Sacace BIOTECHNOLOGIES

IVD For *in Vitro* Diagnostic Use

CE

Chikungunya Real-TM

Handbook

Real Time PCR kit for qualitative detection of Chikungunya virus in clinical material

REF V122-96FRT

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NAME

Chikungunya Real-TM

INTRODUCTION

Chikungunya virus (CHIKV) is a mosquito-borne virus (arbovirus) first found during an outbreak in south Tanzania in 1952. Its transmission route comes mainly from infected mosquitos of the Aedes genus, in particular Ae. aegypti and Ae. Albopictus, even if perinatal vertical transmission has been described as well.

Since 1952, CHIKV caused numerous outbreaks and epidemics in Africa, Asia, Europe, the South Pacific and Americas, involving millions of people, and often separated by periods of more than 10 years.

After the bite of an infected mosquito, symptoms of disease occurs usually between 4 and 8 days but can range from 2 to 12 days. Chikungunya infection is characterised by an abrupt beginning of chills, fever reaching up to 40 °C, vomiting, nausea, headache, arthralgia (joint pain), and in some cases, maculopapular rash. Severe joint and muscular pain is also observed and is the main and the most problematic symptom of chikungunya disease. The pain is so intense that makes movement very difficult and prostrates the patients.

Laboratory diagnosis is generally made by testing serum or plasma to detect virus, viral nucleic acid, or virus-specific immunoglobulin (Ig) M and neutralizing antibodies. Viral culture may detect virus in the first 3 days of disease; however, chikungunya virus should be handled under biosafety level (BSL) 3 conditions. During the first 8 days of disease, chikungunya viral RNA can often be identified in serum. Chikungunya virus antibodies normally develop towards the end of the first week of disease. Therefore, to definitively rule out the diagnosis, convalescent-phase samples should be obtained from patients whose acute-phase samples test negative.

Because of cross-reactivity of Chikungunya antibodies with other arbovirus, the use of serology is limited and controversial, so the Real Time RT-PCR is a better detection method.

INTENDED USE

CHIKUNGUNYA Real-TM PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Chikungunya virus* RNA in the biological material (blood plasma, serum, urine) using real-time hybridization-fluorescence detection of amplified products. The PCR kit is used for studying the biological material, taken from the persons suspected of Chikungunya infection without distinction of form and presence of manifestation.

The results of PCR analysis are taken into account in complex diagnostics of disease. Any diagnosis on the basis of the tests results must be done only by a qualified physician.

PRINCIPLE OF ASSAY

Chikungunya virus detection by the polymerase chain reaction (PCR) is based on the RNA extraction form the test material and subsequent simultaneous carrying out of reverse transcription reaction and amplification of *Chikungunya virus* cDNA fragments and Internal Control (IC) DNA with hybridization-fluorescence detection. The Internal Control allows to identify possible reaction inhibition.

The RNA obtained at the RNA extraction stage is reverse transcribed into DNA by using the Revertase enzyme, and then amplified into cDNA fragments by using specific primers and the enzyme Taq polymerase. In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

At the RT-PCR stage, 2 reactions are carried out simultaneously in one tube – amplification of the *Chikungunya virus* cDNA and the Internal Control (IC) DNA sequences. The amplification results of *Chikungunya virus* cDNA and Internal Control (IC) DNA are detected in 2 different fluorescence detection channels:

- the *CHIKUNGUNYA* virus cDNA is detected in the channel FAM/Green;
- the IC DNA is detected in the JOE/HEX/Yellow channel.

MATERIALS PROVIDED

Reagent	*Volume, ml*	*Quantity*
Chikungunya virus (8-tube lyophilized strips)	-	12 strips
Optical caps (8 caps)	-	12 cap strips
Chikungunya Positive Control* (lyophilized)	-	1 tube
Hydration Buffer	1.8	1 tube
DNA/RNA free water	1.0	1 tube
Negative Control (C–)**	1.0	1 tube

CHIKUNGUNYA Real-TM PCR kit is intended for 96 reactions (including controls).

* *must be used as Positive Control;* ***Positive control must be reconstituted with 100 µl of DNA/RNA free water before use.***

** *must be used as Negative Control*

MATERIALS REQUIRED BUT NOT PROVIDED

- Real Time Thermalcycler
- RNA isolation kit
- Desktop microcentrifuge for “eppendorf” type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Disposable polypropylene PCR tubes or strips
- Tube racks

STORAGE INSTRUCTIONS

All components of the **CHIKUNGUNYA Real-TM** PCR kit are to be stored at 2-8°C. They are stable until the expiration date indicated on the label. The kit can be shipped at room temperature but should be stored at 2-8°C on receipt.

STABILITY

CHIKUNGUNYA Real-TM PCR kit 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. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided.

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.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

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 techniques of DNA amplification (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.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Note: Handle all specimens as if they are potentially infectious agents.

Chikungunya Real-TM PCR kit is intended for analysis of RNA extracted with RNA extraction kits from the following clinical/biological material: **blood plasma, serum, urine**.

- *Blood plasma*. Blood samples are taken after overnight fasting into a tube (special vacuum blood collection system) with EDTA as anticoagulant. The closed tube with blood is rotated several times for thoroughly mixing with the anticoagulant. It can be stored at 2–8 °C. The tubes with the whole blood are to be centrifuged no later than 6 hours from the blood collection at 800-1,600 g (for example, 3,500-5,000 rpm for the Eppendorf microcentrifuge) for 20 min at room temperature. No less than 1 ml of the obtained plasma is transferred into the sterile 2.0-ml tubes using a new filter tip for each sample.

  The blood plasma samples can be stored before the PCR analysis:

  - at the temperature from 2 to 8 °C for 1 day,
  - at the temperature from minus 24 to minus 16 °C – for 1 week,
  - at the temperature ≤ –70 °C – for a long time.

  Only one freeze-thawing cycle is required.

RNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the "SAMPLE COLLECTION, STORAGE AND TRANSPORT" paragraph, could be used.

Sacace Biotechnologies recommends to use the following kits:

- ⇨ **Ribo-Virus** – (Sacace, REF K-2/C)
- ⇨ **DNA/RNA-Prep –** (Sacace, REF K-2-9)
- ⇨ **SaMag Viral Nucleic Acids Extraction kit** – (Sacace, REF SM003)

Please carry out the RNA extraction according to the manufacturer's instructions.

The purified RNA can be stored at 2–8 °C for at most 4 h, at temperatures not higher than minus 16 °C for 1 month, and at temperatures not higher than minus 68 °C for one year.

PROTOCOL: PREPARING TUBES FOR PCR

The total reaction volume is **20 µl**, the volume of the **RNA** sample is **5 µl**.

1. Prepare the necessary quantity of strips for samples and controls (separate tubes if needed).
2. Peel off protective aluminum seal from the strips/plate.
3. Add **15 µl** of **Hydration Buffer** into each reaction tube
4. Add **5 µl** of the extracted **RNA** sample to the corresponding reaction tube.
5. Prepare the **control reactions**:
   - **C+** - Add **5 µl** of the **Positive Control** to the positive control reaction tube
   - **C–** - Add **5 µl** of **Negative Control** to the negative control reaction tube
6. Cap the tubes, gently spin the strip tubes.
7. Insert the tubes into the device

Amplification

1. Create a temperature profile on your instrument as follows:

Step	*Temperature, °C*	*Time*	*Fluorescent signal detection*	*Cycle repeats*
1	45	15 min	–	1
2	95	2 min	–	1
3	95	10 s	–	45
	60	50 s	**FAM/Green, JOE/HEX/Yellow**	

For example SaCycler-96™ (Sacace), ABI 7300/7500, QuantStudio 3/5/6 Flex/7 Flex/12K flex, Viia 7 (Life Technologies), MX3000P/3005P (Agilent);CFX 96 Deep Well / iQ5 (BioRad);
For Rotor-Gene™ 6000/Q (Corbett Research, Qiagen) transfer the reaction volume to RotorGene compatible tubes before starting PCR.
For SmartCycler (Cepheid)) transfer the reaction volume to SmartCycler compatible tubes before starting PCR.

Chikungunya cDNA is detected in the FAM/Green and Internal Control DNA is detected in the JOE/HEX/Yellow channel.

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 6000, RotorGene Q)

Channel	Calibrate / Gain Optimisation	Threshold	More Settings/ Outlier Removal	Slope Correct	Eliminate Cycles before
FAM/Green	5FI - 10FI	0,03	5%	ON	5
JOE/Yellow	5FI - 10FI	0,03	5%	ON	5

Plate- or modular type instruments For result analysis, set the threshold line in the log-linear phase of the curve of positive control, above the signal of the negative control.

DATA ANALYSIS

Analysis of results is performed by the software of the real-time RT-PCR instrument used by measuring fluorescence signal accumulation in 2 channels:

- **The *CHIKUNGUNYA* cDNA is detected in the FAM/Green channel;**
- **The IC DNA is detected in the JOE/HEX/Yellow channel.**

The results are interpreted by the real-time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve.

The result of amplification is considered **negative** if the fluorescence curve is not S-shaped and if it does not cross the threshold line (the Ct value is absent).

RESULTS INTERPRETATION

***Ct* value in**		**Result**
FAM (CHIKV)	**JOE (IC)**	
Ct value defined	Ct value defined	*Chikungunya* RNA **is detected**
absent	Ct value defined	*Chikungunya* RNA **is not detected**
absent	absent	**Invalid***

* If the **invalid** result is obtained, PCR analysis is to be repeated for the corresponding test sample starting from the RNA extraction stage.

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls are correct:

Control	**Stage for control**	***Ct* value in the channel for fluorophore**	
		FAM (CHIKV)	**JOE (IC)**
Negative Control	RT-PCR	Absent	Ct value defined
Positive Control	RT-PCR	Ct value defined	Ct value defined

QUALITY CONTROL PROCEDURE

An Internal Control (IC) is present in this assay in order to identify possible reaction inhibition.

A positive control, negative control are required for every run to verify that the assay is performed correctly.

If the controls are not valid, all of the specimens and controls from that run must be processed beginning from the sample preparation step.

PERFORMANCE

Analitical Sensitivity

The analytical sensitivity (limit of detection or LoD) was evaluated by testing dilutions series of an International Chikungunya virus Standard (1st World Health Organization (WHO) International Standard for Chikungunya Virus (CHIKV) RNA for Nucleic Acid Amplification Techniques (NAT)-Based Assays) from 1,25 x 10^3 to 1,25 x 10^1 International Units per reaction. Every dilution was tested in triplicate as well as the last dilution around the detection limit which was tested 20 times.

The Chikungunya Real TM kit showed a detection limit of 12.5 International Units per reaction.

Analitical Specificity

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

The analytical specificity of **CHIKUNGUNYA Real-TM** PCR kit was proved while studying the following microorganisms' strains: *Zika virus strain MR 766, Dengue 4 virus strain H241, West Nile virus Heja, Dengue 1 virus strain Hawaii, St Louis Encephalitis virus strain 17D, West Nile virus Ug37, Dengue 2 virus strain New Guinea C, West Nile virus strain H160/99, Yellow Fever virus strain 17D, Dengue 3 virus strain H87.*

Target gene: *NSP1* gene

TROUBLESHOOTING

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

1. The *Ct* value determined for the Positive Control (C+) in the channel for the FAM and/or JOE fluorophore is absent. The amplification and detection should be repeated for all samples in which the specific RNA was not detected.
2. The *Ct* value is determined for the Negative Control in the channels for the FAM fluorophore. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The amplification and detection should be repeated for all samples in which the specific RNA was detected.
3. The *Ct* value is determined for the test sample, whereas the area of typical exponential growth of fluorescence is absent (the graphic looks like approximate straight line). It is necessary to check the correctness of the threshold line level or parameters of base line calculation. If the result has been obtained with the correct threshold line (base line) level, the amplification and detection should be repeated for this sample.

KEY TO SYMBOLS USED

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

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