Sacace BIOTECHNOLOGIES

CE 1023

IVD For *in Vitro* Diagnostic Use

For Professional Use Only

HSV/CMV Real-TM

Handbook

Multiplex Real Time PCR Kit for simultaneous detection of Herper Simplex Virus (HSV) and Cytomegalovirus (CMV)

REF V60-100FRT

Σ 100

NAME
HSV/CMV Real-TM

INTRODUCTION
The ***Herpesviridae*** are a large family of DNA viruses that cause diseases in animals, including humans. The members of this family are also known as **herpesviruses.**
The family of **Herpesviridae** is divided in 3 subfamilies:

1. Alphaherpesvirinae (Herpes simplex Virus, Varicella-zoster Virus);
2. Betaherpesvirinae (Cytomegalovirus);
3. Gammaherpesvirinae (virus di Epstein-Barr);

Type	Synonym	Subfamily	Pathophysiology	Site of Latency
HHV-1	Herpes simplex virus-1 (HSV-1)	α (Alpha)	Oral and/or genital herpes (predominantly orofacial), as well as other herpes simplex infections	Neuron
HHV-2	Herpes simplex virus-2 (HSV-2)	α	Oral and/or genital herpes (predominantly genital), as well as other herpes simplex infections	Neuron
HHV-3	Varicella zoster virus (VZV)	α	Chickenpox and shingles	Neuron
HHV-4	Epstein-Barr virus (EBV), lymphocryptovirus	γ (Gamma)	Infectious mononucleosis, Burkitt's lymphoma, CNS lymphoma in AIDS patients, post-transplant lymphoproliferative syndrome (PTLD), nasopharyngeal carcinoma, HIV-associated hairy leukoplakia	B cell
HHV-5	Cytomegalovirus (CMV)	β (Beta)	Infectious mononucleosis-like syndrome, retinitis, etc.	Monocyte, lymphocyte
HHV-6	Roseolovirus, Herpes lymphotropic virus	β	*Sixth disease* (roseola infantum or *exanthem subitum*)	T cells
HHV-7	Roseolovirus	β	*Sixth disease* (roseola infantum or *exanthem subitum*)	T cells
HHV-8	Kaposi's sarcoma-associated herpesvirus (KSHV)	γ	Kaposi's sarcoma, primary effusion lymphoma, some types of multicentric Castleman's disease	B cell

INTENDED USE
The kit **HSV/CMV Real-TM** is an *in vitro* nucleic acid amplification test for simultaneous detection of *herpes simplex virus (HSV)* and *cytomegalovirus* (*CMV*) DNA in clinical materials (urogenital, rectal, and oral swabs; urine; saliva; prostate gland secretion; whole blood and cerebrospinal fluid; and exudate of blisters and erosive-ulcerative lesions of skin and mucosa) by using real-time hybridization-fluorescence detection.

PRINCIPLE OF ASSAY
The kit **HSV/CMV Real-TM** is based on two major processes: isolation of DNA from specimens and Real Time amplification. DNA is extracted from the specimens, amplified using Real-Time amplification and detected fluorescent reporter dye probes specific for *HSV* DNA, CMV DNA and Internal Control. Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition.

MATERIALS PROVIDED

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-1-FL *HSV / 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 (IC)**	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/RNA prep REF K-2-9) protocol).*

MATERIALS REQUIRED BUT NOT PROVIDED

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

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.

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 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. 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. 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.

STORAGE INSTRUCTIONS

All components of the **HSV/CMV Real-TM** PCR kit (except for polymerase (TaqF) and PCR-mix-2-FRT) are to be stored at 2–8 ℃ when not in use. All components of the **HSV/CMV Real-TM** PCR kit are stable until the labeled expiration date. The shelf life of reagents before and after the first use is the same, unless otherwise stated.


Polymerase (TaqF) and PCR-mix-2-FRT are to be stored at ≤ –16 ℃.


PCR-mix-1-FL *HSV / CMV* is to be kept away from light.

STABILITY

HSV/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.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

HSV/CMV Real-TM can analyze DNA extracted from:

- *whole blood* collected in either ACD or EDTA tubes;
- *cerebrospinal fluid* stored in “Eppendorf” tube;
- *urine (a sediment of the first portion of the morning specimen);*
- *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;
- *prostatic liquid* stored in “Eppendorf” tube;
- *seminal liquid: t*ransfer about 30 µl of seminal liquid to a polypropylene tube (1,5 ml) and add 70 µl of sterile saline solution;
- *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℃ for no longer tha n 48 hours, or frozen at -20℃ to -80℃.
Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

The following kit is recommended:

- ⇨ **DNA-Sorb-A** (Sacace, REF K-1-1/A): swabs, urine
- ⇨ **DNA/RNA-Prep** (Sacace, REF K-2-9): whole blood, cerebrospinal fluid samples, tissue

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. Thaw the tube with **PCR-mix-2-FRT.** Vortex the tubes with **PCR-mix-1-FL *HSV / CMV,* PCR-mix-2-FRT, polymerase (TaqF)** then centrifuge briefly (1–2 s). Make sure there are no drops on the walls of the tubes.
2. Prepare the required number of the tubes for amplification of DNA from test and control samples.
3. For N reactions (including 2 controls of amplification), mix in a new tube:
   10*(N+1) µl of PCR-mix-1-FL *HSV / CMV*;
   5.0*(N+1) µl of PCR-mix-2-FRT;
   0.5*(N+1) µl of polymerase (TaqF).

Stir the prepared mixture and then centrifuge briefly (1–2 s). Make sure there are no drops on the walls of the tubes. Transfer **15 µl** of the prepared mix to each tube.

4. Using tips with aerosol barrier add **10 µl** of **DNA** obtained from test or control samples at the DNA extraction stage into prepared tubes.
5. 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, extracted from Negative Control** to the tube labeled C– (Negative Control of Extraction).

HSV DNA is detected in the FAM/Green channel; *CMV* DNA is detected in the JOE/Green/Cy3/Hex channel; Internal Control DNA is detected in the ROX/TexasRed fluorescence channel.

Table.1 Temperature profile

	Rotor-type instruments[1]			**Plate- or modular type instruments[2]**		
Step	***Temperature, °C***	***Time***	**Cycles**	***Temperature, °C***	***Time***	**Cycles**
Hold	**95**	15 min	1	**95**	15 min	1
Cycling 1	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	20 s	
	72	15 s		**72**	15 s	
Cycling 2	**95**	5 s	40	**95**	5 s	40
	60	20 s *fluorescent signal detection*		**60**	30 s *fluorescent signal detection*	
	72	15 s		**72**	15 s	

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

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

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*0.1*	*0 %*	*Off*
JOE/Yellow	*0.1*	*5 %*	*Off*
ROX/Orange	*0.1*	*5 %*	*Off*

Plate- or modular type instruments

For result analysis, set the threshold line at a level corresponding to 10–20% of the maximum fluorescence signal obtained for Pos C+ sample during the last amplification cycle.

RESULTS INTERPRETATION

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line. To set threshold put the line at such level where curves of fluorescence are linear.

- *HSV* DNA is detected in the FAM/Green channel;
- *CMV* DNA is detected in the JOE/Green/Cy3/Hex channel;
- Internal Control DNA is detected in the ROX/Orange/TexasRed fluorescence channel.

Principle of interpretation:

1. The sample is considered to be positive for the *HSV* DNA if its Ct value is defined in the results grid in the FAM/Green channel (the fluorescence curve should cross the threshold line in the region of significant fluorescence increase).
2. The sample is considered to be positive for the *CMV* DNA if its Ct value is defined in the results grid in the JOE/Green/Cy3/Hex channel (the fluorescence curve should cross the threshold line in the region of significant fluorescence increase).
3. The sample is considered to be negative for *HSV* and *CMV* DNA if its Ct value is not defined in the results grid (the fluorescence curve does not cross the threshold line) in FAM/Green and JOE/Green/Cy3/Hex channels and the Ct value does not exceed the boundary value in the results grid in the ROX/Orange/TexasRed channel.
4. The analysis result is considered to be invalid if the Ct value is not defined in the results grid (the fluorescence curve does not cross the threshold line) in the ROX/Orange/TexasRed channel and the Ct value in the results grid in the FAM/Green and JOE/Green/Cy3/Hex channels is negative or exceeds the boundary value. In such cases PCR should be repeated.

The result of the analysis is considered reliable only if the results obtained for both Positive and Negative Controls of Amplification as well as for the Negative Control of Extraction are correct.

Results for controls

Control	Stage for control	Ct FAM (Green)	Ct JOE(Yellow)/HEX/Cy3	Ct Rox/Orange/TexasRed	Interpretation
NCE	DNA isolation	-	-	Pos (< 36)	OK
Pos C+	PCR	Pos (< 33)	Pos (< 33)	Pos (< 33)	OK
NCA	PCR	-	-	-	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.

SPECIFICATIONS

Analytical specificity

Analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific for HSV and CMV primers and probes. The potential cross-reactivity of the kit **HSV/CMV Real-TM** was tested also against the group control. There were not nonspecific test responses during examination of a human DNA as well as a DNA panel of the following microorganisms: *Gardnerella vaginalis, Lactobacillus* spp., *Escherichia coli, Staphylococcus* spp., *Streptococcus* spp., *Candida albicans, Mycoplasma hominis, Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma genitalium, Chlamydia trachomatis, Neisseria* spp., *Neisseria gonorrhoeae, Trichomonas vaginalis, Treponema pallidum, Toxoplasma gondii, VZV, EBV, HHV6, HPV.*

Analytical sensitivity and reproducibility.

The analytical sensitivity of the **HSV/CMV Real-TM** kit was determined using the Standard DNA of the HSV and CMV. This Standard was serially diluted in the DNA-buffer. The following table summarize the results of these experiments.
The analytical sensitivity of the kit **HSV/CMV Real-TM** was not less than 1000 copies/ml.

TROUBLESHOOTING

1. Weak or no signal of the IC (ROX/Orange/TexasRed) 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
    - 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. Any signal on Fam/Green and/or JOE/Yellow/HEX/Cy3 channel 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.

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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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* Rotor-Gene™ Technology is a registered trademark of Qiagen
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* LineGeneK® is a registered trademark of Bioer
* SmartCycler® is a registered trademark of Cepheid

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