CMV Real-TM

Handbook

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

REF TV7-100FRT

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.

MATERIAL PROVIDED

Part N° 1 – **"DNA-Sorb-B":** sample preparation

- **Lysis Solution**, 2 x 15 ml;
- **Washing Solution 1**, 2 x 15 ml;
- **Washing Solution 2**, 2 x 50 ml;
- **Sorbent**, 2 x 1,25 ml;
- **DNA-eluent**, 2 x 5 ml.

Contains reagents for 100 tests.

Part N° 2 – **"CMV Real-TM":** Real Time amplification

- **PCR-mix-1-FRT**, 1,2 ml;
- **PCR-mix-2-FRT**, 2 x 0,3 ml;
- **TaqF Polymerase**, 2 x 0,03 ml;
- **CMV C+**, 0,2 ml;
- **Negative Control C-**, 1,2 ml;*
- **Internal Control IC**, 1,0 ml;**
- **DNA-buffer**, 0,5 ml;

Contains reagents for 110 tests

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator
- Freezer

Zone 2: Real Time amplification:

- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

CMV Real-TM must be stored at 2-8°C. **TaqF Polymerase** and **PCR-mix-2-FRT** must be stored at -16°C. **DNA-sorb-B** must be stored at 2-8°C. The kits can be shipped at 2-8°C but should be stored at 2-8°C and -16°C immediately on receipt.

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. 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.

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

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- 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

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

CMV Real-TM can analyze DNA extracted from:

- *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 -16°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;
- ⇨ **SaMag STD DNA Extraction Kit** (Sacace, REF SM007) for swabs;
- ⇨ **SaMag Viral Nuclei Acid Extraction Kit** (Sacace, REF SM003) for plasma, liquor.

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.

SPECIMEN AND REAGENT PREPARATION

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for **Negative Control of Extraction**.
2. Add to each tube **300 µl** of **Lysis Solution** and **10 µl** of **Internal Control.**
3. Add **100 µl** of **Samples** to the appropriate tube.
4. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Negative Control** provided with the amplification kit) to the tube labeled C*neg*.
5. Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 7-10 sec. If the sample is not completely dissolved it is recommended to re-centrifuge the tube for 5 min at a maximum speed (12000-16000 g.) and transfer the supernatant into a new tube for DNA extraction.
6. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 3 min at room temperature. Repeat this step.
8. Centrifuge all tubes for 30 sec at 8000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet.
9. Add **300 µl** of **Washing Solution 1** to each tube. Vortex vigorously and centrifuge for 30 sec at 8000g. Remove and discard supernatant from each tube.
10. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and centrifuge for 30 sec at 8000g. Remove and discard supernatant from each tube.
11. Repeat step 10 and incubate all tubes with open cap for 5 min at 65°C.
12. Resuspend the pellet in **50 µl of DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
13. Centrifuge the tubes for 1 min at maximum speed (12000-16000 g). The supernatant contains DNA ready for amplification. If amplification is not performed in the same day of extraction, the processed samples can be stored at 2-8°C for at maximum period of 5 days or frozen at –20°/-80°C.

PCR PROTOCOL (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 3 controls), mix in a new tube: **10*(N+1) µl of PCR-mix-1-FRT *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 **CMV C+** 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, SaCycler-96™ (Sacace), 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 SaCycler-96, iQ5, Mx3005P, ABI 7500/7300)

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.

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

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Sacace Biotechnologies Srl
via Scalabrini, 44 – 22100 – Como – Italy Tel +390314892927 Fax +390314892926
mail: ***info@sacace.com*** *web:* ***www.sacace.com***