertase (MMlv) are to be stored at ≤ –16 °C

RT-PCR-mix-1-FRT *Zika virus* is to be kept away from light.

Zika Virus Real-TM PCR kit should be transported at 2–8 °C for no longer than 5 days but should be stored at 2-8 and -20°C immediately on receipt.

STABILITY

Zika Virus Real-TM Test is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Zika Virus Real-TM can analyze RNA extracted from:

- *Plasma* (EDTA anticoagulant)
- *Saliva*
- *Tissue*
- *Amniotic liquid*
- *Swabs*
- *Seminal liquid*
- *Urea*
- *Mosquitoes (homogenate)*

Specimens can be stored at +2-8°C for no longer than 12 hours, or frozen at -20°C to -80°C.
Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

RNA ISOLATION

Sacace Biotechnologies recommends to use the following kits:

⇒ **Ribo-Virus** (Sacace, REF K-2/C) (150 *µl* of sample)
⇒ **DNA/RNA prep** (Sacace, K-2-9) (100 *µl* of sample)
⇒ **Magno Virus** (Sacace, K-2-16/1000) for plasma and cell-free body fluids (1000 *µl* of sample)
⇒ **SaMag Viral Nucleic Acids Extraction Kit** (Sacace, REF SM003) for plasma and cell free body fluids (400 *µl* of sample);

Please carry out the RNA extraction according to the manufacturer's instructions.
During extraction, use the following controls:

- Internal Control ZIKV-rec: *add 10 µl of IC RNA during the RNA isolation procedure directly to the sample/lysis mixture for all samples and controls;*
- Negative Control (C–): *add 100* (400*) µl of Negative Control (C–) to the tube labeled Negative Control of Extraction;*
- Positive Control ZIKV-rec: *add 90 (390) µl of Negative Control (C-) and 10 µl of Positive Control ZIKV-rec to the tube labeled Positive Control of Extraction.*

REAGENTS PREPARATION (REACTION VOLUME 25 µL):

1. Prepare required quantity of reaction tubes.
2. Prepare for each sample in the new sterile tube **Reaction Mix**: add **10 µl** of **RT-PCR-mix-1**, **5 µl** of **RT-PCR-mix-2, 0,25 µl** of **RT-G-mix-2**, **0,50 µl** of **TaqF Polymerase** and **0,25 µl** of **M-MLV Revertase**. Vortex thoroughly and centrifuge for 5 sec. This mix must be used immediately. Don't store the prepared mix!

Reagents volume x 1 reaction (µl)		**10,0**	**5,00**	**0,25**	**0,50**	**0,25**
N RNA samples[1]	N reactions[2]	RT-PCR-mix-1	RT-PCR-mix-2	RT-G-mix-2	TaqF Polymerase	M-MLV Revertase
4	6	60	30	1,5	3,0	1,5
6	8	80	40	2,0	4,0	2,0
8	10	100	50	2,5	5,0	2,5
10	12	120	60	3,0	6,0	3,0
12	14	140	70	3,5	7,0	3,5
...58	60	600	300	15,0	30,0	15,0

[1] specimens plus 1 extraction control (N+1)
[2] specimens plus extraction and amplification controls (N+1+2)

3. Add **15 µl** of **Reaction Mix** into each tube.
4. Add **10 µl** of **extracted RNA** sample including Positive and Negative Controls of Extraction to appropriate tubes with Reaction Mix and mix well by pipetting.
5. Prepare for each panel 2 controls of amplification:
   - NCA: add **10 µl** of **RNA-buffer** to the tube labeled Negative Control of Amplification;
   - C+: add **10 µl** of **Pos Control cDNA/IC ZIKA** to the tube labeled Positive Control of Amplification;

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

Zika cDNA is detected on the JOE(Yellow)/HEX channel, IC DNA on the FAM (Green) channel

AMPLIFICATION

Program the real-time instrument according to manufacturer's manual.

Amplification program*

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Repeats***
1	50	15 min	–	1
2	95	15 min	–	1
3	95	10 sec	–	45
	55	25 sec **	**FAM/Green, JOE/Yellow/HEX**	

**For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen) , SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid)*
*** when using ABI 7300/7500/StepOne instruments set fluorescence acquisition time to 30 seconds*

Rotor-type instruments

Settings

Channel	**Calibrate/Gain Optimisation…**	**Threshold**	**More Settings/ Outlier Removal**	**Slope Correct**	**Eliminate Cycles before**
FAM/Green	*from 5 Fl to 10 Fl*	0.03	5 %	On	5
JOE/Yellow	*from 5 Fl to 10 Fl*	0.03	5 %	On	5

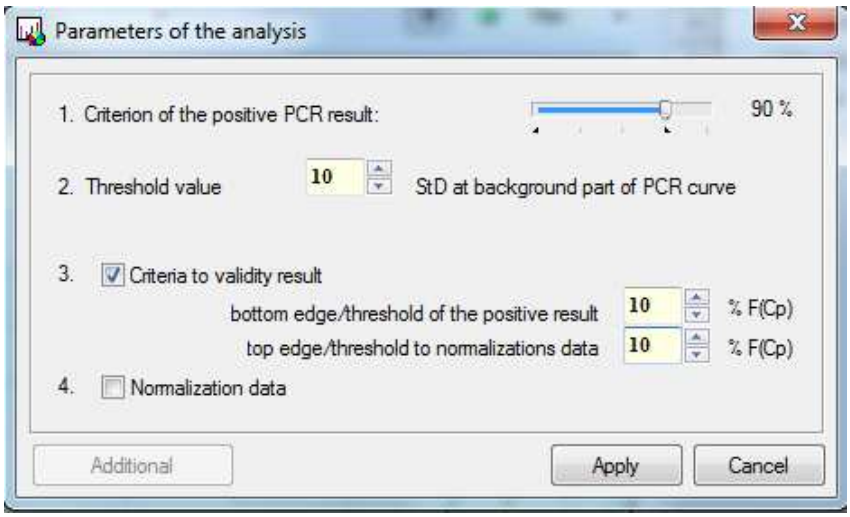
Plate-type instruments

Settings

Channel	Threshold
FAM	The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where log fluorescence curves are linear and do not cross curves of the negative samples.
HEX/Joe/Cy3	

SaCycler-96 (Sacace) instrument analysis settings

Click on the icon for changing the parameter of data analysis  , a new window will show up. The settings must be precisely as in the following picture, then click **"Apply"**:



Set **90%** as *"Criterion of the positive PCR result"*; *"Normalization data"* checkbox must be **deselected.** Select checkbox **"Criteria to validity result"** and insert **10% F(Cp)** for *"bottom edge/threshold of the positive result"* and insert **10% F(Cp)** for *"top edge/threshold to normalizations data"*. Set the threshold line at a level corresponding to 5% of the fluorescence of positive control in the last amplification cycle.

DATA ANALYSIS

Accumulation of ***Zika virus*** **cDNA** amplification product is detected in the **JOE/Yellow/HEX** channel, Internal Control amplification product is detected in the **FAM/Green** channel.
The results are interpreted by the software of the PCR instrument used by the crossing (or not crossing) of the fluorescence curve with the threshold line.
The results of analysis are considered reliable only if the results obtained for Positive and Negative Controls of Amplification as well as for the Negative Control of Extraction are correct.

Boundary Ct values

	Stage for control	Ct in channel (Rotor-type)		Ct in channel (Plate-type)	
		FAM/Green	Yellow	FAM	Joe/HEX
NCE	RNA extraction	< 30	Neg	< 33	Neg
PCE	RNA extraction	< 32	< 32	< 36	< 36
NCA	RT-PCR	Neg	Neg	Neg	Neg
C+	RT-PCR	< 30	< 32	< 33	< 36
Samples	-	< 32	< 42	< 36	< 43

1. The sample is considered **positive** if its Ct value detected in the JOE/Yellow/HEX channel does not exceed the boundary Ct value and the Ct value detected in the FAM/Green channel does not exceed the boundary Ct value specified for the Internal Control. The fluorescence curve should have a typical sigmoid shape and cross the threshold line once in the region of significant fluorescence increase.
2. The sample is considered **negative** if its Ct in the JOE/Yellow/HEX channel is not detected (the fluorescence curve does not cross the threshold line) and the Ct value detected in the FAM/Green channel does not exceed the boundary Ct value specified for the Internal Control.

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.
A negative control of extraction (NCE), negative control of amplification (NCA), positive control of extraction (PCE) and positive control of amplification (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.
If the controls are out of their expected range (see table Boundary Ct values), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of **Zika Virus Real-TM** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

Analytical sensitivity

The kit **Zika Virus Real-TM** allows to detect *ZIKA* RNA in 100% of the tests with a sensitivity of not less than what declared in the following table.

The claimed analytical features of **Zika Virus Real-TM** PCR kit are guaranteed only when the specific extraction kit is used.

Analytical sensitivity table

Type of tested material	Sample volume for extraction, µl	Extraction kit	Analytical sensitivity, copies /ml
Blood plasma	100	DNA/RNA prep	2000
Mosquitoes (homogenate)	100		2000
Urea	100		2000
Saliva	100		2000
Oropharynx swabs	100		2000
Tissue (autopsy/ biopsy) material, placenta	50		10000
Seminal liquid	50		10000
Amniotic fluid	50		10000
Blood plasma	200	Magno Virus	1000
	1000		100
Urea	1000		500

Target region: NS3

TROUBLESHOOTING

The results of analysis are not taken into account in the following cases:

1. If the Ct value of a clinical sample detected in the JOE/Yellow/HEX channel exceeds the boundary Ct value (see table), the result is considered **equivocal**. It is necessary to repeat the analysis twice. If a reproducible positive Ct value is detected, the sample is considered to be **positive**.
2. If any Ct value is detected for the Negative Control of Amplification (NCA) in both channels or the Ct value is detected for Negative Control of Extraction (C–) in the JOE/Yellow/HEX channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered **invalid**. It is necessary to repeat the analysis of all tests and to take measures to detect and eliminate the source of contamination.
3. If the Ct value is absent for the Positive Control of Extraction (PCE), this indicates improper extraction procedure. RNA extraction should be repeated for all samples.
4. If the Ct value is absent for the Positive Control of RT-PCR (C+), this indicates errors in carrying out PCR or an incorrect amplification program. RT-PCR should be repeated for all samples.
5. If the Ct value of a clinical sample is absent or greater than the boundary Ct value for the JOE/Yellow/HEX channel and the Ct value in the FAM/Green channel is greater than the Ct boundary values specified for the Internal Control, the result is **invalid**. Analysis of such samples should be repeated starting from the RNA extraction stage.

KEY TO SYMBOLS USED

IVD	For *in Vitro Diagnostic* Use		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
	Store at	VER	Version
	Manufacturer	**C–**	Negative control of Extraction
	Consult instructions for use	**NCA**	Negative Control of Amplification
	Expiration Date	**IC**	Internal Control
REF	List Number		

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* Rotor-Gene™ is a registered trademark of Qiagen
* MX3005P® is a registered trademark of Agilent Technologies
* ABI® is a registered trademark of Applied Biosystems
* SmartCycler® is a registered trademark of Cepheid

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