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For Professional Use Only

AmpliSens® *HHV*6-screen-titre-FRT

PCR kit

Instruction Manual

AmpliSens®

EC REP

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TABLE OF CONTENTS

1. INTENDED USE

AmpliSens® *HHV*6-screen-titre-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative and quantitative detection of *human herpes virus* type 6 (*HHV*6) DNA in clinical materials (whole human blood, white blood cells, viscera biopsy material, saliva, oropharyngeal swabs and cerebrospinal fluid (liquor)) by using real-time hybridization-fluorescence detection.

The results of PCR analysis are taken into account in complex diagnostics of disease.

2. PRINCIPLE OF PCR DETECTION

*HHV*6 DNA detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific *HHV*6 primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows detection of accumulating product without re-opening the reaction tubes after the PCR run. **AmpliSens® *HHV*6-screen-titre-FRT** PCR kit is a qualitative test based on the use of an endogenous control, the β-globin gene. The DNA target selected as an endogenous internal control is a human genome fragment that is present in a sample in a sufficient quantity equivalent to that of cells in the sample. **AmpliSens® *HHV*6-screen-titre-FRT** PCR kit uses “hot-start,” which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase by using a chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

3. CONTENT

AmpliSens® *HHV*6-screen-titre-FRT PCR kit is produced in 1 form:

AmpliSens® *HHV*6-screen-titre-FRT PCR kit variant FRT-100 F (for use with RG, iQ, Mx)

REF R-V10-T(RG,iQ,Mx)-CE.

AmpliSens® *HHV*6-screen-titre-FRT PCR kit variant FRT-100 F includes:

Reagent		***Description***	***Volume (ml)***	***Quantity***
PCR-mix-1-FRT *HHV*-6 / Glob		colorless clear liquid	0.6	2 tubes
PCR-mix-2-FRT		colorless clear liquid	0.3	2 tubes
Polymerase (TaqF)		colorless clear liquid	0.03	2 tubes
RNA-buffer		colorless clear liquid	0.6	1 tube
DNA calibrators	**KSG1**	colorless clear liquid	0.2	1 tube
	KSG2*	colorless clear liquid	0.2	1 tube
Negative Control (C–)**		colorless clear liquid	1.2	2 tubes
Positive Control DNA *HHV*-6 and human DNA***		colorless clear liquid	0.2	2 tubes

* is used as Positive Control of Amplification (C+).

** is used in the extraction procedure as Negative Control of Extraction (NCA).

*** is used in the extraction procedure as Positive Control of Extraction (PCE).

AmpliSens® *HHV*6-screen-titre-FRT PCR kit is intended for 110 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Hemolytic.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Personal thermocyclers (for example, Rotor-Gene 3000 or Rotor-Gene 6000 (Corbett Research, Australia); Rotor-Gene Q (Qiagen, Germany), iCycler iQ or iQ5 (Bio-Rad, USA), Mx3000P (Stratagene, USA), or equivalent).
- Disposable polypropylene microtubes for PCR (0.1- or 0.2-ml; for example, Axygen, USA).
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.

- Waste bin for used tips.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store and handle amplicons away from all other reagents.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats, protect eyes while samples and reagents handling. Thoroughly wash hands afterward.
- 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 samples and unused reagents in compliance with local authorities' requirements.
- Samples 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 another suitable disinfectant.
- Avoid contact with the skin, eyes, and mucosa. If skin, eyes, or mucosa contact, immediately flush with water and seek medical attention.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Area. 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.

6. SAMPLING AND HANDLING

Obtaining samples of biological materials for PCR-analysis, transportation and storage are described in the manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens® ***HHV*****6-screen-titre-FRT** PCR kit is intended for the analysis of DNA extracted with DNA extraction kits from whole human blood, white blood cells, viscera biopsy material, saliva, oropharyngeal swabs, and cerebrospinal fluid (liquor).

Whole peripheral and umbilical blood

Before extraction, it is necessary to pretreat blood. Add 1.0 ml of Hemolytic (**REF** 137-CE, manufactured by Federal Budget Institute of Science "Central Research Institute for Epidemiology") and 0.25 ml of whole blood to 1.5-ml Eppendorf tube using an individual tip. Carefully mix the contents of the tube by vortexing and incubate it for 10 min with periodic stirring. Centrifuge tubes at 8,000 rpm for 2 min. Remove the supernatant with a vacuum aspirator. Do not disturb the pellet. After washing, the pellet should be white. A small quantity of a pinkish film above the pellet (erythrocyte debris) is allowed. Washing with Hemolytic can be repeated, if necessary. Thus obtained leukocyte pellet should be lysed immediately (in case of RIBO-prep isolation, add 300 µl of Solution for Lysis and then isolate DNA according to the RIBO-prep instruction manual; do not add Solution for Lysis again) or it can be stored at or below - 68 °C for a long time.

Packed white cells of peripheral and/or umbilical blood

It is obtained from peripheral and/or umbilical blood. Blood can be stored for 6 hours after sampling at room temperature. To obtain white cells, centrifuge blood at 800-1,600 g (3,000 rpm) for 20 min. Then, collect the white film formed on the surface of the supernatant and carry out the pretreatment as described for whole peripheral and umbilical blood. White cells of peripheral and umbilical cord blood can be stored at or below –68 °C for a long time.

7. WORKING CONDITIONS

AmpliSens® *HHV6*-screen-titre-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. DNA extraction

It is recommended to use the following nucleic acid extraction kits:

- RIBO-prep, **REF** K2-9-Et-100-CE.
- DNA-sorb-B, **REF** K1-2-100-CE.
- DNA-sorb-C, **REF** K1-6-100-CE (for viscera biopsy material).

Extract DNA according to the manufacturer's instructions.

Transfer **100 µl** of **Negative Control** to the tube labeled C-. Transfer **90 µl** of **Negative Control** and **10 µl** of **Positive Control DNA *HHV*-6 and human DNA** to the tube labeled PCE.

8.2. Preparing PCR

8.2.1 Preparing tubes for PCR (variant FRT-100 F)

The total reaction volume is **25 µl**, the volume of DNA sample is **10 µl**.

1. Before starting work it is necessary to prepare the mixture of **PCR-mix-2-FRT** and

Polymerase (TaqF). Transfer the content of one tube with **Polymerase (TaqF) (30 µl)** into the tube with **PCR-mix-2-FRT (300 µl).** Avoid foaming. Mark each tube with the mixture preparation date.

The prepared mixture is intended for 60 sample analysis. The mixture can be stored at 2–8 ºC until use (for 3 months).

If the mixture cannot be used up for 3 months, prepare the mixture for a smaller number of reactions. For example, mix **150 µl of PCR-mix-2-FRT** and **15 µl of polymerase (TaqF).** Thus obtained mixture is intended for 30 reactions.

2. Prepare the reaction mixture. Note that, for analysis of even one test DNA sample in the qualitative format, it is necessary to run two controls of the PCR amplification stage: positive control (KSG2) and negative control of amplification (RNA-buffer). For assaying even one test DNA sample in the quantitative format, it is necessary to run five controls of the PCR amplification stage: two DNA calibrators (KSG1 and KSG2) in two replicates and the negative control of amplification (RNA-buffer). In addition, take reagents for one extra reaction.
3. Mix **PCR-mix-1-FRT *HHV*-6 / Glob** and the mixture of **PCR-mix-2-FRT** and **polymerase (TaqF)** prepared earlier in an individual tube in the following proportion:
   - **10 µl of PCR-mix-1-FRT *HHV*-6 / Glob,**
   - **5 µl of PCR-mix-2-FRT and polymerase (TaqF)** mixture.

   Calculate the required number of reactions including the test and control samples (see Appendix 1).

If 60 samples are analyzed simultaneously, a simplified scheme of mixture preparation can be used. Transfer the content of one tube with **PCR-mix-2-FRT** and the content of one tube with **polymerase (TaqF)** into the tube with **PCR-mix-1-FRT *HHV*-6 / Glob**.

4. Take the required number of tubes for amplification of test and control DNA samples. Transfer **15 µl** of the prepared mixture to each tube.
5. Add **10 µl** of **DNA samples** obtained from clinical or control samples at the stage of DNA extraction using tips with aerosol barrier.
6. <u>For qualitative analysis:</u>

NCA - Add **10 µl** of **RNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

C+ - Add **10 µl** of **KSG2** to the tube labeled C+ (Positive Control of Amplification).

<u>For quantitative analysis:</u>

NCA - Add **10 µl** of **RNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

DNA calibrators KSG1 and - Add **10 µl** of **KSG1** to the two tubes and add **10 µl** of **KSG2** to the other two tubes

8.2.2 Amplification

Program the real-time amplification instrument according to manufacturer's manual and Guidelines [2].

1. Create a temperature profile on your instrument as follows:

AmpliSens-1 amplification program

	Rotor-type instruments[1]			Plate-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	

Fluorescent signal is detected in the channels designed for the FAM/Green and JOE/Yellow/HEX fluorophores on the 2nd step (60 °C) of stage Cycling 2 (other channels are enabled if several tests are simultaneously carried out in a single run).

2. Adjust the fluorescence channel sensitivity according to *Important Product Information Bulletin.*

3. Insert tubes into the reaction module of the device.

4. Run the amplification program with fluorescence detection.

5. Analyze results after the amplification program is completed.

9. DATA ANALYSIS

The results are interpreted by the software of the used Instrument by the crossing (or not crossing) of the fluorescence curve with the threshold line.

β-globin gene DNA (IC Glob) is detected in the **FAM/Green** fluorescence channel, ***HHV6*** **DNA (Positive Control DNA *HHV*-6 and human DNA)** is detected in the **JOE/HEX/Yellow** fluorescence channel.

See the **Manufacturer's Manual** and **Important Product Information Bulletin** for data analysis settings.

1. *HHV*6 DNA is **detected** in a sample if its Ct value is defined in the results grid in the JOE/HEX/Yellow channel and it does not exceed the boundary value of positive result.
2. *HHV*6 DNA is **not detected** in a sample if its Ct value is not defined in the results grid in the JOE/HEX/Yellow channel (the fluorescence curve does not cross the threshold line)

[1] For example,Rotor-Gene 3000, Rotor-Gene 6000, Rotor-Gene Q or equivalent.
[2] For example, iCycler iQ5, Mx3000P, Mx3000 or equivalent.

and the Ct value in the results grid in the FAM/Green channel does not exceed Ct value indicated in the **Important Product Information Bulletin** for qualitative analysis. For quantitative analysis, the amount of IC Glob DNA should be greater than 2000 copies per reaction in the case of whole blood, white blood cells, and viscera biopsy material and greater than 500 copies per reaction in the case of saliva and oropharyngeal swabs.

For cerebrospinal fluid (liquor), the Ct value in the results grid in the FAM/Green channel can be greater than the boundary Ct value indicated in the **Important Product Information Bulletin** or the amount of IC Glob DNA can be less than 500 copies per reaction in the case of quantitative analysis because cerebrospinal fluid samples may contain a very small number of cells.

3. The result of analysis is **invalid** if the Ct value is not defined in the results grid (the fluorescence curve does not cross the threshold line) or if it is greater than the boundary value in JOE/HEX/Yellow channel and the Ct value in the results grid in the FAM/Green channel exceeds the Ct value indicated in the **Important Product Information Bulletin** for qualitative analysis. For quantitative analysis, the amount of IC Glob DNA is less than 2000 copies per reaction for whole blood, white blood cells, viscera biopsy material and if it is less than 500 copies per reaction for saliva, oropharyngeal swabs. In such cases, PCR should be repeated.
4. The result of analysis is **equivocal** in a sample if its Ct value in JOE/HEX/Yellow channel is greater than the Ct value indicated in the **Important Product Information Bulletin.** It is necessary to carry out the extra analysis for such sample in duplicate. If a reproducible positive Ct value is obtained, the result is considered to be positive. If epy obtained Ct values are not reproducible in duplicate, the negative result is considered to be **equivocal.**
5. For qualitative analysis, if the Ct value in the FAM/Green channel exceeds the Ct value indicated in the **Important Product Information Bulletin,** the result is considered to be **invalid.**

For quantitative analysis, if the amount of IC Glob DNA is less than 2000 copies per reaction in the case of whole blood, white blood cells, and viscera biopsy material or less than 500 copies per reaction in the case of saliva and oropharyngeal swabs, the quantitative positive or negative **result is considered to be invalid.**

The results of analysis are accepted as relevant if the results obtained for all controls (C-, PCE, NCA, C+, KSG1, and KSG2) are correct.

For quantitative analysis, the results for PCE should fall in the range of concentrations indicated in the **Important Product Information Bulletin.**

Results for controls

Control	Stage for control	Ct in channel				Interpre-tation
		FAM/Green		JOE/HEX/Yellow		
		qualitative analysis	quantitative analysis	qualitative analysis	quantitative analysis	
C–	DNA isolation, Amplification	Neg	Neg	Neg	Neg	OK
PCE	DNA isolation, Amplification	Ct < boundary value	Ct < boundary value	Ct < boundary value	Ct value is in borders indicated in the Important product information bulletin	OK
NCA	Amplification	Neg	Neg	Neg	Neg	OK
C+	Amplification	Ct < boundary value	-	Ct < boundary value	-	OK
KSG1, KSG2	Amplification	-	Ct value and rated concentration are defined	-	Ct value and rated concentration are defined	OK

*For boundary Ct values of the samples, Negative Control of Extraction and Positive Control of Amplification, see the **Important Product Information Bulletin**.

In quantitative analysis, if total DNA is isolated from whole human blood, white blood cells, or viscera biopsy material, the logarithmic concentration of *HHV*6 DNA copies per the standard cell quantity (10^5) in control and test samples is calculated by the following formula:

$$\log \{ \frac{\textit{HHV}6 \text{ DNA copies in PCR sample}}{\text{Glob DNA copies in PCR sample}} \times 2*10^5 \} = \log \{ \textit{HHV}6 \text{ DNA copies}/10^5 \text{ of cells} \}.$$

If total DNA is isolated from saliva, oropharyngeal swabs, and cerebrospinal fluid (liquor), the concentration of *HHV*6 DNA per ml of sample ($K_{P\ HHV6\ DNA}$) is calculated by the following formula:

$$K_{P\ HHV6\ DNA} = K_{HHV6\ DNA} \times 100 \text{ (copies/ml)},$$

where $K_{HHV6\ DNA}$ is the number of *HHV*6 DNA copies in the DNA sample.

10. 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+, KGS2) 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), FAM, or ROX/Orange 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 or exceeds the boundary Ct value and the Ct value in the FAM/Green channel exceeds the boundary Ct value specified for Internal Control, 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, 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, the concentration of samples should correspond to the concentration specified in the **Important Product Information Bulletin**, 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.

If you have any further questions or if you encounter problems, please contact our Authorized representative in the European Community.

11. TRANSPORTATION

AmpliSens® *HHV*6-screen-titre-FRT PCR kit should be transported at 2–8 ºC for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *HHV*6-screen-titre-FRT** PCR kit (except for PCR-mix-1-FRT *HHV*-6 / Glob, polymerase (TaqF) and PCR-mix-2-FRT) are to be stored at 2–8 °C

when not in use. All components of the **AmpliSens® *HHV*6-screen-titre-FRT** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

PCR-mix-1-FRT *HHV*-6 / Glob, polymerase (TaqF), and PCR-mix-2-FRT are to be stored at temperature from minus 24 to minus 16 ºC when not in use.

PCR-mix-1-FRT *HHV*-6 / Glob should be kept away from light.

13. SPECIFICATIONS

13.1. Sensitivity

The analytical sensitivity of **AmpliSens® *HHV6*-screen-titre-FRT** PCR kit is the following:

Clinical material	Nucleic acid extraction kit	Sensitivity
Cerebrospinal fluid (liquor), saliva, oropharyngeal swabs, and lavages	RIBO-prep	400 copies per 1 ml
Whole human blood, white blood cells and viscera biopsy material	RIBO-prep	5 DNA copies per 10^5 cells

Linear range of **AmpliSens® *HHV6*-screen-titre-FRT** PCR kit is **500–10.000.000 copies/ml.**

13.2. Specificity

The analytical specificity of **AmpliSens® *HHV6*-screen-titre-FRT** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequence comparison analysis. Nonspecific reactions were absent is tests with DNA of other viruses (herpes simplex virus types 1 and 2, Epstein-Barr human virus, human herpes virus type 8, varicella Zoster virus, parvovirus B19, and others), bacterial pathogens (*Staphylococcus aureus, Streptococcus pyogenes, Streptococcus agalactiae,* and others) and human DNA. The clinical specificity of **AmpliSens® *HHV6*-screen-titre-FRT** PCR kit was confirmed in laboratory clinical trials.

14. REFERENCES

1. Handbook “Sampling, Transportation, and Storage of Clinical Material for PCR Diagnostics”, developed by Federal State Institute of Science “Central Research Institute of Epidemiology” of Federal Service for Surveillance on Consumers’ Rights Protection and Human Well-Being, Moscow, 2008.
2. Guidelines “Real-time fluorescence PCR detection and quantitation of *Human Herpes Virus* type 6 (*HHV6*) DNA in various clinical samples”, developed by Federal Budget

Institute of Science “Central Research Institute for Epidemiology”, Moscow.

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science “Central Research Institute for Epidemiology” ISO 13485-Certified Quality Management System, each lot of **AmpliSens®** ***HHV*6-screen-titre-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

	Catalogue number		Sufficient for
	Batch code		Expiration Date
	*In vitro* diagnostic medical device		Consult instructions for use
	Version		Keep away from sunlight
	Temperature limitation	**NCA**	Negative control of amplification
	Manufacturer	**C–**	Negative control of extraction
	Date of manufacture	**C+**	Positive Control of Amplification
	Authorised representative in the European Community	**IC**	Internal control
	Caution		

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
07.12.11 VV	Specifications, Sensitivity	The linear measurement range was added