AmpliSens® *HHV*8-screen/monitor-FRT PCR kit

Instruction Manual

RUO

For Professional Use Only

KEY TO SYMBOLS USED

REF	Catalogue number	⚠	Caution
LOT	Batch code		Consult instructions for use
RUO	Research Use Only	Σ	Contains sufficient for <n> tests
VER	Version		Use-by Date
	Manufacturer	**IC**	Internal control
	Date of manufacture	**C1, C2**	DNA-calibrators
FBIS CRIE	Federal Budget Institute of Science "Central Research Institute for Epidemiology"	**C–**	Negative control of extraction
	Temperature limit	**NCA**	Negative control of amplification
	Keep away from sunlight	**PCE**	Positive control of amplification
	Keep dry		

1. INTENDED USE

AmpliSens® *HHV*8-screen/monitor-FRT PCR kit is an *in vitro* nucleic acid amplification test for quantitative detection of *human herpes virus* type 8 (*Human gammaherpesvirus* 8, *HHV*8) DNA in the biological material (venous plasma, venous whole blood, oropharyngeal swabs, saliva, biological (pleural, ascitic) fluids, tissue (biopsy, surgical, autopsy) material, paraffin-embedded tissue (biopsy, surgical, autopsy) material) using real-time hybridization-fluorescence detection of amplified products. The material for PCR is DNA samples extracted from test material.

Indications and contra-indications for use of the reagent kit

The reagent kit is used for investigation of biological material taken from the persons with suspected herpes virus infection associated with *HHV*8, without distinction of form and presence of manifestation.

There are no contra-indications with the exception of cases when the material cannot be taken for medical reasons.

NOTE: For research use only. Not for diagnostic procedures

2. PRINCIPLE OF PCR DETECTION

Principle of testing is based on the DNA extraction from the samples of test material together with the exogenous internal control (IC) and simultaneous amplification of DNA fragments of the detected microorganism and DNA of the exogenous and endogenous IC with hybridization-fluorescent detection.

DNA extraction from biological material is performed in the presence of the exogenous internal control sample (Internal Control-FL (IC)) which allows to control all stages of the PCR study for each sample and assess the effect of inhibitors on PCR study results. While the DNA extraction from biological material containing cells (venous whole blood, oropharyngeal swabs, tissue (biopsy, surgical, autopsy) material, paraffin-embedded tissue (biopsy, surgical, autopsy) material) the DNA fragment of the human β-globin gene (endogenous internal control) is amplified. Endogenous internal control (IC Glob) allows not only to control all stages of the PCR study for each sample, but also to evaluate the adequacy of material sampling and storage.

Amplification of DNA fragments with the use of specific primers and Taq-polymerase enzyme are performed with the DNA samples obtained at the extraction stage. In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

The quantitative analysis of *HHV*8 DNA is based on the linear dependence between the initial concentration logarithm of DNA target in a test sample and the cycle of beginning of fluorescence signal exponential growth (cycle threshold (*Ct*)). For the quantitative analysis amplification of DNA from the test samples is carried out simultaneously with DNA-calibrators – samples with the known concentration of the DNA target. Values for DNA calibrators and positive control are assigned according to the Manufacturer's measurement procedure. Based on the amplification results of DNA-calibrators a calibration line is plotted and it is used for the estimation of concentration of the DNA target in the test samples.

AmpliSens® *HHV*8-screen/monitor-FRT PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by using chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

Variant FRT-100 FN contains the system for prevention of contamination by amplicons using the enzyme uracil-DNA-glycosylase (UDG) and deoxyuridine triphosphate (dUTP).

The results of amplification are registered in the following fluorescence channels:

Table 1

Channel for fluorophore	FAM	JOE	ROX
DNA-target	β-globin gene region DNA (IC Glob)	*HHV*8 DNA	IC DNA
Target gene	β-globin gene	Minor capsid protein gene	Artificially synthesized sequence

3. CONTENT

AmpliSens® *HHV*8-screen/monitor-FRT PCR kit is produced in 3 forms:

variant FRT-100 FN, REF H-3581-1-1-CE;

variant FRT-100 FN in bulk[1], REF H-3581-1-1-CE-B;

variant FRT-L, REF H-3582-1-14-CE.

Variant FRT-100 FN includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL *HHV*8	clear liquid from colorless to light lilac colour	1.2	1 tube
PCR-buffer-H	colorless clear liquid	0.6	1 tube
C1 *HHV*8	colorless clear liquid	0.2	1 tube
C2 *HHV*8	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	2 tubes
Positive Control *HHV*8**	colorless clear liquid	0.1	1 tube
Internal Control-FL (IC)***	colorless clear liquid	1.0	1 tube

* must be used in the extraction procedure as Negative Control of Extraction.
** must be used in the extraction procedure as Positive Control of Extraction.
*** add **10 µl** of **Internal Control-FL (IC)** during the DNA extraction procedure directly to the sample/lysis mixture (see **RIBO-prep** or **MAGNO-sorb** protocols).

Variant FRT-100 FN is intended for 110 reactions (including controls).

Variant FRT-L includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix *HHV*8-Lyo	white powder	–	96 tubes of 0.2 ml
C1 *HHV*8	colorless clear liquid	0.5	1 tube
C2 *HHV*8	colorless clear liquid	0.5	1 tube
TE-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	2 tubes
Positive Control *HHV*8**	colorless clear liquid	0.1	1 tube
Internal Control-FL (IC)***	colorless clear liquid	1.0	1 tube

* must be used in the extraction procedure as Negative Control of Extraction.
** must be used in the extraction procedure as Positive Control of Extraction.
*** add **10 µl** of **Internal Control-FL (IC)** during the DNA extraction procedure directly to the sample/lysis mixture (see **RIBO-prep** or **MAGNO-sorb** protocols).

Variant FRT-L is intended for 96 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

For sampling and pretreatment

- Transport medium with mucolytic agent.
- Transport medium for storage and transportation of respiratory swabs.
- Reagent for pretreatment of whole peripheral and umbilical blood.
- 96% ethanol, 70% ethanol.
- o-Xylene, chemically pure (C.P.), or dewaxing agent.
- Buffered formalin for tissue fixation or specialized solutions designed for tissue fixation for subsequent molecular-biological analysis.
- Vacuum tubes for sampling, storage and transportation of blood samples.
- Flocked-swab for collecting, transportation and storage of biological samples.
- Disposable sterile scalpels or disposable scarifiers.
- Plastic container (50-60 ml) for storage and transportation of biological samples.
- Disposable screwed polypropylene 2.0-ml tubes with conical bottom and support skirt (self standing) and attached caps with sealing ring.
- Disposable tightly closed polypropylene 1.5 and 2.0-ml tubes for sampling and pretreatment.
- Sterile pipette tips with aerosol filters (up to 200 and 1,000 µl).
- Tube racks.
- Sterile tools (individual for each sample) for homogenization (porcelain mortar and pestle) or homogenizer for pretreatment of tissue material.
- Thermo-shaker for microtubes and PCR plates.
- Medical centrifuge with equipment.
- Desktop centrifuge up to 12,000 g (suitable for Eppendorf tubes).
- Vortex mixer.
- Vacuum aspirator with flask for removing supernatant.
- Pipettes (adjustable).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir to throw off and inactivate the material.
- Disposable powder-free gloves and a laboratory coat.

[1] In bulk form contains unlabeled tubes. Tubes with identical reagent are packed in one bag with label.

For DNA extraction and amplification

- DNA extraction kit.
- Sterile pipette tips with aerosol filters (up to 100 and 1,000 µl).
- Tube racks.
- Vortex mixer.
- PCR box.
- Real-time instruments (for example, Rotor-Gene Q (QIAGEN, Germany); CFX 96 (Bio-Rad, USA)).
- Disposable polypropylene tubes for variant FRT-100 FN:
  a) screwed or tightly closed 1.5-ml tubes for reaction mixture preparation.
  b) thin-walled 0.2-ml PCR tubes with optical transparent domed or flat caps or strips of eight 0.2-ml tubes with optical transparent caps if a plate-type instrument is used;
  c) thin-walled 0.2-ml PCR tubes with flat caps or strips of four 0.1-ml Rotor-Gene PCR tubes if a rotor-type instrument is used.
- Pipettes (adjustable).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.
- Disposable powder-free gloves and a laboratory coat.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol filters and use a new tip for every procedure.
- Store all extracted positive material (specimens, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use the PCR kit if the internal packaging was damaged or its appearance was changed.
- Do not use the PCR kit if the transportation and storage conditions according to the Instruction Manual were not observed.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid inhalation of vapors, samples and reagents contact with the skin, eyes, and mucous membranes. Harmful if swallowed. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice if necessary.
- While observing the conditions of transportation, operation and storage, there are no risks of explosion and ignition.
- Safety Data Sheets (SDS) are available on request.
- The PCR kit is intended for single use for PCR analysis of specified number of samples (see the section “Content”).
- The PCR kit is ready for use in accordance with the Instruction Manual. Use the PCR kit strictly for intended purpose.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where 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

AmpliSens® *HHV*8-screen/monitor-FRT PCR kit is intended for analysis of the DNA extracted with DNA extraction kits from the biological material (venous plasma, venous whole blood, oropharyngeal swabs, saliva, biological (pleural, ascitic) fluids, tissue (biopsy, surgical, autopsy) material, paraffin-embedded tissue (biopsy, surgical, autopsy) material).

Sampling

Venous plasma. To obtain the venous plasma samples, blood should be taken after overnight fasting or in 3 hour after eating by a disposable 0.8-1.1 mm diameter needle into the tube with EDTA (special vacuum system Vacuette® (lavender caps – 6 % EDTA)). After blood sampling the tube should be smoothly rotated several times for the thoroughly mixing with the anticoagulant. During 6 hours after blood sampling plasma should be transferred into a new tube. To do this the tubes with venous whole blood should be centrifuged at 3,000 rpm for 10 min at room temperature (from 18 to 25 °C). No less than 1 ml of obtained plasma is transferred by separate filter tips into sterile dry 2.0-ml tubes.
The venous plasma samples can be stored before PCR analysis:
– at the temperature from 2 to 8 °C – for 5 days;
– at the temperature from minus 24 to minus 16 °C – for 3 months;
– at the temperature not more than minus 68 °C – for a long time.
Only one freeze-thaw cycle is allowed.
Venous whole blood should be taken after overnight fasting or in 3 hours after eating by a disposable 0.8-1.1 mm diameter needle into the tube with EDTA (special vacuum system Vacuette® (lavender caps – 6 % EDTA)). After blood sampling the tube should be smoothly rotated several times for the thoroughly mixing with the anticoagulant. (Otherwise, blood will coagulate and DNA extraction will be impossible!). Place the tube in the rack after rotating.
Venous whole blood samples can be stored before pretreatment:
– at the temperature from 18 to 25 °C – for 2 hours;
– at the temperature from 2 to 8 °C – for 3 days after sampling of biological material.
Freezing of venous whole blood samples is unallowable!
Oropharyngeal swab is collected using sterile dry viscose swab with rotating movements from the surface of the palatine arches and the posterior wall of the oropharynx.
When the material is collected, insert the swab tip into a sterile disposable tube with 500 µl of transport medium for storage and transportation of respiratory swabs. Carefully break off the polystyrene stick at a distance of no more than 0.5 cm from the working part and leave the working part of the swab with the biological material in the tube. Close the tube with the solution and the working part of the swab.
Oropharyngeal swabs can be stored before PCR analysis:
– at the temperature from 18 to 25 °C – for 6 hours;
– at the temperature from 2 to 8 °C – for 3 days;
– at the temperature from minus 24 to minus 16 °C – for 3 months;
– at the temperature not more than minus 68 °C – for a long time.
Only one freeze-thawing cycle is acceptable.
Saliva should be obtained after rinsing the oral cavity with water. Take saliva into sterile dry 2.0-ml tubes or in sterile plastic container (50-60 ml) in an amount not less than 0.5 ml.
The samples of test material can be stored before PCR analysis:
– at the temperature from 18 to 25 °C – for 6 hours;
– at the temperature from 2 to 8 °C – for 24 hours;
– at the temperature from minus 24 to minus 16 °C – for 3 months;
– at the temperature not more than minus 68 °C – for a long time.
Only one freeze-thaw cycle is allowed.
Pleural fluid. Pleural puncture (pleurocentesis or thoracentesis) is performed using a special instrument, a needle or a plastic catheter, which pierces the chest wall and allows the fluid to be drawn out. At least 0.5 ml of pleural fluid is taken into disposable sterile plastic tubes with a volume of at least 2 ml or into containers.
Pleural fluid can be stored before PCR analysis:
– at the temperature from 2 to 8 °C – for 24 hours;
– at the temperature from minus 24 to minus 16 °C – for 3 months;
– at the temperature not more than minus 68 °C – for a long time.
Only one freeze-thawing cycle is acceptable.
Ascitic fluid. Laparocentesis is performed by piercing the abdominal wall with a special instrument such as metal tube and inserted triangular needle. After the puncture, the needle is removed and the fluid is drawn out. At least 1.0 ml of ascitic fluid is taken into disposable sterile plastic tubes with a volume of at least 2 ml or into containers.
Ascitic fluid can be stored before PCR analysis:
– at the temperature from 2 to 8 °C – for 24 hours;
– at the temperature from minus 24 to minus 16 °C – for 3 months;
– at the temperature not more than minus 68 °C – for a long time.
Only one freeze-thawing cycle is acceptable.
Tissue (biopsy, surgical, autopsy) material. The material is taken from the area of probable location of infection agent, from the lesional tissue or the area surrounding lesional tissue.
Tissue samples not more than 5 mm in diameter are placed into 2.0-ml disposable sterile tubes with 500 µl of transport medium with mucolytic agent. The tube is to be closed tightly. Tissue samples over 5 mm in diameter are placed into disposable plastic 50-ml containers with a wide neck.
The tissue (biopsy, surgical, autopsy) material can be stored before pretreatment:
– at the temperature from 2 to 8 °C – for 24 hours;
– at the temperature from minus 24 to minus 16 °C – for 3 months;
– at the temperature not more than minus 68 °C – for a long time.
Only one freeze-thawing cycle is acceptable.
Paraffin-embedded tissue (biopsy, surgical, autopsy) material is collected, transported and stored for molecular-biological analysis according to the national legislation.

NOTE: Do not use acidic formalin to fix the tissue material.

Sampling is performed by two methods:
1. Cut paraffin blocks on a microtome for paraffin sections using a separate disposable knife for each type of biological material. Transfer the samples (4-5 slices with a total size of 2-3 mm^3 excluding the volume of paraffin) using tweezers to a sterile 2.0-ml disposable screw cap tube with attached cap and sealing ring.
2. Remove 2-3 mm^3 of tissue fragments from the paraffin block using a sterile disposable scalpel or scarificator (excluding the volume of paraffin). Transfer the specimens to a sterile 2.0-ml disposable screw cap tube with attached cap and sealing ring.

Paraffin-embedded tissue (biopsy, surgical, autopsy) material can be stored before pretreatment at the temperature from 18 to 25 °C – for a long time while preventing the paraffin blocks from melting.

Pretreatment

Pretreatment of venous plasma, oropharyngeal swabs, saliva, pleural fluid and ascitic fluid samples is not required.
Venous whole blood is to be pretreated.
Transfer 250 µl of venous whole blood to the disposable 1.5-ml tube. Add 1.0 ml of Hemolytic. Gently vortex the tubes and leave them for 10-15 minutes at room temperature (from 18 to 25 °C), stirring occasionally. Centrifuge at 8,000 rpm for 3 min. Remove the supernatant using vacuum aspirator leaving 100 µl of the pellet. After washing the cell pellet should be white, only a small pinkish bloom on the pellet is allowed (the remains of the destroyed erythrocytes). The washing using hemolytic may be repeated if necessary. The obtained pellet must be immediately lysed (in case of extraction using **RIBO-prep** reagent kit add **300 µl of Solution for Lysis** and then extract DNA in accordance with the *Instruction Manual* enclosed to the RIBO-prep reagent kit without adding **Solution for Lysis** once again).
The pretreated with solution for lysis venous whole blood samples can be stored before extraction at the temperature from minus 24 to minus 16 °C for one year.
Only one freeze-thaw cycle is allowed.
Tissue (biopsy, surgical, autopsy) material is to be pretreated.
Tissue (biopsy, surgical, autopsy) material not more than 5 mm in diameter which is placed in 2.0-ml disposable sterile tubes with transport medium with mucolytic agent is not to be pretreated.
Place tissue (biopsy, surgical, autopsy) material with diameter of 5 to 10 mm into a sterile porcelain mortar and grind it with a pestle. Add 1 ml of transport medium with mucolytic agent to the obtained homogenate and mix thoroughly with a pestle. Use 100 µl of the suspension for DNA extraction.
The pretreated samples can be stored before extraction:
– at the temperature from minus 24 to minus 16 °C – for 3 months;
– at the temperature not more than minus 68 °C – for a long time.
Only one freeze-thaw cycle is allowed.
Paraffin-embedded tissue (biopsy, surgical, autopsy) material is to be pretreated.
The collected samples of paraffin-embedded tissue (biopsy, surgical, autopsy) material should be deparaffinized using special reagents such as o-xylene, and then a series of washes with a decreasing concentration of ethanol solution (similar to standard histological processing). If commercial deparaffinization solutions are used, proceed according to the instructions manual. Take 100 µl of a sample without stromal tissue fragments for DNA extraction.
The pretreated samples can be stored before extraction:
– at the temperature from minus 24 to minus 16 °C – for 3 months;
– at the temperature not more than minus 68 °C – for a long time.
Only one freeze-thaw cycle is allowed.

Interfering substances and limitations of using test material samples

The next samples are inapplicable for analysis:
– venous whole blood samples and venous plasma, collected in the tubes with heparin as anticoagulant,
– venous whole blood samples, containing blood clot or which has been exposed to freezing,
– paraffin-embedded tissue material samples fixed with acidic formalin.

In order to control the DNA extraction efficiency and possible reaction inhibition the Internal Control (Internal Control-FL (IC)) is used in the PCR kit. The Internal Control is added in each biological sample at the extraction stage. The presence of internal control signal after the amplification testifies the effectiveness of nucleic acid extraction and the absence of PCR inhibitors.

Potential interfering substances

Endogenous and/or exogenous substances that may be present in the biological material (venous plasma, venous whole blood, oropharyngeal swabs, saliva, biological (pleural, ascitic) fluids, tissue (biopsy, surgical, autopsy) material, paraffin-embedded tissue (biopsy, surgical, autopsy) material) used for the study were selected to assess potential interference.
Samples without adding and with the addition of endogenous and exogenous potential interfering substances were tested. The concentration of each potential interfering substance is shown in Table 2. Samples of test material contained quality control sample (QCS) with *HHV*8 DNA concentration of $1x10^3$ copies/ml.

Table 2

Test material	Type of potential interferent	Potential interferent	Tested concentration in a sample	Nucleic acid extraction kit	Interference presence
Venous plasma	Endogenous substances	Whole blood	5 %	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
	Exogenous substances	Prospidine	200 mg/ml	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
		Lithium heparin	from 12 to 30 IU/ml	RIBO-prep	Detected
				MAGNO-sorb	Detected
		Potassium EDTA	2.0 mg/ml	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
Venous whole blood	Endogenous substances	Total bilirubin	210 µmol/l (upper limit of normal – 21 µmol/l)	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
		Total cholesterol	77.6 mmol/l (upper limit of normal – 7.8 mmol/l)	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
		Triglycerids	37.6 mmol/l (upper limit of normal – 3.7 mmol/l)	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
		Hemoglobin	250 g/l (upper limit of normal – 170 g/l)	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
	Exogenous substances	Lithium heparin	from 12 to 30 IU/ml	RIBO-prep	Detected
				MAGNO-sorb	Detected
		Potassium EDTA	2.0 mg/ml	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
		Prospidine	200 mg/ml	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
Oropharyngeal swab	Endogenous substances	Whole blood	5 %	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
		Mucin	0.15 mg/ml	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
	Exogenous substances	Chlorhexidine aqueuos solution	2.5 %	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
Saliva	Endogenous substances	Whole blood	5 %	RIBO-prep	Not detected
		Mucin	0.15 mg/ml	RIBO-prep	Not detected
	Exogenous substances	Chlorhexidine aqueuos solution	2.5 %	RIBO-prep	Not detected
Biological (pleural, ascitic) fluids	Endogenous substances	Albumine	500 mg/l	RIBO-prep	Not detected
		Whole blood	5 %	RIBO-prep	Not detected
	Exogenous substances	Prospidine	200 mg/ml	RIBO-prep	Not detected
Tissue (biopsy, surgical, autopsy) material	Endogenous substances	Whole blood	5 %	RIBO-prep	Not detected
	Exogenous substances	Prospidine	200 mg/ml	RIBO-prep	Not detected
Paraffin-embedded tissue (biopsy, surgical, autopsy) material	Exogenous substances	Acidic formalin	—	RIBO-prep	Detected
		Buffered formalin	—	RIBO-prep	Not detected

7. WORKING CONDITIONS

AmpliSens® *HHV*8-screen/monitor-FRT PCR kit should be used at the temperature from 20 to 28 °C and relative humidity from 15 to 75 %.

8. PROTOCOL

8.1. DNA extraction

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

- **RIBO-prep** for DNA extraction from venous plasma, venous whole blood, oropharyngeal swabs, saliva, biological (pleural, ascitic) fluids, tissue (biopsy, surgical, autopsy) material, paraffin-embedded tissue (biopsy, surgical, autopsy) material;
- **MAGNO-sorb** for DNA extraction from venous plasma, venous whole blood, oropharyngeal swabs.

If using the RIBO-prep reagent kit extract the DNA according to the manufacturer's protocol.

The volumes of reagents and samples when the DNA is extracted by the RIBO-prep reagent kit:

The DNA extraction for each sample is carried out in the presence of **Internal Control-FL (IC).**

Add **10 µl** of **Internal Control-FL (IC)** to each tube.

The volume of the test sample is **100 µl.**

Add **100 µl** of **Negative Control (C–)** into the tube labeled C– (Negative Control of Extraction).

Add **10 µl** of **Positive Control *HHV*8** and **90 µl** of **Negative Control (C–)** into the tube labeled PCE (Positive Control of Extraction).

The volume of elution:

- **50 µl** (in case of using variant FRT-100 FN for the amplification);
- **100 µl** (in case of using variant FRT-L for the amplification).

If using the MAGNO-sorb reagent kit extract the DNA according to the manufacturer's protocol.

The volumes of reagents and samples when the DNA is extracted by the MAGNO-sorb reagent kit:

The DNA extraction for each sample is carried out in the presence of **Internal Control-FL (IC)**.

Add **10 µl** of **Internal Control-FL (IC)** to each tube.

The volume of the test sample is **200 µl**.

Add **200 µl** of **Negative Control (C–)** into the tube labeled C– (Negative Control of Extraction).

Add **20 µl** of **Positive Control *HHV*8** and **180 µl** of **Negative Control (C–)** into the tube labeled PCE (Positive Control of Extraction).

The volume of elution:

- **50 µl** (in case of using variant FRT-100 FN for the amplification);
- **100 µl** (in case of using variant FRT-L for the amplification).

8.2. Preparing PCR

8.2.1 Preparing tubes for PCR

The type of tubes depends on the PCR instrument used for analysis. Use disposable filter tips for adding reagents, DNA and control samples into tubes.

Variant FRT-100 FN

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

1. Calculate the required quantity of each reagent for reaction mixture preparation. For one reaction:
   - **10 µl** of **PCR-mix-FL *HHV*8,**
   - **5 µl** of **PCR-buffer-H.**

   Prepare the reaction mixture for the total number of test and control samples plus some extra reaction. See numbers of control samples in item 7.

NOTE: Prepare the reaction mixture just before use.

2. Thaw the tubes with **PCR-mix-FL *HHV*8** and **PCR-buffer-H**. Thoroughly vortex all the reagents of the PCR kit and sediment the drops by vortex.
3. In a new tube prepare the reaction mixture. Mix the required quantities of **PCR-mix-FL *HHV*8** and **PCR-buffer-H**. Sediment the drops by vortex.
4. Take the required number of the tubes or strips for PCR of DNA of test and control samples.
5. Transfer **15 µl** of the prepared reaction mixture to each tube. Discard the unused reaction mixture.
6. Add **10 µl** of **DNA samples** obtained by extraction of test and control samples.

NOTE: Avoid transferring the sorbent together with the DNA samples extracted by magnetic separation.

7. Carry out the control amplification reactions:

C1 – Add **10 µl** of **C1 *HHV*8** to the two tubes labeled **C1**.

C2 – Add **10 µl** of **C2 *HHV*8** to the two tubes labeled **C2**.

C– – Add **10 µl** of **the sample extracted from the Negative Control (C–) reagent** to the tube labeled C– (Negative control of Extraction).

PCE – Add **10 µl** of **the sample extracted from the Positive Control *HHV*8 reagent** to the tube labeled PCE (Positive control of Extraction).

NOTE: It is also necessary to carry out Negative Control of Amplification (NCA) at suspicion on possible contamination.

NCA – Add **10 µl** of **TE-buffer** to the tube labelled NCA (Negative Control of Amplification).

Variant FRT-L

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

Use disposable filter tips for adding reagents, DNA and control samples into tubes.

1. Take the required number of the tubes with ready-to-use lyophilized reaction mixture **PCR-mix *HHV*8-Lyo** for amplification of DNA from test and control samples (see the number of control samples in point 3).
2. Add **25 µl** of **DNA samples** obtained by extraction of test and control samples.

NOTE: Avoid transferring the sorbent together with the DNA samples extracted by magnetic separation.

NOTE: Mix the tubes thoroughly by pipetting avoiding foaming.

3. Carry out the control amplification reactions:

C1 – Add **25 µl** of **C1 *HHV*8** to the two tubes labeled **C1**.

C2 – Add **25 µl** of **C2 *HHV*8** to the two tubes labeled **C2**.

C– – Add **25 µl** of **the sample extracted from the Negative Control (C–) reagent** to the tube labeled C– (Negative control of Extraction).

PCE – Add **25 µl** of **the sample extracted from the Positive Control *HHV*8 reagent** to the tube labeled PCE (Positive control of Extraction).

NOTE: It is also necessary to carry out Negative Control of Amplification (NCA) at suspicion on possible contamination.

NCA – Add **25 µl** of **TE-buffer** to the tube labelled NCA (Negative Control of Amplification).

NOTE: Mix the tubes thoroughly by pipetting avoiding foaming.

8.2.2. Amplification

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

Table 3

AmpliSens unified amplification program for rotor-type[2] and plate-type[3] instruments

Step	Temperature, °C	Time	Fluorescent signal detection	Cycles
1	50	15 min	–	1
2	95	15 min	–	1
3	95	10 s	–	45
	60	20 s	FAM, JOE, ROX	

NOTE: Any combination of the tests (including tests with reverse transcription and amplification) can be performed in one instrument simultaneously with the use of the unified amplification program. If several tests in "multiprime" format are carried out simultaneously, the detection is enabled in other used channels except for the specified ones. If only the tests for DNA detection are performed in one instrument then the first step of reverse transcription (50 °C – 15 min) can be omitted for time saving.

Fluorescent signal is detected in the channels for the **FAM**, **JOE** and **ROX** fluorophores. If several tests are carried out simultaneously, the detection is enabled in other used channels.

2. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin*.
3. Insert tubes into the reaction module of the device.

NOTE: It is recommended to sediment drops from walls of tubes by short centrifugation before placing them into the instrument.
Insert empty tubes at the edges of reaction module in case of incomplete filling of plate-type instrument.

4. Run the amplification program with fluorescence detection.
5. Analyze results after the amplification program is completed.

[2] For example, Rotor-Gene Q (QIAGEN, Germany).
[3] For example, CFX 96 (Bio-Rad, USA).

9. DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in three channels:

Table 5

Channel for the fluorophore	FAM	JOE	ROX
Amplification product	Human DNA (IC Glob)	*HHV*8 DNA	Internal Control-FL (IC) DNA

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a *Ct* value of the DNA sample in the corresponding column of the results grid.

Based on the obtained *Ct* values and specified concentration values of DNA-calibrators C1 and C2, a calibration line is automatically plotted and the concentration values of *HHV*8 DNA, human DNA (IC Glob) and Internal Control-FL (IC) DNA in copies/reaction are calculated. The obtained values are used for calculation of *HHV*8 DNA copies in 1 ml of test and control samples:

$$\frac{\text{number of } \textit{HHV}\text{8 DNA copies per reaction}}{\text{number of Internal Control-FL (IC) DNA copies per reaction}} \times A \times B = \text{copies /ml}$$

where;

A is the coefficient taking into account the volume of extraction. It is calculated by the formula:

$$A = \frac{100}{\text{extraction volume (µl)}}$$

B is the number of copies of IC in 1 ml of test sample. The coefficient takes into account the DNA loss during extraction.

The obtained values of *HHV*8 DNA concentration (during extraction from venous whole blood and tissue (biopsy, surgical, autopsy) material) can be normalized to the standard number of human cells (number of *HHV*8 DNA copies per 10^5 human cells). Normalized *HHV*8 DNA concentration values are calculated according to the formula:

$$\lg\left(\frac{\text{number of } \textit{HHV}\text{8 DNA copies per reaction}}{\text{number of IC Glob DNA copies per reaction}} \times 2*10^5\right) = \lg(\text{number of } \textit{HHV}\text{8 copies per } 10^5 \text{ of human cells})$$

Normalized concentration values reflect the number of pathogen cells in relation to the number of human cells. The value of the human DNA concentration allows to evaluate the quality of the collected biological material.

A conversion factor is used to express the relative concentration of *HHV*8 DNA in lg copies per standard number of cells:

10^5 cells = $2x10^5$ human genomes

NOTE: The values of calibrators' concentrations and coefficient B are specified in the *Important Product Information Bulletin* enclosed to the given lot of PCR kit and couldn't be used for result calculation in analysis with the use of another lot reagents.

Principle of interpretation is the following:

Table 6

Results Interpretation for the test samples

Result	Interpretation
Invalid	The *Ct* value in the channel for the ROX fluorophore is absent or determined greater than the boundary value. The PCR analysis (beginning with the DNA extraction stage) should be repeated for this sample
Invalid (when analyzing venous whole blood analysis and tissue (biopsy, surgical, autopsy) material)	IC Glob DNA concentration is less than 2,000 copies/reaction and the value of calculated concentration is absent in the channel for the JOE fluorophore. The PCR analysis (beginning with the DNA extraction stage) should be repeated for this sample. If IC Glob DNA is absent in the test sample the biological material sampling and PCR analysis should be repeated
Invalid (when analyzing oropharyngeal swabs)	IC Glob DNA concentration is less than 500 copies/reaction and the value of calculated concentration is absent in the channel for the JOE fluorophore. The PCR analysis (beginning with the DNA extraction stage) should be repeated for this sample. If IC Glob DNA is absent in the test sample the biological material sampling and PCR analysis should be repeated
*HHV*8 DNA is not detected	The *Ct* value for *HHV*8 DNA is absent or greater than the boundary value, the *Ct* value determined in the channel for the ROX fluorophore is less than the boundary value
Less than 500 *HHV*8 DNA copies/ml	The concentration of detected *HHV*8 DNA is less than the lower limit of measurement range of the PCR kit
X x 10^y *HHV*8 DNA copies/ml	The concentration of detected *HHV*8 DNA falls within the measurement range of the PCR kit
Greater than $1x10^7$ *HHV*8 DNA copies/ml	The concentration of detected *HHV*8 DNA is greater than the upper limit of measurement range of the PCR kit. If the accurate quantification is required, the DNA sample is to be diluted by TE-buffer reagent (for example, 10-fold dilution) and the PCR-analysis is to be repeated from the amplification stage. The result obtained after repeated analysis should be multiplied by the coefficient of the sample dilution

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The result of the analysis is considered reliable only if the results obtained for the controls of extraction and amplification are correct (see Table 7).

Table 7

Results for controls

Control	Stage for control	*Ct* value in the channel for fluorophore		
		FAM	JOE	ROX
PCE	DNA extraction	< boundary value	< boundary value; concentration value is within the range	< boundary value
C–	DNA extraction	Absent	Absent	< boundary value
NCA	PCR	Absent	Absent	Absent
C1	PCR	*Ct* value and calculated concentration are determined	*Ct* value and calculated concentration are determined	*Ct* value and calculated concentration are determined
C2	PCR	*Ct* value and calculated concentration are determined	*Ct* value and calculated concentration are determined	*Ct* value and calculated concentration are determined

NOTE: Boundary *Ct* values and the concentration range for Positive Control *HHV*8 are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

10. TROUBLESHOOTING

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

1. The *Ct* value determined for the Positive Control of Extraction (PCE) in any of the specified channels for fluorophores (see table 7) is greater than the boundary value or absent. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples.
2. The calculated concentration of the Positive Control *HHV*8 does not fit in the range specified in the *Important Product Information Bulletin*. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected.
3. For the Negative Control of Extraction (C–):
   a) The *Ct* values are determined in the channels for the FAM and/or JOE fluorophores. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected;
   b) The *Ct* value is absent or more than the boundary value in the channel for the ROX fluorophore. This means that the Negative Control of Extraction (C–) did not perform the contamination control function. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples.
4. If the *Ct* value is determined for the Negative Control of amplification (NCA) in the channels for the FAM and/or JOE, and/or ROX fluorophores. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The amplification and detection should be repeated for all samples in which specific DNA was detected.
5. The *Ct* values are absent for the DNA-calibrators C1 and C2 in either of the specified channels for fluorophores (see table 7). The amplification and detection should be repeated for all the samples.
6. The correlation coefficient R^2 is less than 0.98 when plotting the calibration line. Check the correctness of set concentrations of calibrators in accordance with the *Important Product Information Bulletin*. If the improper result has been obtained again the amplification and detection for all the samples should be repeated.
7. If the *Ct* value is determined for the test sample, whereas the area of typical exponential growth of fluorescence is absent (the graphic looks like approximate straight line). It is necessary to check the correctness of selected threshold line level or parameters of base line calculation. If the result has been obtained with the correct level of threshold line (base line), the amplification and detection should be repeated for this sample.

11. TRANSPORTATION

AmpliSens® *HHV*8-screen/monitor-FRT PCR kit should be transported at 2–8 °C for no longer than 5 days. PCR kit can be transported at 2–25 °C for no longer than 3 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *HHV*8-screen/monitor-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-mix-FL *HHV*8 and PCR-buffer-H included in variant FRT-100 FN).

All components of the **AmpliSens® *HHV*8-screen/monitor-FRT** PCR kit are stable until the expiry date stated on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: PCR-mix-FL *HHV*8 and PCR-buffer-H are to be stored at the temperature from minus 24 to minus 16 °C

NOTE: PCR-mix-FL *HHV*8 is to be kept away from light

NOTE: PCR-mix *HHV*8-Lyo is to be kept in packages with a desiccant away from light

13. SPECIFICATIONS

13.1. Measurement range and limit of detection

Table 8

Biological material	Transport medium	Nucleic acid extraction kit	The volume of sample for extraction, µl	PCR kit	Limit of detection, copies/ml	Measurement range, copies/ml
Venous plasma	—	RIBO-prep	100	Variant FRT-100 FN, FRT-L	300	500 – $1x10^7$
		MAGNO-sorb	200			
Venous whole blood	—	RIBO-prep	100			
		MAGNO-sorb	200			
Oropharyngeal swabs	Transport medium for storage and transportation of respiratory swabs	RIBO-prep	100			
		MAGNO-sorb	200			
Saliva	—	RIBO-prep	100			
Biological (pleural, ascitic) fluids	—	RIBO-prep	100			
Tissue (biopsy, surgical, autopsy) material	Transport medium with mucolytic agent	RIBO-prep	100			
Paraffin-embedded tissue (biopsy, surgical, autopsy) material	—	RIBO-prep	100			

The claimed features are achieved while respecting the rules specified in the section "*Sampling and Handling*".

13.2. Analytical specificity

The analytical specificity of **AmpliSens® *HHV*8-screen/monitor-FRT** PCR kit is ensured by the selection of specific primers and probes as well as stringent reaction conditions. The primers and probes have been checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

The PCR kit detects *HHV*8 DNA fragment (test sample with concentration of *HHV*8 DNA no less than 10^4 copies/ml, specificity was confirmed by direct sequencing of nucleotide sequences).

The analytical specificity was proved by investigation of the human DNA and DNA/RNA of the following microorganisms/strains:

– strains from ATCC® (American Type Culture Collection, USA): *Acinetobacter baumannii* (ATCC® 19606™), *Enterococcus faecalis* (ATCC® 29212™), *Escherichia coli* (ATCC® 25922™), *Haemophilus influenzae* (ATCC® 33930™), *Klebsiella pneumoniae* (ATCC® 27736™), *Listeria grayi* (ATCC® 25401™), *Listeria innocua* (ATCC® 33090™), *Listeria monocytogenes* (ATCC® 7644™), *Moraxella catarrhalis* (ATCC® 25240™), *Pseudomonas aeruginosa* (ATCC® 15442™), *Staphylococcus aureus* (ATCC® 29213™), *Staphylococcus aureus* (MRSA) (ATCC® 43300™), *Staphylococcus epidermidis* (ATCC® 12228™), *Staphylococcus haemolyticus* (ATCC® 29970™), *Staphylococcus saprophyticus* (ATCC® 49907™), *Streptococcus agalactiae* (ATCC® 12386™), *Streptococcus pyogenes* (ATCC® 19615™) in concentration no more than $1x10^8$ copies/ml and no less than $1x10^4$ copies/ml;

– clinical isolates of a panel of strains and isolates held by FBIS CRIE: *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Enterovirus* spp., *Human alphaherpesvirus* 1, *Human alphaherpesvirus* 2, *Human alphaherpesvirus* 3, *Human betaherpesvirus* 5, *Human betaherpesvirus* 6A/B, *Human betaherpesvirus* 7, *Human gammaherpesvirus* 4, *Human gammaherpesvirus* 8, *Human polyomavirus* 1, *Human polyomavirus* 2, *Mycobacterium tuberculosis*, *Pneumocystis jirovecii*, *Primate erythroparvovirus* 1, *Streptococcus pneumoniae*, *Toxoplasma gondii* in concentration no more than $1x10^7$ copies/ml and no less than $1x10^4$ copies/ml;

– human DNA in concentration of 0.2 mg/ml.

The nonspecific responses were not observed while testing the DNA samples of the above mentioned microorganisms, as well as human DNA.

The information about interfering substances is specified in the *Interfering substances and limitations of using test material samples*.

13.3. Reproducibility and repeatability

Repeatability and reproducibility were determined by testing of positive model samples. Positive samples were dilutions of quality control sample (QCS) containing *HHV*8 DNA to final concentrations of $1x10^4$ copies/ml and $1x10^6$ copies/ml (see Tables 9, 10).

Repeatability conditions included testing in the same laboratory, by the same operator, using the same equipment within a short period of time. Reproducibility conditions included testing different lots of PCR kit in different laboratories, by different operators, on different days, using different equipment. The results are presented in Tables 9, 10.

Table 9

Reproducibility

AmpliSens® *HHV*8-screen/monitor-FRT PCR kit	Nucleic acid extraction kit	Detected pathogen	Expected concentration value, copies/ml	Number of repeats	Average value of concentration measurement, lg	Standard deviation (SD)	Coefficient of variation (CV), %
Variant FRT-100 FN	RIBO-prep	*HHV*8	6.0	30	5.8	0.15	2.6
			4.0	30	3.9	0.18	4.6
	MAGNO-sorb		6.0	30	5.9	0.12	2.1
			4.0	30	3.9	0.13	3.3
Variant FRT-L	RIBO-prep	*HHV*8	6.0	30	6.0	0.29	4.9
			4.0	30	4.1	0.30	7.5
	MAGNO-sorb		6.0	30	6.2	0.34	5.5
			4.0	30	4.1	0.32	8.0

Table 10

Repeatability

AmpliSens® *HHV*8-screen/monitor-FRT PCR kit	Nucleic acid extraction kit	Detected pathogen	Expected concentration value, copies/ml	Number of repeats	Average value of concentration measurement, lg	Standard deviation (SD)	Coefficient of variation (CV), %
Variant FRT-100 FN	RIBO-prep	*HHV*8	6.0	10	5.8	0.07	1.1
			4.0	10	3.9	0.09	2.3
	MAGNO-sorb		6.0	10	5.8	0.03	0.6
			4.0	10	3.9	0.07	1.8
Variant FRT-L	RIBO-prep	*HHV*8	6.0	10	6.0	0.18	3.0
			4.0	10	4.1	0.13	3.0
	MAGNO-sorb		6.0	10	6.2	0.23	3.7
			4.0	10	4.1	0.19	4.8

13.4. Trueness

The trueness was determined by testing of positive model samples. Positive samples were dilutions of quality control sample (QCS) containing *HHV*8 DNA to final concentrations of $1x10^4$ copies/ml and $1x10^6$ copies/ml (see Table 11).

Table 11

Trueness

AmpliSens® *HHV*8-screen/monitor-FRT PCR kit	Nucleic acid extraction kit	Detected pathogen	Number of repeats	Expected concentration value, lg	Average value of concentration measurement, lg	Bias (B), %
Variant FRT-100 FN	RIBO-prep	*HHV*8	30	6.0	5.8	-3.3
			30	4.0	3.9	-2.5
	MAGNO-sorb		30	6.0	5.9	-1.7
			30	4.0	3.9	-2.5
Variant FRT-L	RIBO-prep	*HHV*8	30	6.0	6.0	0.0
			30	4.0	4.0	0.0
	MAGNO-sorb		30	6.0	6.1	1.7
			30	4.0	4.0	0.0

14. REFERENCES

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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 the **AmpliSens® *HHV*8-screen/monitor-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

AmpliSens®

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