IVD For *in Vitro* Diagnostic Use

CE

HHV6 Real-TM Quant

Handbook

Real Time PCR kit for quantitative detection of Human Herpes Virus 6

REF V10-100FRT

REF TV10-100FRT

Σ 100

NAME

HHV6 Real-TM Quant

INTENDED USE

kit **HHV6 Real-TM Quant** is an *in vitro* Real Time amplification test for quantitative detection of *Human Herpes Virus 6* in the biological materials. DNA is extracted from samples, amplified using real time amplification with fluorescent reporter dye probes specific for pol-gene of HHV6 and Internal Control (IC). Test contains an IC (β-globine gene) which serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition.

PRINCIPLE OF ASSAY

kit **HHV6 Real-TM Quant** is based on two major processes: isolation of DNA from specimens and Real Time amplification. Amplification results of HHV6 DNA are detected on the Joe/HEX/Yellow and β-globine gene used as Internal Control is detected on the Fam/Green channel. If the sample is not correctly prepared or well stored (insufficient quantity < 2,0 x 10^4 genomes/sample) the Internal Control will not be detected or it comes very low.

Kit contains quantitative standards for simultaneous detection of DNA *HHV6* and β-globine gene in one tube which allows to avoid labouring procedure of leukocytes extraction from blood and their calculation.

Calculate the concentration of *HHV6* DNA in standard quantity of cells, for example in 10^6 human cells using the following formula:

Copies *HHV6* DNA in 10^6 cells* = copies *HHV6* DNA in reaction/copies human DNA in reaction x $2*10^6$

$*10^6$ cells contains $2*10^6$ human genomes

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V10-100FRT)

Part N° 2 – **"HHV6 Real-TM Quant":** Real Time amplification

- **PCR-mix-1 "HHV6/Glob"**, 2 x 0,6 ml
- **PCR- buffer-FRT**, 2 x 0,3 ml
- **TaqF Polymerase**, 2 x 0,03 ml
- **Negative Control**, 1,2 ml;*
- **Pos HHV6 & Human DNA C+,** 2 x 0,2 ml;**
- **DNA-buffer (C-)**, 0,5 ml
- **Quantitation Standard HHV6 & Glob:**
  - **QS1**, (concentration 10^4 copies/sample) 0,2 ml,
  - **QS2**, (concentration 10^2 copies/sample) 0,2 ml,

Contains reagents for 120 test.

Module No.2: Complete Real Time PCR test with DNA purification kit (TV10-100FRT)

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

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

Contains reagents for 100 tests.

Part N° 2 – **"HHV6 Real-TM Quant":** Real Time amplification

- **PCR-mix-1 "HHV6/Glob"**, 2 x 0,6 ml
- **PCR- buffer-FRT**, 2 x 0,3 ml
- **TaqF Polymerase**, 2 x 0,03 ml
- **Negative Control**, 1,2 ml;*
- **Pos HHV6 & Human DNA C+,** 2 x 0,2 ml;**
- **DNA-buffer (C-)**, 0,5 ml
- **Quantitation Standard HHV6 & Glob:**
  - **QS1**, (concentration 10^4 copies/sample) 0,2 ml,
  - **QS2**, (concentration 10^2 copies/sample) 0,2 ml,

Contains reagents for 120 test.

** 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 Pos HHV6 & Human DNA C+ 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

HHV6 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

HHV6 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

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic 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

HHV6 Real-TM Quant can analyze DNA extracted from:

- *whole blood* collected in either ACD or EDTA tubes;
- *buffy coat;*
- *sputum:* add 1 volume of sputum to 1 volumes of saline water and vortex vigorously. Centrifuge at 10000g/min for 10 min. Discard the supernatant and leave about 100 µl of solution for DNA extraction.
- *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.
- *Saliva*
- *Cerebrospinal fluid (liquor)*
- *Viscera biopsy material*

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)

⇒ **Ribo Virus 50**– spin column extraction kit (Sacace, REF K-2-C)

⇒ **SaMag Viral Nucleic Acids Extraction Kit** (Sacace, REF SM003) for cerebrospinal fluid and cell free body fluids;

⇒ **SaMag STD DNA Extraction Kit** (Sacace, REF SM007) for swabs.

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 HHV6 & Human DNA C+** 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 + 6 controls (4 standards, 1 Neg. control, 1 Pos. Control).
2. Prepare in the new sterile tube for each sample **10*N µl** of **PCR-mix-1 "HHV6/Glob"**, **5,0*N** of **PCR-Buffer-FRT** and **0,5*N µl** of **TaqF Polymerase.** Vortex and centrifuge for 2-3 sec.
3. Add **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA** sample to appropriate tube. Mix by pipetting.
4. Prepare for qualitative run 1 positive control and 1 negative control:
   - add **10 µl** of **QS2** to the tube labeled C*pos*;
   - add **10 µl** of **DNA-buffer** to the tube labeled C*neg*;

5. For quantitative analysis prepare 1 negative control (add **10 µl** of **DNA-buffer** to the tube labeled C*neg)* and 4 tubes for standards. Perform QS1 and QS2 standards twice by adding **10 µl** of **Quantitation Standards HHV6 & Glob** (QS1, QS2) into 4 labelled tubes;

Amplification

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

	Rotor-type Instruments[1]			**Plate- or modular type Instruments[2]**		
Step	***Temperature, °C***	***Time***	**Repeats**	***Temperature, °C***	***Time***	**Repeats**
1	**95**	15 min	1	**95**	15 min	1
2	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	20 s	
	72	15 s		**72**	15 s	
3	**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, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in FAM/Green and JOE/Yellow/Hex/Cy3 fluorescence channels.

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	*0.03*	*10 %*	*On*
JOE/Yellow	*from 4 Fl to 8 Fl*	*0.03*	*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; 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.

RESULT ANALYSIS:

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

Internal Control (Human DNA) is detected on the FAM/Green channel and *HHV6* on the Joe/HEX/Yellow channel .

Calculate the concentration of copies HHV6 DNA in 10^6 cells using the following formula:

$$HHV6\ copies\ \text{in}\ 10^6\ cells = \frac{HHV6\ DNA\ copies/reaction}{human\ DNA\ copies/reaction} \times 2\times 10^6$$

In quantitative analysis, if total DNA is isolated from whole human blood, white blood cells, or viscera biopsy material, the logarithmic concentration of HHV6 DNA copies per the standard cell quantity (10^5) in samples is calculated using the following formula:

$$\log\left(\frac{copies\ HHV6\ DNA}{copies\ human\ DNA} \times 200000\right) = \log\left(HHV6\ \text{in}\ 100000\ cells\right)$$

If total DNA is isolated from saliva, oropharyngeal swabs, and cerebrospinal fluid (liquor), the concentration of HHV6 DNA per ml of sample (Copies HHV6 DNA) is calculated using the following formula:

Copies *HHV6* DNA = Calc *HHV6* DNA x 100 (copies/ml),

where **Calc HHV6 DNA** is the number of HHV6 DNA copies/reaction calculated in Joe/HEX/Yellow channel.

The calculation can be made manually or using Microsoft® Excel program:

Name	Type	Copies *HHV*-6 (Joe/Yellow)/ reaction	Copies Glob (Fam/Green)/ reaction	lg *HHV*-6/10*5 cells	copies *HHV*-6/10*6 cells
A	B	C	D	E=LOG(C/D*200000)	F=C/D*2000000
1	Unknown	2907	56303	4.0	1.03E+05
1	Unknown	2958	52432	4.1	1.13E+05
2	Unknown	306	57845	3.0	1.06E+04
2	Unknown	309	52206	3.1	1.18E+04
3	Unknown	37	66964	2.0	1.11E+03
3	Unknown	39	69013	2.1	1.13E+03
C-	Unknown				
C-	Unknown				
C+	Unknown	296	9519	3.8	6.22E+04
C+	Unknown	269	9455	3.8	5.69E+04
QS1	Standard	9968	9894		
QS1	Standard	10024	10106		
QS2	Standard	97	105		
QS2	Standard	102	98		

The difference between 2 values of “Pos HHV6 & Human DNA C+” in one run doesn’t have to be higher than 25%.

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 6* primers and probes. The specificity of the kit **HHV6 Real-TM Quant** was 100%. The potential cross-reactivity of the kit **HHV6 Real-TM Quant** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **HHV6 Real-TM Quant** allows to detect *Human Herpes Virus 6* DNA in 100% of the tests with a sensitivity of not less than 200 copies/ml or 5 copies of HHV6 DNA per 10^5 cells. The linear range of **HHV6 Real – TM Quant** PCR kit is 500-10.000.000 HHV6 DNA copies/ml. If the result is less than 500 copies/ml, the results should be indicated as “less than 500 HHV6 copies/ml”.

Target region: polymerase gene HHV6

TROUBLESHOOTING

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

1. The appearance in the results grid of a Ct value in JOE/Yellow/HEX and FAM/Green channels for the negative control of amplification (NCA) and a Ct value in the JOE/Yellow/HEX channel for the negative control of extraction (C–) indicates contamination of reagents or samples. Repeat PCR analysis of all samples in which *HHV6* DNA was detected starting from the DNA extraction stage.
2. In qualitative analysis, if the Ct value for the positive control of amplification (C+, QS2) in the JOE/Yellow/HEX (*HHV6*) or FAM/Green channel is absent in the results grid, repeat amplification of all samples in which *HHV6* DNA was not detected.
3. If the Ct value for the positive control of extraction (PCE, **Positive Control DNA *HHV*-6 and human DNA**) in JOE (HHV6) or FAM channel is absent, the results of analysis of all samples are considered invalid. Repeat analysis of all samples starting from the DNA amplification stage.
4. If the Ct value for the sample in the JOE/Yellow/HEX channel is absent repeat analysis of the sample starting from the DNA extraction stage. This may be caused by errors in preparation of clinical material, which entailed the loss of DNA, or by the presence of inhibitors.
5. If the Ct value for a clinical sample in the JOE/Yellow/HEX channel (HHV6 DNA) exceeds the boundary Ct value (>36), the result of analysis of such samples is considered **equivocal**. Repeat analysis of such samples in duplicate. If a positive result is obtained in both replicates, the result of analysis is considered as **positive**. If the results in two replicates are discrepant, the result of analysis of such samples is considered **equivocal**.
6. If the number of copies per reaction in DNA calibrators in quantitative tests exceeds the specified value by more than 30%, check the order of placing the tubes in the rotor (DNA calibrators should be inserted into the cells named “Standard” in the table of samples and cell no. 1 in rotor-type instruments should not be empty (fill it with any test tube).
7. If the correlation coefficient **R** in the ***Standard Curve*** window in quantitative tests is less than 0.9, this indicates error in calibration. Check whether the settings for DNA calibrators are correct and change them, if necessary. If this does not help, run PCR for all samples and calibrators.

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	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

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