Sacace BIOTECHNOLOGIES

CE 1023

IVD For *in Vitro* Diagnostic Use

For Professional Use Only

CMV Real-TM

Handbook

Real Time PCR kit for the qualitative detection of *Cytomegalovirus (CMV)*

REF V7-100FRT

Σ 100

NAME
CMV Real-TM

INTENDED USE
CMV Real-TM PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of human cytomegalovirus (*CMV*) DNA in the clinical materials (urogenital swabs, urine samples, saliva, whole human blood) by using real-time hybridization-fluorescence detection.

The results of PCR analysis are taken into account in complex diagnostics of disease.

PRINCIPLE OF ASSAY
CMV DNA detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using special primers. In real-time PCR the amplified product is detected using fluorescent dyes. These dyes are usually linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. The real-time monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run. **CMV Real-TM** PCR kit is a qualitative test that contains the Internal Control (IC). It must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

MATERIALS PROVIDED

Reagent	***Description***	***Volume (ml)***	***Quantity***
PCR-mix-1-FL *CMV*	colorless clear liquid	1.2	1 tube
PCR-mix-2-FRT	colorless clear liquid	0.3	2 tubes
Polymerase (TaqF)	colorless clear liquid	0.03	2 tubes
Positive Control complex (C+)	colorless clear liquid	0.2	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C-)*	colorless clear liquid	1.2	1 tube
Internal Control-FL**	colorless clear liquid	1.0	1 tube

**must be used in the isolation procedure as Negative Control of Extraction.*
***add 10 µl of Internal Control during the DNA isolation directly to the sample/lysis mixture (see DNA-Sorb-A REF K-1-1/B protocol).*

ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Transport medium.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers up to 200 µl.
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 2 ml reaction tubes.
- PCR box.
- Real Time PCR instrument.
- 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. Biosafety Level 2 should be used for materials that contain or are suspected of containing infectious agents.
7. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant.
8. 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.
9. Material Safety Data Sheets (MSDS) are available on request.
10. Use of this product should be limited to personnel trained in the techniques of DNA amplification.
11. 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.
12. 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.

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

⚠ 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 techniques of DNA amplification (EN375). 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 **CMV Real-TM** PCR kit (except for Polymerase (TaqF) and PCR-mix-2-FRT) are to be stored at 2–8 ºC when not in use. The kit can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.
The shelf life of reagents before and after the first use is the same, unless otherwise stated.

STABILITY

CMV Real-TM 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

CMV Real-TM can analyze DNA extracted from:

- *Whole peripheral and umbilical blood* should be collected to a tube with 6% EDTA solution at a ratio 20:1 (20 portions of blood per 1 portion of EDTA) after overnight fasting.

  

  Do not freeze the whole blood samples!

- *plasma* collected in EDTA tubes;
- *liquor* stored in "Eppendorf" tube;
- *tissue:* 1,0 gr homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles and dissolved in 1,0 ml of saline water or PBS sterile. Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml tube;
- *cervical, urethral, conjunctival swabs*:* insert the swab into the nuclease-free 1,5 ml tube and add 0,2 mL of Transport medium. Vigorously agitate swabs in medium for 15-20 sec.

Specimens can be stored at +2-8°C for no longer than 48 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.

DNA ISOLATION

The following kits are recommended:

- ⇨ **DNA-Sorb-B** (Sacace, REF K-1-1/B) for plasma, liquor, tissue, etc;
- ⇨ **DNA/RNA-Prep** (Sacace, REF K-2-9) for plasma, liquor, tissue, etc;
- ⇨ **DNA-Sorb-A** (Sacace, REF K-1-1/A) for swabs;

Please carry out DNA extraction according to the manufacture's instruction.
Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture.

REAGENTS PREPARATION (REACTION VOLUME 25 µL):

The total reaction volume is **25 µl**, the volume of DNA sample is **10 µl**.

1. Prepare the required number of the tubes for amplification of DNA from test and control samples.
2. For carrying out N reactions (including 2 controls), mix in a new tube: **10*(N+1) µl of PCR-mix-1-FL *CMV*, 5.0*(N+1) µl of PCR-mix-2-FRT** and **0.5*(N+1) µl of polymerase (TaqF).** Mix the content of the tube by vortexing and then centrifuge shortly. Transfer **15 µl** of the prepared mix into each tube.
3. Using tips with aerosol barrier, add **10 µl** of **DNA** obtained from test or control samples at the DNA extraction stage into the prepared tubes.
4. Carry out the control amplification reactions:

NCA - Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

C+ - Add **10 µl** of **Positive Control complex** to the tube labeled C+ (Positive Control of Amplification).

C– - Add **10 µl** of **sample, isolated from Negative Control** to the tube labeled C- (Negative Control of Extraction).

Cytomegalovirus is detected on the FAM (Green) channel, *IC DNA* on the JOE(Yellow)/HEX/Cy3 channel

AMPLIFICATION

Program the real-time instrument according to the manual provided by the manufacturer.

Amplification program for rotor-type instruments[1]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling2	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow	
	72	15 s		

Amplification program for plate or modular type instruments[2]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
1	95 °C	15 min	–	1
2	95 °C	5 s	–	5
	60 °C	20 s	–	
	72 °C	15 s	–	
3	95 °C	5 s	–	40
	60 °C	30 s	FAM, HEX/Cy3/JOE	
	72 °C	15 s		

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*

[2] *For example, iQ5™ (BioRad); Mx3005P™ (Stratagene), Applied Biosystems® 7300/7500/StepOne Real Time PCR (Applera), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

INSTRUMENT SETTINGS

Rotor-type instruments (for example RotorGene 3000/6000/Q)

Channel	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*0.1*	*5 %*	*On*
JOE/Yellow	*0.1*	*5 %*	*On*

Plate-type instruments (for example iQ5, Mx300P, ABI 7500/7300)

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 fluorescence curves are linear and do not cross curves of the negative samples.

DATA ANALYSIS

The fluorescent signal intensity is detected in two channels:

– The signal from the *CMV* DNA amplification product is detected in the FAM channel;

The signal from the Internal Control amplification product is detected in the JOE/Yellow/HEX channel.

Interpretation of results

The results are interpreted by the software of the instrument by the crossing (or not crossing) of the fluorescence curve with the threshold line.

Principle of interpretation:

— *CMV* DNA is **detected** in a sample if its Ct value is present in the FAM channel. The fluorescence curve should cross the threshold line in the area of exponential fluorescence growth.

— *CMV* DNA is **not detected** in a sample if its Ct value is absent in the FAM channel (fluorescence curve does not cross the threshold line) and the Ct value in the JOE channel is less than 33.

— The result is **invalid** if the Ct value of a sample in the FAM channel is absent while the Ct value in the JOE channel is either absent or greater than 33. It is necessary to repeat the PCR analysis of such samples.

Results for controls

Control	Stage for control	Ct value on channel		Interpretation
		FAM	JOE	
C–	DNA extraction	Neg	Pos (< 33)	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	Pos (< 33)	Pos (< 33)	OK

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 amplification control (NCA), positive amplification control (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 Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

PERFORMANCE CHARACTERISTICS

Analytical Sensitivity of **CMV Real-TM** PCR kit is the following:

Clinical material	Transport medium	Nucleic acid extraction kit	Sensitivity, GE/ml*
Urogenital swabs	Transport Medium for Swabs or with Mucolytic	DNA-sorb-A	10^3
Urine (pretreatment is required)	–	DNA-sorb-A	$2x10^3$

***** Genome equivalents (GE) of the microorganism per 1 ml of a clinical sample placed in the transport medium specified.

13.2. Specificity

The analytical specificity of **CMV 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. The clinical specificity of **CMV Real-TM** PCR kit was confirmed in laboratory clinical trials.

Target region: MAJOR IMMEDIATE-EARLY (MIE) gene

TROUBLESHOOTING

1. Weak or no signal of the IC (Joe/Hex/Cy3 channel) for the Negative Control of extraction.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting of the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - Improper DNA extraction
        - ⇒ Repeat analysis starting from the DNA extraction stage
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and select for the IC detection the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the DNA extraction procedure.
2. Weak or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
3. Fam signal with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips between tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

REFERENCES

- PCR detection of cytomegalovirus DNA in serum as a diagnostic test for congenital cytomegalovirus infection. C T Nelson, A S Istas, M K Wilkerson, and G J Demmler. J Clin Microbiol. 1995 December; 33(12): 3317–3318.

- Detection of Cytomegalovirus DNA in Peripheral Blood of Patients Infected with Human Immunodeficiency Virus. D. Shibata, W. John Martin, Maria D. Appleman, Dennis M. Causey, J. M. Leedom, N. Arnheim. J Infect Dis. (1988) 158 (6): 1185-1192.

- Multiplex PCR for six herpesviruses after hematopoietic stem cell transplantation. Sawada A, Koyama-Sato M, Yasui M, Kondo O, Ishihara T, Takeshita Y, Okamura T, Nishikawa M, Inoue M, Kawa Pediatr Int. 2011 Aug 2. doi: 10.1111/j.1442-200X.2011.03437.

- Cytomegalovirus Infections in Non-immunocompromised and Immunocompromised Patients in the Intensive Care Unit. Florescu DF, Kalil AC. Infect Disord Drug Targets. 2011 Jun 16.

- Comparison of PCR, Antigenemia Assay, and Rapid Blood Culture for Detection and Prevention of Cytomegalovirus Disease after Lung Transplantation. Adriana Weinberg, Tony N. Hodges, Shaobing Li, Guanyung Cai, M. R. Zamora. Journal of Clinical Microbiology, February 2000, p. 768-772, Vol. 38, No. 2

- Optimization of Quantitative Detection of Cytomegalovirus **DNA in Plasma by Real-Time PCR.** Michael Boeckh, MeeiLi Huang, James Ferrenberg, Terry Stevens-Ayers, Laurence Stensland, W. Garrett Nichols, and Lawrence Corey. Journal of Clinical Microbiology, March 2004, p. 1142-1148, Vol. 42, No. 3

- Quantification of Human Cytomegalovirus DNA by Real-Time PCR. Elyanne Gault,Yanne Michel, Axelle Dehée, Chahrazed Belabani, Jean-Claude Nicolas, Antoine Garbarg-Chenon. J Clin Microbiol. 2001 February; 39(2): 772–775

- Definitions of Cytomegalovirus Infection and Disease in Transplant Recipients. Per Ljungman, Paul Griffiths, Carlos Paya, …Clin Infect Dis. (2002) 34 (8): 1094-1097

KEY TO SYMBOLS USED

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

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