

HHV6 DNA QUANTITATION (QT)

**Real -Time PCR
for the HHV6 A-B genome
Quantification**

-for “in vitro” diagnostic use only-



DIA.PRO

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REF. HHV6DNAQT.CE
25/50/100/150 Tests

HHV6 DNA

A. INTENDED USE

The **HHV6 DNA Quantitation (QT)** Real-Time PCR kit coded **HHV6DNAQT.CE** is intended for the quantitative detection of Human herpesvirus type 6 DNA in human plasma/blood with a simultaneous control of the extraction/amplification reaction through an **Internal Control (IC)**.

HHV6DNAQT.CE assay was standardised against the 1st WHO International Standard for HHV6 (NIBSC code 15/266) to express samples concentration also in International Unit (IU/ml).

B. INTRODUCTION

Human herpesvirus Type 6 is a beta herpes virus that can exist in two variants, A and B, and infects nearly 90% of the population before two years of age. Furthermore, there is evidence of HHV6 infection in approximately 10% of febrile infants less than 90 days old. Primary infections in children often result in fever followed by development of exanthema subitum, also known as roseola infantum.

After primary infection, latency is established in myeloid and bone marrow progenitors and exists for the life time of the host. The virus periodically re-activates from this latent state, with HHV6 DNA detectable in healthy adults. Re-activation in immunocompetent individuals are often asymptomatic but in an immunocompromised clinical status it can lead to serious complications.

HHV6 reactivates in HIV-seropositive patients but specific clinical syndromes associated with reactivation are rare. Cases of pneumonitis and encephalitis are described most frequently. There is a greater evidence of disease association in organ transplanted recipients. Disease associated with HHV6 reactivation range from undifferentiated febrile illness to interstitial pneumonitis, hepatitis, encephalitis and transplanted organ rejection.

Molecular based assay such as real time PCR assays were demonstrated to be a useful tool for HHV6 detection because of high sensitivity, specificity, easy to use and quick method.

C. PRINCIPLE OF THE TEST

The HHV6DNAQT.CE Kit is based on a Real Time chemistry which uses specific Primers and Probes,

HHV6 DNA, recovered from the biological sample under investigation through an extraction step, and amplified using Real Time amplification system. The amplified product, is detected and quantified, against the standard curve using a fluorescent reporter dye probe specific for a HHV6 unique genomic sequence.

Heterologous Internal Control (IC) serves as an extraction/amplification control for each individually processed specimen aiming to the identification of reaction inhibitors.

A standard curve is supplied allowing the determination of the viral load.

D. COMPONENTS

The standard format of the product code HHV6DNAQT.CE contains reagents for 50 tests.

Component	Contents	HHV6DNAQT.CE 50 Reactions
A CODED: ALL/MM5 COLOR CODE: CLEAR	Master mix	N° 1 vials / 0.825 ml

B CODED: HHV6/CB COLOR CODE: YELLOW	Lyophilised Primers/Probes	N° 2 vials (Dissolve with the volume of ALL/C indicated on the vial label)
C CODED: ALL/C COLOR CODE: RED	MG Water	N° 4 vials /1.5 ml
NTC CODED: ALL/NTC COLOR CODE: WHITE	Negative Control	N° 1 vials /1.5 ml
STD Quantitation Standard (1.65x10 ⁵ copies/µl) CODED: HHV6/STD COLOR CODE: RED	Lyophilised Quantitative Standard	N°6 vials (Dissolve with the volume of ALL/C indicated on the vial label)
I.C. Internal Control CODED: ALL/IC COLOR CODE: GREEN	Lyophilised Internal Control	N° 2 vials (Dissolve with the volume of ALL/C indicated on the vial label)
Package Insert	Instruction for Use	1

Important note: Upon request, Dia.Pro can supply reagents for 25, 100, 150 tests, as reported below :

1. Component A	n°1 vial/0.4 ml	n°2 vials/0.825 ml	n°3 vials/0.825 ml
2. Component B	n°1 vial	n°4 vials	n°6 vials
3. Component C	n°2 vial/1.5 ml	n°4 vial/1.5 ml	n°6 vial/1.5 ml
4. NTC	n°1 vial/1.5 ml	n°1 vial/1.5 ml	n°1 vial/1.5 ml
6. IC	n°1 vial	n°4 vials	n°6 vials
7. STD	n°3 vial	n°4 vials	n°6 vials
8. Pack. insert	n°1	n°1	n°1
Number of tests	25	100	150
Code	HHV6DNAQT.CE.25	HHV6DNAQT.CE.100	HHV6DNAQT.CE.150

E. STORAGE AND STABILITY

The kit HHV6DNAQT.CE must be stored at +2...8 °C .

Once dissolved **Component B** (coded HHV6/CB) and **Component IC** (coded ALL/IC) are stable for 4 months at

-20°C. Once dissolved **Component STD** (coded HHV6/STD) is stable for 15 days at -20°C. Component STD stored at -20°C for a time ≤ 15 days can be used only in conjunction with Component B and ALL/IC frozen at -20 for a time ≤ 30 days.

If the components are to be used only intermittently, they should be frozen in aliquots, repeated thawing and freezing should be avoided. Only one defreezing is allowed.

F. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (0.5 µl < volume <1000 µl)
2. DNA extraction kit
3. MG EtOH
4. Thermal Block
5. Microcentrifuge
6. Tube racks
7. Sterile filtered tips with aerosol barrier
8. Nuclease-Free Microtubes
9. 0,2 ml Microtubes or Pcr Microplates recommended from the Real-Time PCR instruments manufacturers
10. Disposable gloves, powder-free

11. Real-Time PCR Thermalcycler (*)
12. Absorbent paper tissues.
13. Vortex or similar mixing tools.

(*) **Attention:** A valid calibration of the pure dyes (Pure Spectra Component File) and of the background (Background Component File) must be done routinely.

G. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible for the laboratory.
2. The technical personnel must be deeply trained in the use of Real-Time thermalcyclers, in the manipulation of Molecular Biology reagents and in the Real-Time PCR amplification protocols.
3. The kit has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
4. All the personnel involved in performing the assay have to wear protective laboratory clothes, powder-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
5. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
6. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-borne microbial agents,
7. Components A and B are light sensitive. Protect them from strong light exposition.
8. Avoid vibration of the bench surface where the test is undertaken.
9. Upon receipt, store the kit at 2..8°C into a temperature controlled refrigerator or cold room.
10. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.
11. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
12. Avoid cross-contamination between samples by using disposable tips and changing them after each sample.
13. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
14. Do not use the kit after the expiration date stated on the external container label.
15. Treat all specimens as potentially infective. All human serum/blood/plasma specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
16. Store and extract specimens separately from the other reagents and use a separate room for their handling
17. Dissolve the lyophilised reagents with the correct amount, (stated in the labels) of Component C (Coded: ALL/C) supplied in the kit.
18. Carry on all the working operations as quickly as possible maintaining the components on ice or in a cooling block.
19. The laboratory Workflow must proceed in a unidirectional way, beginning in the Extraction Area and moving to the Amplification and Data Analysis Areas. Do not return samples, equipment and reagents to the area where previous steps have been performed.
20. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.

21. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from sample extraction procedures, has to be treated as potentially infective material and inactivated before waste. Do not put in contact the extraction waste with bleach.

22. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.

23. Other waste materials generated (example: tips used for samples) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

H. SPECIMEN: PREPARATION AND RECOMMENDATIONS

1. Blood is drawn aseptically by venepuncture and plasma is prepared using standard techniques of preparation of samples for clinical laboratory analysis.

2. No influence has been observed in the preparation of the sample with citrate, EDTA.

Attention: Heparin (>10 IU/ml) affects the PCR reactions.

Samples, which has been collected in tubes containing heparin as an anticoagulant should not be used. Also, samples of heparinised patients must not be used.

3. Avoid any addition of preservatives to samples.

4. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results.

5. Haemolysed (red) and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.

6. Plasma, if not used immediately, must be aliquoted and stored at -20°..-80°C after collection. Samples can be stored frozen at -80°C for several months. Any frozen samples should not be frozen/thawed more than once as this may affect the test result.

7. The plasma samples for DNA extraction must be collected according to the common laboratory procedures, transported and stored at +2 / +8 °C for a maximum period of 4 hours. The plasma samples can be stored frozen at -20°C for a maximum period of 30 days or at -70 °C for longer periods.

8. We recommend you, for optimal storage of samples, to split them in several aliquots (minimum volume 300 µl) and store them frozen at -20°C for a maximum period of 30 day or -70°C for longer periods. Avoid repeated freezing / thawing cycles.

9. When using frozen samples, thaw the samples just immediately before the extraction in order to avoid possible cases of nucleic acid degradation.

10. The whole peripheral blood samples for DNA extraction must be collected in EDTA according to laboratory advices, transported and stored at +2 / +8 °C for a maximum period of 3 days. Do not freeze the whole peripheral blood samples to avoid cell lysis and viral titer loss

I. PREPARATION OF COMPONENTS AND WARNINGS

Master Mix:

Component A. Ready to use. Mix well on vortex before use and centrifuge briefly to collect the whole volume.

WARNING: *Component A is light sensitive. Protect it from strong light exposition.*

Primers/Probes:

Component B.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.

- Dissolve homogenously the Lyophilized Component B with the volume of water (Component C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

WARNING: Component B is light sensitive. Protect it from strong light exposition.

MG Water :

Component C. Ready to use.

Negative Control :

NTC. Ready to use.

Standard Curve:

STD.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized STD with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex
- Prepare the Nucleases-Free vials for the preparation of the Standard Curve
- Set up an STD 1:10 serial dilution in Component C (Code: ALL/C) to obtain the standard curve points as described in the table below:

Standard curve preparation		
STD	Calibrator 165000copies/ µl	Add the Volume of Component C (Code: ALL/C) as written on the vial label
STD 1	16500 copies/ µl	10 µl (STD) + 90 µl Component C (Code: ALL/C)
STD 2	1650 copies/ µl	10 µl (STD 1) + 90 µl Component C (Code: ALL/C)
STD 3	165 copies/ µl	10 µl (STD 2) + 90 µl Component C (Code: ALL/C)
STD 4	16.5 copies/ µl	10 µl (STD 3) + 90 µl Component C (Code: ALL/C)

Internal Control:

I.C.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding the powder dispersion.
- Dissolve homogenously the Lyophilized I.C. with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

L. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. **Micropipettes** have to be calibrated and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-5%.
2. **Extraction Device:** The HHV6DNAQT.CE Kit has been validated for the use in combination with QIAamp DNA Minikit Code: 51306 (QIAGEN), Nucleospin Blood kit Code: 740951 (Macherey Nagel) and OMNIA LH 75 PRO workstation (extraction reagents supplied with the instrument). The end users must strictly follow the Instruction for use supplied by the manufacturers.
3. **Real-Time Thermocyclers and Instrument Softwares:** The HHV6DNAQT.CE Kit has been validated for the use in combination with the Real Time Thermal cyclers ABI7500, software SDS version 1.3.1 (Applied Biosystems), CFX96, software CFX manager version 1.7 (Biorad), Gentier real-time PCR System 96E, and OEM derived Versions, software Realab system (Tianlong).
For the use of the HHV6DNAQT.CE Kit the end users must strictly follow the Instruments Instruction for use supplied by the manufacturers.
Important note: new versions software for those real-time PCR instrument should be evaluated for possible needs of a specific analysis setting (see section P).

M. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that on the bottom of the Lyophilized components vials is present a well formed aggregate. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box.
3. Dissolve the Lyophilized Components with the appropriate amount of Component C (Molecular Grade water) as described in the proper section (I).
4. Turn the Thermalcyclers on, check settings and be sure to use the right assay protocol.
5. Follow strictly the Instruments Manual supplied by the manufacturers for the correct setting of the Real-Time Thermalcyclers.
6. Check that the micropipettes are set to the required volume.
7. Check that all the other equipment is available and ready to use.
8. In case of problems, do not proceed further with the test and advise the supervisor.

N. ASSAY PROCEDURE

The assay has to be carried out according to what reported below.

N.1 DNA extraction

The extraction step of the HHV6 genomic DNA has to be carried out exclusively in combination with the following kits:

Manual Extraction tools

Material	Description	Kit code	manufacturer
Plasma/Blood	Nucleospin Blood	740951	MN™
Plasma/Blood	QIAamp DNA mini kit®	51306	Qiagen™

Automatic Extraction tool

Material	Description	Kit code	Manufacturer
Plasma/Blood	Extraction kit supplied with instrument	Masmec	OMNIA LH 75 PRO

The DNA isolation must be carried out only according to the Instruction Manual (QIAGEN™, MN™, Masmec).

Important note: The IC of the HHV6DNAQT.CE Kit is used in the isolation procedure as extraction control to evaluate if the DNA extraction procedure has been performed correctly (see section Q).

The following volumes have to be strictly used in the extraction procedures:

Description	Sample volume µl	IC volume µl	Elution volume µl
Nucleospin Blood	200	5	100
QIAamp DNA mini kit®	200	5	100
OMNIA LH 75 PRO	200	5	100

The DNA extracted from the samples, not used in the run, has to be stored frozen (-20°C.....-80°C).

For this application

- Nucleospin Blood and QIAamp DNA mini kit : add 5 µl of I.C. to the lysis buffer and sample mixture and proceed following the instruction manual supplied by the manufacturer of the Extraction Kit.

N.2 Setting up of the reaction

For PCR set-up follow the procedure reported in the next paragraph.

N.2.1 Preparing the PCR

Important: An example of dispensation scheme is reported in Section O. Please, refer to it before starting the operations described here below.

- Prepare the components as described in Section I;
- Prepare the required number of reaction tubes or a 96-well reaction plate for the samples under evaluation and for the Standard curve (prepared as described in Section I).

Important note: Use only optical tubes or microplates suggested by the Real-Time thermocyclers manufacturers.

- Consider that the samples, if possible, should be tested in duplicate;
- Include at least 1 tube for the NTC (negative control)
- Prepare the **Amplification Mix** for **Samples, NTC and standard curve** as table below:

Preparation of the Amplification Mix

(IC as Extraction/Amplification control)

Number of Reactions		x1	x12
A	Master mix	12.5 µl	150 µl
B	Primers/probes	2 µl	24 µl
C	MG Water	0.5 µl	6 µl
Tot vol.		15 µl	180 µl

N.2.2 Amplification procedure

- Dispense 15 ul of the amplification mix in each reaction tube or microplate well
- Add 10 ul of the **Samples, NTC and standard curve** to the reaction tubes.
- Close firmly the reaction tubes
- Centrifuge briefly the reaction tubes at 2000 rpm
- Don't leave the reaction tubes at room temperature (RT) for more than 30 minute and at light exposure (cover the tubes).
- Load the tubes in the Real-Time Thermacycler Thermoblock Holder.
- After the setting operations described in the Sections N3 (Instrument Programming) start the Thermacycler run.

Important note: The Components Lyophilized after dissolution in Component C (Code: ALL/C) are stable no more than 3 hours kept in ice or at 2°...8° °C.

At the end of the working day discard adequately the material leftover of the STD Dilution Points.

The not used volume of Component B, STD and I.C. can be freeze at -20°C and used as described in Section E.

N.3 Instrument programming

For programming the instrument refer to the Instrumentation Instruction Manual provided by the manufacturers.

N.3.1 Thermal Profile

The thermal profile is reported in the table below:

Step	Cycle	Temp.	Time
1	1	50°C	2 min
2	1	95°C	10 min
3	50	95°C	15 sec
		60°C (*)	1 min

IMPORTANT NOTE: (*) step for the real time data collection

WARNING: Keep attention to program the Real-Time Thermacycler with the correct Thermal Profile following the Instrument Manual supplied by the Instrument manufacturer.

N.3.2 Selection of the Detectors

Detectors have to be selected according to what described in the table below and in agree with the Instruction manuals of the Real-Time thermal cyclers used.

Detectors	HHV6	Internal Control (IC)	Passive Reference
Instrument	Fluorophore	Fluorophore	
ABI 7500 SDS	FAM - none	JOE - none	ROX
BIORAD CFX96®	FAM	VIC	-
TIANLONG GENTIER	FAM	JOE	-

According to the Instruction Manual of the Real-Time thermal cycler suggested (ABI 7500 Applied Biosystems, BioRad CFX96, , Gentier Tianlong) choose the sample type and load the Detectors as reported in the table below:

Sample Type	STD	SAMPLE (Unknown)	NTC
	Detectors	Detectors	Detectors
ALL INSTRUMENTS	HHV6	HHV6 IC	HHV6

WARNING: Keep attention to program the Real-Time Thermal cycler with the correct settings following the Instrument Manual supplied by the manufacturer.

O. ASSAY SCHEME

An example of dispensation scheme for Quantitative Analysis is reported below:

Microplate or tubes			
	1	2	3
A	STD 1 16500 copies/ µl	Sample 4	
B	STD 2 1650 copies/ µl	Sample 5	
C	STD 3 165 copies/ µl	Sample 6	
D	STD 4 16.5 copies/ µl	Sample 7	
E	NTC	Sample 8	
F	Sample 1	Sample 9	
G	Sample 2	Sample 10	
H	Sample 3	Sample 11	

Legenda: NTC = Negative Control STD 1,2,3,4 = HHV6 DNA Standard Curve, Sample 1,2,3,... = Samples under evaluation.

P. INTERNAL QUALITY CONTROL

P.1 Pre- Analysis setting

Before starting the analysis

- Set the “Baseline” (the background fluorescence level) as reported in the table below:

“Baseline”	
ABI™PRISM® 7500 SDS	Auto Baseline
BIORAD™ CFX96®	Auto Baseline
Tialong Gentier 96E	Baseline Gain setting

- Set manually the FAM/JOE/VIC fluorescence “Threshold”

FAM fluorescence “Threshold”	
ABI™PRISM® 7500 SDS	0.15
BIORAD™ CFX96	300
Tialong Gentier 96E	120

JOE/VIC fluorescence “Threshold”	
ABI™PRISM® 7500 SDS	0.10
BIORAD™ CFX96®	200
Tialong Gentier 96E	100

P.2 Data Analysis

A check is carried out on the STD calibrators any time the kit is used in order to verify the quality of the run. The following requirements must be fulfilled.

Check FAM	Requirements
STD 1	Ct (Threshold Cycle) < 24.0

Check FAM	Requirements
Slope	-3.9 < Slope < -3.1

Check FAM	Requirements
Efficiency	R ² > 0.98

Q. INTERPRETATION OF THE RESULTS AND TROUBLESHOOTING

For each samples FAM fluorescence (positive/negative Ct value) and Internal Control JOE/VIC fluorescence are assumed to validate HHV6 detection as described in the table below:

HHV6 FAM	Internal Control JOE/VIC	Assay Result
SAMPLE POSITIVE	20 < Ct < 40	CORRECT
	Ct > 40 or undetermined	INVALID*
SAMPLE NEGATIVE	20 < Ct < 40	CORRECT
	Ct > 40 or undetermined	INVALID*

IMPORTANT: High Initial concentration of HHV6 DNA in the sample (Positive FAM Signal) can lead to REDUCED Fluorescent Signal of Internal Control I.C. due to the reagents Competition.

* Problems may be occurred during the extraction step (presence of inhibitors or initial sample containing an insufficient number of cells) leading to an incorrect result. The test procedure must be repeated starting from the Extraction step using a fresh sample coming from the patient.

For each positive samples detected by kit code HHV6DNAQT.CE a correct quantitation of the viral load can be applied as reported in the table below:

HHV6 Viral load (IU/ml)	Interpretation of results
Quantity > 5.6E+07	HHV6 Viral Load > ULOQ
$2.5E+02 \leq Qty \leq 5.0E+06$	QUANTITATION IU/ml or copies/ml*
Quantity < 2.5E+02	HHV6 Viral Load < LLOQ

Important note: For samples quantitation refer to Section R

ULOQ = Upper Limit Of Quantification

LLOQ = Lower Limit Of Quantification

The results obtained with the HHV6DNAQT.CE Kit must be interpreted by the responsible of the laboratory keeping in consideration the clinical symptoms of the patients and the other laboratory markers of Infection.

The following results are possible:

Troubleshooting table

	FAM	JOE/VIC	Result	CHECK
SAMPLE unknown	+	+	CORRECT RESULT <u>Positive</u>	IMPORTANT: High Initial concentration of HHV6 DNA (Positive FAM Signal) can lead to REDUCED Fluorescent Signal of Internal Control I.C. due to the reagents Competition.
SAMPLE unknown	+/-	-	ATTENTION ! POSSIBILITY OF: Inhibition, error in the procedure or malfunctioning of the Instruments	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected FAM for the HHV6 detection and JOE/VIC for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube 7. That the Extraction procedure have been executed correctly;
SAMPLE unknown	-	+	CORRECT RESULT <u>Negative</u>	
STD	+	-	CORRECT RESULT	Negative JOE/VIC Signal is correct because the internal control was used as extraction control
STD	-	-	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected FAM for the HHV6 detection 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube
NTC	-	-	CORRECT RESULT	Negative JOE/VIC signal is correct because IC was used as extraction control
NTC	+	+/-	ATTENTION ! POSSIBILITY OF: Contamination	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the work space and Instruments are decontaminated at regular intervals; 4.that the kit has been stored correctly;

Important notes:

1. Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.
2. When test results are transmitted from the laboratory to an informatics centre, attention has to paid to avoid erroneous data transfer.

If the results of the test match the CORRECT ASSAY RESULT requirements stated above, proceed to the next section.

If one of more of the problems described in the table above happen, after checking, report any residual problem to the supervisor for further actions.

R. QUANTITATION

The STD calibrators are treated like purified samples and it uses the same volume, 10µl.

The STD calibrators concentration is expressed in copies/µl.

The **Viral Genome Concentration per mL** for each patient specimen is calculated applying the following formula:

$$\text{Results (copies/ml)} \equiv \frac{\text{copies/}\mu\text{l}(\text{run data}) \times \text{Elution sample volume (}\mu\text{l)}}{\text{Sample Extraction volume (ml)}}$$

Example:

$$\text{Results (copies/ml)} \equiv \frac{1500 \times 100}{0.2}$$

$$\text{Results (copies/ml)} \equiv 7.5 \text{ E}+05$$

To convert samples viral load expressed in copies/ml to IU/ml use the appropriate conversion factor as reported in the table below:

Extraction tools	Conversion Factor	Result (IU/ml) (*)
Manual Extraction	0.95	copies/ml ÷ 0.95
Automatic Extraction	1.40	copies/ml ÷ 1.40

*calibrated on 1st WHO International Standard (NIBSC code 15/266)

S. PERFORMANCES

Evaluation of Performances has been conducted in accordance to what reported in the Internal Technical Specifications or ITS. The performance evaluation was carried out in DiaPro's laboratories on materials supplied by the reference clinical labs.

S.1 ANALYTICAL SENSITIVITY

Analytical sensitivity may be expressed as **limit of detection** and as **limit of quantitation**.

Limit of detection (LOD): it is the lowest amount of target that can be detected by a test system with a stated probability.

For the NAT tests it is expressed as the smallest analyte concentration that tested in multiple repetitions gives positive results.

The **limit of detection (LOD)** is determined by testing serial dilutions containing known concentrations of the analyte.

The **LOD** is the lowest concentration of analyte that can be consistently detected (e.g. in ≥ 95% of samples under routine laboratory conditions).

In the kit code HHV6DNAQT.CE the **LOD** has been determined by testing serial dilutions (8 replicates for three different runs) of a positive sample. The results were analyzed by a **Probit** analysis, to determine the detection limit at 95%.

The results of the **PROBIT** Analysis are the following:

LOD Limit of Detection (p=0.05)	
TIANLONG GENTIER 96E	127 IU/ml
ABI™ PRISM® 7500 SDS	154 IU/ml
BIORAD™ CFX96®	153 IU/ml

S.1.1 Limit of quantitation

The **Limit of quantitation** was determined by measuring the **linearity**, the **dynamic range** and the **reproducibility**. The **Linearity** is the measure of the degree to which a curve approximates a straight line. It is expressed with the **SLOPE** value.

The **dynamic range** is the span of analyte concentrations for which the final output value (Ct threshold cycle) of the system is directly proportional to the analyte concentration, with acceptable trueness and precision.

The boundaries of the dynamic range are the lower and upper limits of quantitation (**Limit of quantitation**).

In the kit code HHV6DNAQT.CE a limiting dilution curve of 1st WHO International Standard for HHV6 (NIBSC code 15/266) with defined IU/ml were prepared. The dilution points were tested after DNA purification (extraction step) and their Ct (threshold cycle) determined.

The upper **limit of quantitation** is $7.75\log_{10}$ (5.6E+07 IU/ml) and the lower limit of quantitation is $2.40\log_{10}$ (2.5E+02 IU/ml).

IMPORTANT NOTE: For samples quantitation refer to [section Q and R](#)

S.2 ANALYTICAL SPECIFICITY

Analytical specificity is the ability of a method to detect and quantify only the DNA target.

The analytical specificity of HHV6 DNA assay has been studied as follow:

1. The primer/probe Set has been choose analysing the genome target sequence with an appropriate software
2. The primer/probe Set and the target genome sequence has been controlled by the "BLAST" software, in order to check if any of the nucleotide sequences deposited in the worldwide genomic banks has any homology with HHV6, and by the "ClustalX" software, in order to compare the genome target sequences of the different genotypes of HHV6.
3. The specificity was improved through the selection of stringent reaction conditions.
4. Samples coming from patients suffering infections due to potential interfering organisms were obtained from a reference Clinical Centre and tested.

The results are reported in the following table:

Organism	Result
CMV	negative
VZV	negative
EBV	negative
HHV8	negative
HSV1	negative
HSV2	negative
JC virus	negative
BK virus	negative

S.3 DIAGNOSTIC SPECIFICITY AND SENSITIVITY

S.3.1 Diagnostic Specificity:

Diagnostic specificity is the probability that the device gives a negative result in the absence of the target marker. So that **true negative** sample is a specimen known to be negative for the target marker and correctly classified by the device.

This parameter was studied by examining 8 HHV6 DNA negative plasma samples and 5 HHV6 DNA negative whole blood samples:

TRUE NEGATIVES	13
FALSE POSITIVES	0
TOTAL SAMPLES	13
SPECIFICITY %	100

On the basis of the results obtained **Diagnostic Specificity of the system has been calculated in the 100%.**

S.3.2 Diagnostic Sensitivity

Diagnostic sensitivity is the probability that the device gives a positive result in the presence of the target marker. So that **true positive** sample is a specimen known to be positive for the target marker and correctly classified by the device.

For the kit code HHV6DNAQT.CE this parameter was studied by examining QCMD 2019 and QCMD 2021 human herpes virus 6 panel samples Then it was been calculated the percentage (%) of positive samples.

TRUE POSITIVES	16
FALSE NEGATIVES	0
TOTAL SAMPLES	16
SENSITIVITY %	100

On the basis of the results obtained **Diagnostic Sensitivity of the system has been calculated in the 100%.**

Diagnostic Sensitivity	100 %
Diagnostic Specificity	100 %

S.4 PRECISION

Precision shows the degree of the system's reliability. Every measurement procedure has an inherent random variation called "random error". Random error does not have a number value but it is determined by dispersion of measurement as standard deviation (DevST) and coefficient variation (CV%). Usually precision of an assay refers to the agreement between replicate measurements of the same material.

In the kit code HHV6DNAQT.CE, **precision** was expressed as intra-assay variability and inter-assay variability. 4 dilution points (STD) in 3 replicates were tested In the same run (intra-assay) and in three different runs (inter-assay).

On the basis of the results obtained Intra and inter-assay variability were then calculated.

In absence of established International parameters we have identified the following value of acceptability for the HHV6DNAQT.CE Kit:

Intra-Assay Coefficient Variation (CV%) ≤ 10%.
Inter-Assay Coefficient Variation (CV%) ≤ 10%.

T. LIMITATIONS

The user of this kit is advised to carefully read and understand this package insert. Strict adherence to the protocol is necessary in order to obtain reliable test results. In particular, accurate sample and reagent pipetting, application of a correct workflow along with careful programming of thermocycling step is essential for accurate and reproducible HHV6 DNA detection and quantitation.











The determination of the HHV6 DNA in a patient sample has extensive medical, social, Psychological and economic implications.

It is recommended that confidentiality, appropriate counselling and medical evaluation be considered an essential aspect of the testing sequence.

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5. Symbols

LEGENDA			
	Product code		Storage temperature
	In Vitro Diagnostic Device		See use instructions
	Lot number		Manufacturer
	Expiry date		Number of tests
	CE conformity mark		Date of manufacturing

All the IVD Products manufactured by the company are under the control of a certified Quality Management System in compliance with ISO 13485 rule. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

Manufacturer
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