HHV7 Real-TM Quant

Handbook

Real Time PCR kit for quantitative detection of Human Herpes Virus 7

REF V17-100FRT

REF TV17-100FRT

100

NAME

HHV7 Real-TM Quant

INTENDED USE

kit **HHV7 Real-TM Quant** is an *in vitro* Real Time amplification test for quantitative detection of *Human Herpes Virus 7* in the biological materials. DNA is extracted from samples, amplified using real time amplification with fluorescent reporter dye probes specific for HHV7, an exogenous Internal Control (IC) and an endogenous Internal Control for the amplification of the human β-globine gene. The Internal Control serves as an amplification control for each individually processed specimen and to identify any possible reaction inhibition.

PRINCIPLE OF ASSAY

kit **HHV7 Real-TM Quant** is based on two major processes: isolation of DNA from specimens and Real Time amplification. Amplification results of HHV7 DNA are detected on the Joe/HEX/Yellow, β-globine gene DNA used as Internal Control is detected on the Fam/Green channel and the exogenous Internal Control DNA is detected on the ROX/Orange channel.

Extraction of DNA from blood plasma, whole blood and saliva is performed in the presence of an exogenous internal control (IC), which allows to monitor all stages of the PCR test for each sample and to evaluate the effect of inhibitors on the final results of the PCR. Furthermore, when DNA is extracted from whole blood, the DNA portion of the human β-globine gene (endogenous internal control) is amplified. Endogenous internal control (IC glob) allows to control all the steps of the PCR-study as well as the collection and storage of the sample.

The quantitative determination of HHV7 DNA is possible on the basis of a linear relationship between the initial concentration of the target DNA in the tested sample and the Ct. For the quantitative test, the amplification of DNA from the samples is carried out simultaneously with the DNA calibrators with a known concentration of DNA. Based on the results of the amplification of DNA calibrators, is made a calibration line that allows to calculate the concentration of the target DNA in the clinical samples.

At the stage of amplification, three reactions are simultaneously performed in the same tube - amplification of HHV7 DNA, the amplification of the DNA sequence of the exogenous IC and the amplification of the endogenous IC human β-globine gene. The results of the targets are detected on 3 different fluorescence channels.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V17-100FRT)

"HHV7 Real-TM Quant": Real Time amplification

Reagent	ml	Quantity
PCR-mix-1 *HHV7*	1,2	1 vial
PCR- buffer-FRT (with Taq polymerase)	0,6	1 vial
QS 1 *HHV7*	0,2	1 vial
QS 2 *HHV7*	0,2	1 vial
DNA-buffer (C-)	0,2	1 vial
Internal control (IC)*	1,0	1 vial
Negative control**	1,2	2 vials
Positive control *HHV7****	0,1	1 vial

Contains reagents for 110 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TV17-100FRT)

Part N° 1 – **"DNA-sorb-B":** Sample preparation

Reagent	ml	Quantity
Lysis Solution	15	2 vials
Sorbent	1,25	2 vials
Washing Solution 1	15	2 vials
Washing Solution 2	50	2 vials
DNA-eluent	5	2 vials

Contains reagents for 100 tests.

Part N° 2 – **"HHV7 Real-TM Quant":** Real Time amplification

Reagent	ml	Quantity
PCR-mix-1 *HHV7*	1,2	1 vial
PCR- buffer-FRT (with Taq polymerase)	0,6	1 vial
QS 1 *HHV7*	0,2	1 vial
QS 2 *HHV7*	0,2	1 vial
DNA-buffer (C-)	0,2	1 vial
Internal control (IC)*	1,0	1 vial
Negative control**	1,2	2 vials
Positive control *HHV7****	0,1	1 vial

Contains reagents for 110 tests.

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

** *must be used during the sample preparation procedure as Negative control: add 100 µl of C– (Negative control) to the tube labeled Cneg;*

*** *add 90 µl of C– (Negative control) and 10 µl of Positive control HHV7 to the tube labeled Cpos.*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 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
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

HHV7 Real-TM Quant must be stored at - 20°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 -20°C immediately on receipt.

STABILITY

HHV7 Real-TM Quant 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. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

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

RUO For Research Use Only

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.

**** Only for Module No.2***

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

HHV7 Real-TM Quant can analyze DNA extracted from:

- *whole blood* collected in either ACD or EDTA tubes;
- *Blood plasma;*
- *Saliva*

It is recommended to process samples immediately after collection. Store samples at 2–8 °C for no longer than 24 hours, or freeze at –20/80°C. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA 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:

⇒ **DNA-Sorb-B** (Sacace, REF K-1-1/B)

⇒ **SaMag Viral Nucleic Acids Extraction Kit** (Sacace, REF SM003) for cell free body fluids.

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

SPECIMEN AND REAGENT PREPARATION (reagents supplied with the module no.2)

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60°C until disappearance of ice crystals.
2. Prepare required quantity of 1.5 ml polypropylene tubes.
3. Add to each tube **300 µl** of **Lysis Solution**.
4. Add **100 µl** of **Samples** to the appropriate tube.
5. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Negative control**) to labeled C*neg*.
   - add **90 µl of C– (Negative control)** and **10 µl** of **Pos HHV7** to the tube labeled *Cpos*.
6. Vortex the tubes, incubate 5 min at 65°C and centrifuge for 5 sec.
7. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
8. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically
9. Centrifuge all tubes for 1 min at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
10. Add **300 µl** of **Washing Solution 1** to each tube. Vortex vigorously and centrifuge for 1 min at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
11. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and centrifuge for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
12. Repeat step 11.
13. Incubate all tubes with open cap for 5 min at 65°C.
14. Resuspend the pellet in **50 µl** of **DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
15. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g). The supernatant contains DNA ready for amplification. The amplification can be performed on the same day of extraction.

PROTOCOL:

Reaction volume = 25 µl

1. Prepare required quantity of tubes (N + controls + 4 tubes for standards)
2. Prepare the **Reaction Mix**. In a new sterile tube for each sample **10*N µl** of **PCR-mix-1 HHV7**, **5,0*N** of **PCR-Buffer-FRT**. Vortex and centrifuge for 2-3 sec. The Reaction Mix should be used immediately after preparation.
3. Add **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA** sample to appropriate tube. Mix by pipetting.
4. For each analysis it is necessary to include the followings:
    - add **10 µl** of extracted **Positive control *HHV7*** to the tube labeled C*pos;*
    - add **10 µl** of extracted **Negative control** to the tube labeled C*neg;*
    - add **10 µl** of **DNA-buffer (C-)** to the tube labeled *Cneg-amp;*
    - add **10 µl** of **QS1** into **2 tubes** each labeled Standard 1 (prepare 2 tubes of QS1);
    - add **10 µl** of **QS2** into **2 tubes** each labeled Standard 2 (prepare 2 tubes of QS2).

Amplification

1. Close tubes and transfer them into the Real Time Thermal Cycler.
2. Program position of the samples, controls and standards.
3. Program the instruments as follows:

Step	Temperature, °C	Time	Fluorescence channels	Repeats
1	50	15 min	–	1
2	95	15 min	–	1
3	95	10 sec	–	45
	60	25 sec	**FAM/Green, JOE/Yellow/HEX, ROX/Orange**	

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)

Fluorescence is detected at the 3th step (60 °C) in FAM/Green JOE/Yellow/Hex/Cy3, ROX/Orange fluorescence channels.

INSTRUMENT SETTINGS

Fluorescence channel	FAM/Green	JOE/HEX/Yellow	ROX/Orange
Detecting	human β-globine gene	*HHV7* DNA	exogenous Internal Control

Rotor-type instruments

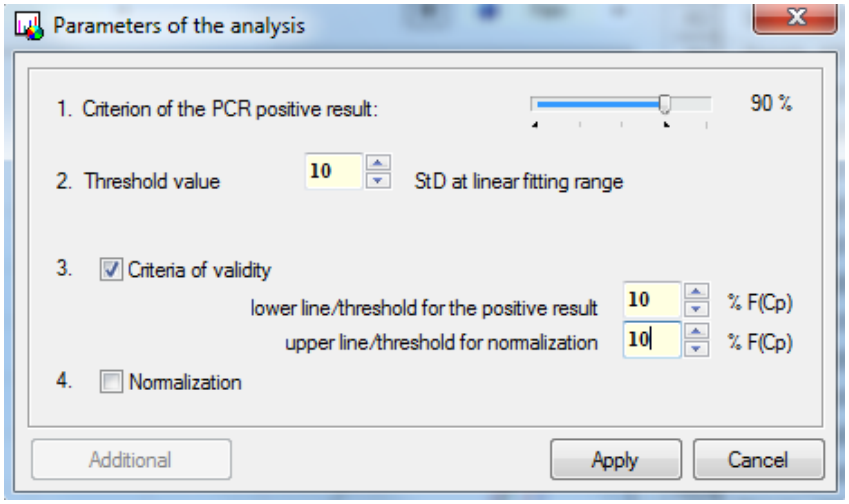
Channel	Calibrate/Gain Optimisation…	Threshold	Eliminate cycles before	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	*0.03*	*5*	*10 %*	*On*
JOE/Yellow	*from 5 Fl to 10 Fl*	*0.03*	*5*	*10 %*	*On*
ROX/Orange	*from 5 Fl to 10 Fl*	*0.03*	*5*	*10 %*	*On*

Plate-type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; Set the threshold line at a level corresponding to 10–20% of the maximum fluorescence signal obtained for Pos Control HHV7 sample during the last amplification cycle.

Parameters of the analysis for SaCycler-96 instrument:

Select “Criteria of validity” and “Criterion of the PCR positive result” as indicated in the below picture:



Criterion of the PCR positive result: **90%**

Criteria of validity:

Lower line/threshold: **10%**

Upper line/threshold: **10%**

Normalization:

deselected

RESULT ANALYSIS:

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

If total DNA is isolated from acellular samples like plasma, the calculation of the amount of HHV7 DNA in 1 ml of the test sample is carried out according to the formula:

$$\frac{\text{DNA copies of HHV7 DNA per reaction}}{\text{DNA copies of IC DNA per reaction}} \times \text{coefficient B} = \text{HHV7 GE/ml}$$

Coefficient B is the number of IC in 1 ml of the test material. The coefficient takes into account the loss of DNA during the extraction process.
Values of the DNA concentration of calibrators and coefficient B are lot specific and are indicated in the Data Card included in the kit.

If total DNA is isolated from cellular samples like whole blood, the obtained HHV7 DNA concentration can be normalized to the standard number of human cells (the number of HHV7 GE per 10^5 human cells). Calculation of normalized values of DNA concentration HHV7 is carried out according to the formula:

$$\frac{\text{DNA copies of HHV7 per reaction}}{\text{DNA copies of human } \beta\text{-globine gene per reaction}} \times 2*10^5 = \text{HHV7 DNA GE per } 10^5 \text{ human cells}$$

The normalized values of concentrations reflect the amount of the pathogen relative to human cells. To express the relative concentration of HHV7 DNA in GE, the conversion factor used for the standard number of cells is the following: 10^5 cells = 2 * 10^5 human genomes.

Interpretation of results for the samples under study

Result	Interpretation
Invalid	The value of Ct on ROX channel is absent or is higher than the boundary value. It is necessary to repeat the PCR test of the sample, starting from the DNA extraction step.
Invalid (Only when testing whole blood)	The concentration of human β-globine is less than 2000 copies/reaction, and there is no calculated concentration value on JOE channel. It is necessary to repeat the PCR test of the sample, starting from the DNA extraction step.
HHV7 DNA not detected	The Ct value for HHV7 DNA on JOE channel is absent, while the Ct value on ROX channel is less than the boundary value. The result is interpreted as HHV7 DNA is not detected.
Less than 1000 GE/ml	HHV7 DNA is detected below the linear range of the reagent kit. The result is interpreted as less than 1000 GE HHV7/ml.
X x 10^y GE/ml	The concentration value (GE/ml) is within the linear range of the reagent kit. The result is interpreted as HHV7 DNA detected with concentration of X x 10^y GE/mL.
More than 1x10^7 GE/ml	The HHV7 DNA is detected with a concentration higher than the linear range of the reagent kit. The result is interpreted as more than 1x10^7 HHV7 GE/ml. If is necessary to quantify exactly the result, it is possible to dilute the extracted sample with the Negative Control (for example, 100 times) and repeat the testing from the amplification step. The obtained result must be then multiplied by the dilution factor of the sample.

The result is considered reliable if the controls of for DNA extraction and amplification are in accordance with the results in table below**:

Control	Stage	Fluorophore channel		
		FAM	JOE	ROX
PCE	DNA extraction	Ct value less than the boundary value	Ct value less than the boundary value; the concentration value is within the range indicated in DataCard	Ct value less than the boundary value
NCE	DNA extraction	No Ct value or > value indicated in Boundary Ct value	No Ct value	Ct value less than the boundary value
NCA	PCR	No Ct value or > value indicated in Boundary Ct value	No Ct value	No Ct value
QS1*	PCR	Ct depending on the concentration	Ct depending on the concentration	Ct depending on the concentration
QS2*	PCR	Ct depending on the concentration	Ct depending on the concentration	Ct depending on the concentration

* *The concentrations of the QS1 and QS2 are lot specific and reported on the Data Card included in the kit.*

** *Boundary Ct values are indicated on the DataCard included in the kit*

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific *Human Herpes Virus 7* primers and probes.

The analytical specificity of the kit was also tested with the DNA of the following microorganisms: Acinetobacter baumanii, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Listeria grayi (murrayi), Listeria monocytogenes, Listeria innocua, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus aureus (MRSA), Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pyogenes, Moraxella catarrhalis, HSV I (human herpes simplex virus type 1), HSV II (human herpes simplex virus type 2), CMV (human cytomegalovirus), EBV (Epstein-Barr virus), HHV6 (human herpesvirus type 6), HHV8 (human herpesvirus type 8), VZV (varicella zoster virus), JCV (John Cunningham virus), Parvovirus B19 (parvovirus B19), Toxoplasma gondii, Candida Albicans, as well as human genomic DNA.

When testing the DNA samples of the above microorganisms and human DNA, nonspecific reactions were not detected. The potential cross-reactivity of the kit **HHV7 Real-TM Quant** was tested against the group control. It was not observed any cross-reactivity with other pathogens. The specificity of the kit **HHV7 Real-TM Quant** was 100%.

Analytical sensitivity

The kit **HHV7 Real-TM Quant** allows to detect *Human Herpes Virus 7* DNA in 100% of the tests with a sensitivity as specified in the below table:

Clinical material	Sample extraction volume, µl	Extraction kit	Detection limit, GE/ml	Linear range, GE/ml
Blood plasma	100	DNA-Sorb-B	$5x10^2$	$1x10^3$ – $1x10^7$
Whole blood				
Saliva				

TROUBLESHOOTING

1. Positive extraction control (PC), with the Ct on FAM and/or JOE and/or ROX channels is absent or exceeds the boundary value. It is necessary to repeat the PCR for all samples starting from the DNA extraction step.
2. The concentration of HHV7 does not fall within the range indicated in the Data Card. It is necessary to repeat the PCR for all samples, starting from the DNA extraction step.
3. Negative extraction control (NCE), with the Ct on FAM and/or JOE channels. Probable contamination with amplification products or contamination of the reagents at any stage of the PCR. It is necessary to identify and eliminate the source of contamination and to repeat the PCR for all samples, starting from the DNA extraction step.
4. Negative control of amplification (NCA), with the Ct on FAM and/or JOE and/or ROX. Probable contamination with amplification products or contaminations of the reagents, at any stage of the PCR test. It is necessary to identify and eliminate the source of contamination and to repeat the amplification step for all samples.
5. Calibrators QS1 and QS2 with absent values of Ct for any of the indicated fluorescence channels. Repeat amplification and detection for all samples.
6. When carrying out a quantitative PCR analysis, the correlation coefficient R^2 of the standards is less than 0.98. It is necessary to take care during the PCR preparation. Pipette the correct amount in both the repetitions of standards and. If the results are unsatisfactory, it is necessary to repeat the amplification and detection for all samples.
7. The value of the threshold cycle is determined for the sample under study, but there is no characteristic exponential rise of the fluorescence curve (the graph is approximately a straight line). It is necessary to check the set up of the selected threshold line level or baseline parameters and if necessary re-amplify and detect the sample.

KEY TO SYMBOLS USED

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

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