AmpliSens® *EBV / CMV / HHV6A/B*-screen-FRT PCR kit

RUO

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number	Σ	Contains sufficient for <n> tests
LOT	Batch code		Use-by-date
RUO	Research Use Only		Consult instructions for use
VER	Version		Keep away from sunlight
	Temperature limit	NCA	Negative control of amplification
	Manufacturer	C–	Negative control of extraction
	Date of manufacture	C+	Positive control of amplification
	Caution	PCE	Positive control of extraction

1. INTENDED USE

AmpliSens® *EBV / CMV / HHV6A/B*-screen-FRT PCR kit is not a medical device. PCR kit is intended for an *in vitro* nucleic acid amplification test for qualitative and quantitative detection of *Epstein-Barr virus* (*Lymphocryptovirus humangamma4*, *EBV*) DNA, *human cytomegalovirus* (*Cytomegalovirus humanbeta5, CMV*) DNA and *human Herpes virus* 6A/B (*Roseolovirus humanbeta6a/Roseolovirus humanbeta6b, HHV*6A/B)[1] DNA in biological material (whole venous blood, umbilical cord blood, leukocytes of venous, umbilical cord blood, plasma of venous, umbilical cord blood, oropharyngeal swab, saliva, cerebrospinal fluid, amniotic liquid, transsudates, bronchoalveolar lavage fluid, sputum, 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 the analysis of biological material taken from persons with suspected herpesvirus infection, without distinction of form and presence of disease 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 and the simultaneous amplification of DNA fragments of the detected microorganism and DNA of the human β-globin gene with hybridization-fluorescence detection. DNA of the β-globin gene is used as an endogenous internal control (IC Glob) and allows not only to control all stages of the PCR study for each sample, but also to evaluate the adequacy of the material and its 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.

For the quantitative analysis amplification of DNA is carried out from the test samples simultaneously with DNA-calibrators (samples with the known concentration of the DNA target). 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® *EBV / CMV / HHV6A/B*-screen-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 ºC for 15 min.

The PCR kit contains the system for prevention of contamination by amplicons using the enzyme uracil-DNA-glycosylase (UDG) and deoxyuridine triphosphate (dUTP).

In the amplification step, four DNA targets are amplified simultaneously in a single tube. The results of amplification are registered in the following fluorescence channels.

Table 1

Channel for fluorophore	FAM	JOE	ROX	Cy5
DNA-target	β-globin gene site (IC Glob) DNA	*Lymphocryptovirus humangamma4* (*EBV*) DNA	*Cytomegalovirus humanbeta5* (*CMV*) DNA	*Roseolovirus humanbeta6a/ Roseolovirus humanbeta6b* (*HHV6A/B*) DNA
Target gene	β-globin gene	LMP-gene	exon 4 of MIE (major immediate early) gene	DNA polymerase catalytic subunit

3. CONTENT

AmpliSens® *EBV / CMV / HHV6A/B* -screen-FRT PCR kit is produced in 1 form:

variant FRT-100 FN REF R-V48(RG,iQ,Mx)-CE.

Variant FRT-100 FN includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-1-FRT *EBV / CMV / HHV*6 / Glob	clear liquid from colorless to light lilac colour	0.6	2 tubes
PCR-buffer-H	colorless clear liquid	0.3	2 tubes
TE-buffer	colorless clear liquid	0.2	1 tube
DNA calibrator KSG1	colorless clear liquid	0.2	1 tube
DNA calibrator KSG2	colorless clear liquid	0.2	1 tube
Negative Control (C-)*	colorless clear liquid	1.2	4 tubes
Positive Control DNA *EBV / CMV / HHV*6 and human DNA**	colorless clear liquid	0.5	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 (PCE).

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

The software in Microsoft® Excel format for data processing and result generation.

4. ADDITIONAL REQUIREMENTS

Sampling and pretreatment

- Transport medium.
- 0.9 % sodium chloride solution (sterile saline solution).
- 70 % ethanol solution.
- Vacuum tubes or disposable system for collecting venous blood with EDTA as an anticoagulant.
- Swabs for collecting biological material, single use, sterile.
- Disposable tubes with screw cap, volume 5; 10; 15 ml.
- Disposable tightly closed polypropylene 1.5-ml and 2-ml tubes.
- Plastic container (30-60 ml) for storage and transportation of biological samples.
- Whole blood pretreatment reagent.
- Reagent for pretreatment of sputum.
- 0.9 % of sodium chloride (sterile saline solution) or phosphate buffered saline (PBS) (137 mM sodium chloride; 2,7 mM potassium chloride; 10 mM sodium monophosphate; 2 mM potassium diphosphate; pH=7.5±0.2).
- Sterile pipette tips with aerosol filters up to 200 and 1000 µl.
- Sterile pipette tips up to 200 µl.
- Tube racks.
- Sterile tools (individual for each sample) for homogenization (porcelain mortar and pestle) or a homogenizer for pretreatment of tissue material.
- PCR box.
- Laboratory centrifuge with accessories.
- Vortex mixer.
- Desktop centrifuge up to 12,000 g (suitable for Eppendorf tubes).
- Shaker.
- 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.
- Disposable powder-free gloves and a laboratory coat.
- Reservoir to throw off and inactivate the material.

For DNA extraction and amplification

- DNA extraction kit or the "open type" automated station for DNA extraction with MAGNO-sorb Nucleic Acid Extraction kit.
- Set of consumables for used automated station according to the manufacturer's manual.
- Disposable polypropylene tubes:
  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.
- Disposable tips for variable volume pipettes up to 100, 200 and 1000 µl.
- Tube racks.
- PCR box.
- Vortex mixer.
- Pipettes (adjustable).
- Real-time instruments (for example, Rotor-Gene 6000 (Corbett Research, Australia); Rotor-Gene Q (QIAGEN, Germany), CFX 96 (Bio-Rad, USA)).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Disposable powder-free gloves and a laboratory coat.
- Reservoir for used tips.

[1] *Lymphocryptovirus humangamma4, EBV,* previously *Human gammaherpesvirus 4; Cytomegalovirus humanbeta5*, *CMV,* previously *Human betaherpesvirus* 5; *Roseolovirus humanbeta6a/Roseolovirus humanbeta6b*, *HHV6A/B*, previously *Human betaherpesvirus 6A/B.*

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 a biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagent spills using a disinfectant, such as 0.5 % sodium hypochlorite or other 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® *EBV / CMV / HHV6A/B*-screen-FRT PCR kit is intended for analysis of the DNA extracted with DNA extraction kits from the biological material:

– whole venous, umbilical cord blood,
– leukocytes of venous, umbilical cord blood,
– plasma of venous, umbilical cord blood,
– oropharyngeal swab,
– saliva,
– cerebrospinal fluid,
– amniotic liquid,
– transsudates,
– bronchoalveolar lavage fluid,
– sputum,
– tissue (biopsy, surgical, autopsy) material.

Whole venous, umbilical cord blood; leukocytes of venous, cord blood

It is recommended to take venous blood after overnight fasting or 3 hours after a meal from the ulnar vein in a sitting position. Umbilical cord blood should be taken during cordocentesis. Collect a blood sample into a tube with 6% EDTA.

Heparin cannot be used as an anticoagulant!

Immediately after sampling, gently turn the closed tube with blood upside down several times so that the blood in the tube with the anticoagulant is thoroughly mixed. After smooth mixing, place the test tube in a rack.

NOTE: The obtaining of blood leukocytes is described in the *Pretreatment* subsection

Whole venous, umbilical cord blood samples can be stored before the pretreatment:

− at the temperature from 18 to 25 °C – for 2 hours;
− at the temperature from 2 to 8 °C – within 3 days from the moment of taking biological material;

Freezing whole blood samples is unacceptable!

Venous and umbilical cord blood plasma

It is recommended to take venous blood after overnight fasting or 3 hours after a meal from the ulnar vein in a sitting position. Umbilical cord blood should be taken during cordocentesis. Collect a blood sample into a tube with 6% EDTA or 6% EDTA and gel.

Heparin cannot be used as an anticoagulant!

Immediately after sampling, gently turn the closed tube with blood upside down several times so that the blood in the tube with the anticoagulant is thoroughly mixed. After smooth mixing, place the test tube in a rack.

To obtain blood plasma, centrifuge tubes with whole blood at 600 g for 10 minutes at a temperature of 18 to 25 °C. Next, transfer at least 1 ml of the blood plasma aliquot into 2- or 5-ml test tubes using a tip with a filter. Blood plasma must be transferred to a new tube within 6 hours of taking the blood sample.

Venous and umbilical cord blood plasma samples can be stored before the 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 higher than minus 68 °C – for a long time.

Only one freeze-thawing cycle is required.

Oropharyngeal swab

Use the working part of the swab move with rotational movements along the surface of the tonsils, palatine glands and the posterior wall of the oropharynx. Transfer the swab to a tube with 0.5 ml of transport medium. Break off the working part of the swab with the test material and leave it in the tube with the transport medium. Tightly close the tube with the cap, ensuring that there is no gap or wrinkling of the inner part of the cap. If it is impossible to break, the working part of the swab should be immersed in the transport medium and pressed against the inner side of the tube. Rotate for 5–10 s, then remove the swab and close the tube tightly.

It is not allowed to use scissors to cut the working part of the probe!

Oropharyngeal swab samples can be stored before the 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 not higher than minus 68 °C - for a long time.

Only one freeze-thawing cycle is required.

Saliva

Rinse the mouth three times with 0.9% sodium chloride solution or boiled water. Collect at least 1–2 ml saliva into a tube or container and close the cap tightly.

Saliva samples can be stored before the 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 higher than minus 68 °C - for a long time.

Only one freeze-thawing cycle is required.

Cerebrospinal fluid

Puncture the lumbar, suboccipital area or cerebral ventricles with puncture needles and collect at least 1 ml cerebrospinal fluid by aspiration method into a tube or container. Close the tube or container tightly with a cap.

If necessary, the native material can be concentrated. Mix the cerebrospinal fluid sample by vortex and sediment the drops from the walls of the tube and the inside of the cap by centrifugation for 3–5 s. Transfer 1 ml of material into a 1.5-ml tube using a tip with a filter. Centrifuge for 5 minutes at 8,000 g (for example, 10,000-11,000 rpm for the MiniSpin Eppendorf microcentrifuge). Remove the supernatant using a non-filter tip and a vacuum aspirator, leaving 100 µl of supernatant and precipitate. Mix the resulting sample thoroughly using a vortex, sediment drops from the walls of the tube and the inside of the cap by centrifugation for 3–5 s and use for DNA extraction.

Cerebrospinal fluid samples can be stored and transported before the PCR-analysis:

– at the temperature from 2 to 8 °C - for 1 day;
– at the temperature from minus 24 to minus 16 °C - for 3 months;
– at the temperature not higher than minus 68 °C - for a long time.

Only one freeze-thawing cycle is required.

Amniotic fluid

Collect at least 1–2 ml of amniotic fluid by aspiration method into a tube when performing the amniocentesis procedure. Tightly close the tube with the cap.

If necessary, the native material can be concentrated. Mix the amniotic fluid sample by vortex and sediment the drops from the walls of the tube and the inside of the cap by centrifugation for 3–5 s. Transfer 1 ml of material into a 1.5 ml test tube using a tip with a filter. Centrifuge for 10 minutes at 10,000 g (for example, 12,000 rpm for the MiniSpin Eppendorf microcentrifuge). Remove the supernatant using a non-filter tip and a vacuum aspirator, leaving 100–200 µl of supernatant and precipitate. Mix the resulting sample thoroughly using a vortex, sediment drops from the walls of the tube and the inside of the cap by centrifugation for 3–5 s and use for DNA extraction.

Amniotic fluid samples can be stored before the PCR-analysis:

– at the temperature from 2 to 8 °C – for 1 day;
– at the temperature from minus 24 to minus 16 °C – for 1 month;
– at the temperature not higher than minus 68 °C – for a long time.

Only one freeze-thawing cycle is required.

Transudates

Collect at least 0.1–0.3 ml of transudate into a test tube during puncture of the skin, pre-treated with a 70% solution of ethanol. Tightly close the tube with the cap.

Transudates samples can be stored before the PCR-analysis:

– at the temperature from 2 to 8 °C – for 3 days;
– at the temperature from minus 24 to minus 16 °C – for 7 days;
– at the temperature not higher than minus 68 °C – for a long time.

Only one freeze-thawing cycle is required.

Bronchoalveolar lavage fluid

Collect 5-50 ml of bronchoalveolar lavage fluid into a tube or container during bronchoscopy. Tightly close the tube or container with a cap.

To control contamination with the studied microorganisms or their DNA, it is recommended to carry out preliminary swabbing from bronchoscopes prepared for the bronchoscopy procedure. For this purpose, rinse the bronchoscope channel and hose with 1 ml of 0.9% sodium chloride solution. Transfer the resulting wash into a test tube for subsequent testing. Tightly close the tube with the cap.

Bronchoalveolar lavage fluid samples can be stored before the pretreatment:

– at the temperature from 2 to 8 °C – for 3 days;
– at the temperature from minus 24 to minus 16 °C – for 12 months;
– at the temperature not higher than minus 68 °C – for a long time.

Only one freeze-thawing cycle is required.

Sputum

Collect at least 1 ml (optimally 3–5 ml) of sputum into a container and close the cap tightly. Mucoid or mucopurulent sputum can be considered a high-quality material. If the patient does not produce sputum or produces it only sporadically and in scanty quantities, then the night before and early in the morning on the day of collection of biological material, he should be given an expectorant or use irritating inhalations. When receiving induced sputum, it should be noted in the accompanying document that the material was obtained after aerosol inhalation.

Sputum samples can be stored before the pretreatment:

– 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 12 months;
– at the temperature not higher than minus 68 °C – for a long time.

Only one freeze-thawing cycle is required.

Tissue (biopsy, surgical, autopsy) material

Biological material should be taken closest to the place of the lesion: from the area of the suspected location of the infectious agent, damaged tissue, or the area bordering the damage. Transfer 3–5 samples with a diameter of more than 5 mm into a container, less than 5 mm into a tube with 0.5 ml of **Transport medium with mucolytic agent**. Tightly close the container or tube.

Tissue (biopsy, surgical, autopsy) material samples can be stored before the pretreatment/ 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 higher than minus 68 °C – for a long time.

Only one freeze-thawing cycle is required.

Pretreatment

Pretreatment for the *samples of venous and umbilical cord blood plasma; oropharyngeal swabs; saliva; cerebrospinal fluid; amniotic fluid; transudates; tissue (biopsy, surgical, autopsy) material less than 5 mm in size* is not required.

Samples of whole venous and umbilical cord blood; leukocytes of venous, umbilical cord blood; bronchoalveolar lavage fluid; sputum; tissue (biopsy, surgical, autopsy) material larger than 5 mm in size are to be pretreated.

Whole venous, umbilical cord blood

Transfer 250 µl of whole blood into a 1.5-ml tube using a filter tip. Add 1 ml of **Hemolytic** reagent. Gently mix the contents of the tube by vortex and leave for 10–15 minutes at the temperature of 18–25 °C, periodically stirring on the vortex. Centrifuge for 3 minutes at 4,000 g (for example, 8,000 rpm for the MiniSpin Eppendorf microcentrifuge). Collect the supernatant using a tip without a filter and a vacuum aspirator, leaving 100 µl of supernatant and precipitate. After washing, the cell precipitate should be white; only a small pinkish coating is allowed above the precipitate (remains of destroyed erythrocytes). The washing using **Hemolytic** may be repeated if necessary. The obtained leukocyte pellet must be immediately lysed (for example, in the case of DNA extraction using the **RIBO-prep** reagent kit, add 300 µl of the **Solution for Lysis** and subsequently extract DNA in accordance with the *Instruction Manual* for the **RIBO-prep** reagent kit, without adding the **Solution for Lysis** once again).

Pretreated samples of whole venous and umbilical cord blood are to be stored at the temperature from minus 24 to minus 16 °C for a year before PCR-analysis.

Only one freeze-thawing cycle is required.

Leukocytes of venous and umbilical cord blood

Methods for obtaining leukocyte fraction of blood

Method 1. Transfer 1.5 ml of whole blood into a 2-ml tube using a filter tip. Centrifuge for 10 min at 50 g (for example, 800 rpm for the MiniSpin Eppendorf microcentrifuge). Then transfer 500–600 µl of the supernatant (the upper layer of plasma with leukocytes) into another 2-ml tube using a filter tip and centrifuge again for 10 minutes at 10,000 g (for example, 12,000 rpm for the MiniSpin Eppendorf microcentrifuge). At the end of

centrifugation, remove the supernatant using a non-filter tip and a vacuum aspirator, leaving 200 µl of supernatant and precipitate. The resulting sample is used for DNA extraction.

Method 2. Add **500 µl** of the **Hemolytic** reagent and 200 µl of the whole blood into 1.5-ml tubes using a tip with a filter. Close the tubes tightly and carefully vortex. Incubate for 3 minutes at 18–25 °C, then gently vortex again and leave for another 3 minutes. Mix by vortex, then centrifuge for 2 minutes at 4,000 g (for example, 8,000 rpm for the MiniSpin Eppendorf microcentrifuge). After centrifugation, remove the supernatant using a tip without a filter and a vacuum aspirator, without picking up the precipitate. Add 500 µl of the **Hemolytic** reagent to the resulting precipitate. Close the tubes and vortex the contents of the tubes until the cells are completely resuspended. Then incubate the tubes for 3 minutes at a temperature of 18–25 °C and mix the content of the tubes again using a vortex. Then centrifuge for 2 minutes at 4,000 g (for example, 8,000 rpm for the MiniSpin Eppendorf microcentrifuge). After centrifugation, remove the supernatant using a tip without a filter and a vacuum aspirator, without picking up the precipitate.

NOTE: The leukocyte fraction obtained after pretreatment of whole venous or umbilical cord blood must be immediately lysed or frozen.

Samples of pretreated leukocytes of venous and umbilical cord blood can be stored before the PCR-analysis:

- at the temperature from minus 24 to minus 16 °C - for 1 year;
- at the temperature not higher than minus 68 °C - for a long time.

Bronchoalveolar lavage fluid

Mix a sample of bronchoalveolar lavage fluid by vortex and sediment the drops from the walls of the tube and the inside of the cap by centrifugation for 3–5 s. Using a filter tip, remove 1 ml of material and transfer to a 1.5 ml tube. Centrifuge for 10 minutes at 7–10 thousand g. Using a tip without a filter and a vacuum aspirator, remove the supernatant, leaving 100–200 µl of supernatant above the sediment, then thoroughly mix the content by vortex and sediment drops from the walls of the tube and the inside of the cap by centrifugation for 3–5 s. The resulting sample is used for DNA extraction.

Pretreated bronchoalveolar lavage fluid samples can be stored before the PCR-analysis:

- at the temperature from 2 to 8 °C – no more than 3 days;
- at the temperature from minus 24 to minus 16 °C - no more than 12 months;
- at the temperature not higher than minus 68 °C - for a long time.

Only one freeze-thawing cycle is required.

Sputum

Add **Mucolysin** reagent to the container with sputum in 1:5 ratio (1 part of sputum to 5 parts of the reagent), guided by the calibration of the container. During the process of liquefying sputum (20–30 minutes), the container must be shaken periodically using a shaker. Then transfer 1 ml of liquefied sputum, using a tip with a filter, into a 1.5 ml tube and centrifuge for 10 minutes at 5-7 thousand g.

Remove the supernatant using a non-filter tip and a vacuum aspirator, leaving 100–200 µl of supernatant and precipitate. After thorough vortex mixing and sedimentation of droplets from the walls of the test tube and the inside of the cap by centrifugation for 3–5 s obtained sample is used for DNA extraction.

DNA extraction from 100 µl of liquefied sputum without the centrifugation stage is allowed.

Pretreated sputum samples can be stored before the PCR-analysis:

- at the temperature from 18 to 25 °C – for 6 hours;
- at the temperature from 2 to 8 °C – no more than 3 days;
- at the temperature from minus 24 to minus 16 °C - no more than 12 months;
- at the temperature not higher than minus 68 °C - for a long time.

Only one freeze-thawing cycle is required.

Tissue (biopsy, surgical, autopsy) material

Tissue material (sample size less than 5 mm in diameter) placed in 1.5–2 ml tubes with 0.5 ml Transport medium with mucolytic agent

Before DNA extraction procedure, mix the contents of the tube thoroughly on a vortex and sediment drops of material from the tube walls and the inner part of the cap by centrifugation (1,500–3,000 rpm for 5 s). No other sample preparation procedures are required.

Tissue material (sample size 5–10 mm in diameter)

Place the sample in a chilled porcelain mortar and, if necessary, crush using scissors and a scalpel. Add 0.5-1 ml of cooled 0.9% sterile sodium chloride solution (sterile saline solution) or phosphate-buffered saline solution, or Transport Medium with Mucolytic Agent. Homogenize by rubbing thoroughly with a porcelain pestle to obtain a homogeneous suspension. Transfer 100 µl of the resulting suspension into 1.5 ml tubes using a filter tip for subsequent DNA extraction.

Tissue material (sample size in diameter more than 10 mm)

Homogenize 30–50 mg (µl) of tissue material by trituration using pre-cooled, sterile porcelain mortars and pestles. When using a homogenizer, transfer tissue fragments into 2-ml tubes with 1–2 stainless steel homogenizer balls. From the grated tissue, prepare a 10%-suspension in a chilled sterile 0.9% sodium chloride solution (sterile saline solution) or phosphate-buffered saline solution. To do this, add 9 volumes of sterile 0.9% sodium chloride solution or phosphate-buffered saline to 1 volume of grated tissue material. Transfer 50–100 µl of the resulting suspension into 1.5-ml tubes using a filter tip for subsequent DNA extraction.

Pretreated tissue (biopsy, surgical, autopsy) material samples can be stored before the PCR-analysis:

- at the temperature from minus 24 to minus 16 °C – for 3 months;
- at the temperature not higher than minus 68 °C - for a long time.

Only one freeze-thawing cycle is required.

Interfering substances and limitations of using test material samples

In order to control the DNA extraction efficiency, the amplification reaction and the quality of material collection, the reagent kit provides a quantitative determination of human DNA contained in a biological sample. At the end of the amplification reaction, the presence of a sufficient amount of human DNA in the sample indicates the adequacy of the collected material, the effectiveness of nucleic acid extraction and the absence of PCR inhibitors.

The next samples are inapplicable for analysis:

- whole blood samples collected in the tubes with heparin as anticoagulant,
- whole blood samples containing a blood clot or which has been exposed to freezing.

Potential interfering substances

Endogenous and exogenous substances that may be present in the biological material used for the study were selected to assess potential interference (see Table 2).

Model samples of biological material without adding and with the addition of potential interfering substances were tested. The concentration of each potential interfering substance is specified in Table 2. Quality control samples (QCS) with *EBV, CMV,* and *HHV6A/B* DNA were added to model samples to concentrations of each target equal to the detection limit.

Table 2

Type of tested material	Type of potential interferent	Potential interferent	Tested concentration in a sample	Nucleic acid extraction kit	Interference presence
Whole (venous, umbilical cord) 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
		Triglycerides	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
Leukocytes of venous, umbilical cord blood	Endogenous substances	Total bilirubin	210 µmol/l (upper limit of normal – 21 µmol/l)	RIBO-prep	Not detected
		Total cholesterol	77.6 mmol/l (upper limit of normal – 7.8 mmol/l)	RIBO-prep	Not detected
		Triglycerides	37.6 mmol/l (upper limit of the norm – 3.7 mmol/l)	RIBO-prep	Not detected
		Hemoglobin	250 g/l (upper limit of normal – 170 g/l)	RIBO-prep	Not detected
	Exogenous substances	Lithium heparin	from 12 to 30 IU/ml	RIBO-prep	Detected
		Potassium EDTA	2.0 mg/ml	RIBO-prep	Not detected
Plasma of venous, umbilical cord blood	Endogenous substances	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
Oropha-ryngeal 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	Aqueous solution of chlorhexidine bigluconate	2.5%	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
Saliva	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	Aqueous solution of chlorhexidine bigluconate	2.5%	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
Cerebro-spinal fluid	Endogenous substances	Glucose	10 mmol/l (upper limit of normal – 3.89 mmol/l)	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
		Leukocytes	500 cells/mm^3 (upper limit of normal – 20 cells/mm^3)	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
Amniotic liquid	Endogenous substances	Whole blood	5 %	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
Transsu-dates	Endogenous substances	Albumin	500 mg/l	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
		Whole blood	5 %	RIBO-prep	Not detected
				MAGNO-sorb	Not detected
Broncho-alveolar lavage fluid	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
Sputum	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
Tissue (biopsy, surgical, autopsy) material	Endogenous substances	Hemoglobin	250 g/l (upper limit of normal – 170 g/l)	RIBO-prep	Not detected
				MAGNO-sorb	Not detected

7. WORKING CONDITIONS

AmpliSens® *EBV / CMV / HHV6A/B*-screen-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 for different types of test material:

Nucleic acid extraction kits	RIBO-prep	MAGNO-sorb	
Sample volume, µl	100	200	1000
Types of test material	- whole venous, umbilical cord blood; - leukocytes of venous, umbilical cord blood; - plasma of venous, umbilical cord blood; - oropharyngeal swabs; - saliva; - cerebrospinal fluid; - amniotic liquid; - transsudates; - bronchoalveolar lavage fluid; - sputum; - tissue (biopsy, surgical, autopsy) material	- whole venous, umbilical cord blood; - plasma of venous, umbilical cord blood; - oropharyngeal swabs; - saliva; - cerebrospinal fluid; - amniotic liquid; - transsudates; - bronchoalveolar lavage fluid; - sputum; - tissue (biopsy, surgical, autopsy) material	- plasma of venous, umbilical cord blood; - cerebrospinal fluid; - amniotic liquid; - transsudates

NOTE: Extract the DNA according to the manufacturer's protocol.

MAGNO-sorb nucleic acid extraction kit can be used in combination with "open type" automatic nucleic acid extraction stations. The DNA extraction is carried out in accordance with the *Instruction Manual* to MAGNO-sorb reagent kit.

The volumes of reagents and samples when the DNA is extracted by **RIBO-prep** nucleic acid extraction kit:

NOTE: The addition of Internal Control-FL (IC) is not required.

The volume of the test sample is **100 µl**.
Add **100 µl of Negative Control (C–)** to the tube labeled C– (Negative Control of Extraction).
Add **10 µl of Positive Control DNA *EBV / CMV / HHV*6 and human DNA** and **90 µl of Negative Control (C–)** to the tube labeled PCE (Positive Control of Extraction).
The volume of elution is **50 µl**.

The volumes of reagents and samples when the DNA is extracted by **MAGNO-sorb** nucleic acid extraction kit from 200 µl:

NOTE: The addition of Internal Control-FL (IC) is not required.

The volume of the test sample is **200 µl.**
Add **200 µl of Negative Control (C–)** to the tube labeled C– (Negative Control of Extraction).
Add **20 µl of Positive Control DNA *EBV / CMV / HHV*6 and human DNA** and **180 µl of Negative Control (C–)** to the tube labeled PCE (Positive Control of Extraction).
The volume of elution is **100 µl**.

The volumes of reagents and samples when the DNA is extracted by **MAGNO-sorb** nucleic acid extraction kit from 1000 µl:

NOTE: The addition of Internal Control-FL (IC) is not required.

The volume of the test sample is **1000 µl.**
Add **1000 µl of Negative Control (C–)** to the tube labeled C– (Negative Control of Extraction).
Add **100 µl of Positive Control DNA *EBV / CMV / HHV*6 and human DNA and 900 µl of Negative Control (C–)** to the tube labeled PCE (Positive Control of Extraction).
The volume of elution is **100 µl**.

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

8.2.1 Preparing tubes for PCR

1. Calculate the required quantity of each reagent to prepare the reaction mixture. For one reaction **10 µl of PCR-mix-1-FRT *EBV / CMV / HHV*6 / Glob** and **5 µl of PCR-buffer-H** is required. Prepare the mixture for total number of test and control samples (see numbers of control samples in item 4) plus one extra reaction (see Table 3).

Table 3

Scheme of reaction mixture preparation

		Volume of reagents for the specified number of reactions, µl	
Reagent volume for 1 reaction (µl)		**10.0**	**5.0**
Quantity of test samples		**PCR-mix-1-FRT *EBV / CMV / HHV*6 / Glob**	**PCR-buffer-H**
For quantitative analysis	**For qualitative analysis**		
1	**4**	**90**	**45**
2	**5**	**100**	**50**
3	**6**	**110**	**55**
4	**7**	**120**	**60**
5	**8**	**130**	**65**
6	**9**	**140**	**70**
7	**10**	**150**	**75**
8	**11**	**160**	**80**
9	**12**	**170**	**85**
10	**13**	**180**	**90**
11	**14**	**190**	**95**
12	**15**	**200**	**100**
13	**16**	**210**	**105**
14	**17**	**220**	**110**
15	**18**	**230**	**115**
16	**19**	**240**	**120**
17	**20**	**250**	**125**
18	**21**	**260**	**130**
19	**22**	**270**	**135**
20	**23**	**280**	**140**
21	**24**	**290**	**145**
22	**25**	**300**	**150**
23	**26**	**310**	**155**
24	**27**	**320**	**160**
25	**28**	**330**	**165**
30	**33**	**380**	**190**

NOTE: Prepare the reaction mixture just before use.

2. Thaw the test tube with **PCR-mix-1-FRT *EBV / CMV / HHV*6 / Glob.** Mix the contents of the tubes with **PCR-mix-1-FRT *EBV / CMV / HHV*6 / Glob** and **PCR-buffer-H,** sediment the drops by vortex.
3. Prepare the reaction mixture in a separate test tube. Mix the required amount of **PCR-mix-1-FRT *EBV / CMV / HHV*6 / Glob** and **PCR-buffer-H,** and sediment the drops by vortex.
4. Take the required number of tubes or strips for amplification of test and control DNA samples.
5. Transfer **15 µl** of the prepared mixture into each tube. Discard the unused reaction mixture.
6. Add **10 µl** of **DNA** obtained at the DNA extraction stage to the tubes with the reaction mixture.

NOTE: Avoid transferring sorbent together with the DNA sample in case of extraction using the magnetic separation.

7. Carry out the control reactions:

For qualitative analysis:

NCA – Add **10 µl** of **TE-buffer** to the tube labeled NCA (Negative Control of Amplification).
C+ – Add **10 µl** of **DNA calibrator KSG2** to the tube labeled C+ (Positive Control of Amplification).
C– – Add **10 µl** of **the sample extracted from the Negative Control reagent** to the tube labeled C– (Negative control of Extraction).
PCE – Add **10 µl** of **the sample extracted from the Positive Control DNA *EBV / CMV / HHV*6 and human DNA reagent** to the tube labeled PCE (Positive control of Extraction).

For quantitative analysis:

NCA – Add **10 µl** of **TE-buffer** to the tube labeled NCA (Negative Control of Amplification).
Calibrator K1 – Add **10 µl** of **DNA calibrator KSG1** to two tubes labeled **K1**
Calibrator K2 – Add **10 µl** of **DNA calibrator KSG2** to two tubes labeled **K2**
C– – Add **10 µl** of **the sample extracted from the Negative Control reagent** to the tube labeled C– (Negative control of Extraction).
PCE – Add **10 µl** of **the sample extracted from the Positive Control DNA *EBV / CMV / HHV*6 and human DNA reagent** to the tube labeled PCE (Positive control of Extraction).

8.2.2. Amplification

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

Table 4

AmpliSens unified amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	**50**	**15 min**	**–**	**1**
2	**95**	**15 min**	**–**	**1**
3	**95**	**10 s**	**–**	**45**
	60	**20 s**	**FAM, JOE, ROX, Cy5**	

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.

Table 5

«AmpliSens-1» program

	Rotor-type instruments[2]			Plate-type instruments[3]		
Step	**Temperature, °C**	**Time**	**Cycles**	**Temperature, °C**	**Time**	**Cycles**
1	**95**	**15 min**	**1**	**95**	**15 min**	**1**
2	**95**	**5 s**	**5**	**95**	**5 s**	**5**
	60	**20 s**		**60**	**20 s**	
	72	**15 s**		**72**	**15 s**	
3	**95**	**5 s**	**40**	**95**	**5 s**	**40**
	60	**20 s Fluorescence detection**		**60**	**30 s Fluorescence detection**	
	72	**15 s**		**72**	**15 s**	

Fluorescent signal is detected in the channels for the FAM, JOE, ROX, Cy5 fluorophores (if other tests are performed simultaneously, the detection is assigned in other used channels).

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

NOTE: It is recommended to sediment drops from walls of tubes by short centrifugation (1–3 s) before placing them into the instrument.
Insert empty tubes at the edges of reaction module in case of incomplete filling of plate-type instrument.

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

[2] For example, Rotor-Gene 6000, Rotor-Gene Q or equivalent.

[3] For example, CFX 96 or equivalent.

9. DATA ANALYSIS

Fluorescence signal accumulation curves indicating the accumulation of the amplification product are analyzed in four channels:

Table 6

Channel for the fluorophore	FAM	JOE	ROX	Cy5
Amplification product	β-globin gene DNA fragment (IC Glob)	*EBV* DNA	*CMV* DNA	*HHV6A/B* DNA

Analysis and interpretation of the results obtained are performed using the software of the real-time PCR instrument using the algorithm below or by the AmpliSens® *EBV / CMV / HHV6A/B*-screen software based on Microsoft® Excel.

NOTE: Operation of the AmpliSens® *EBV / CMV / HHV6A/B*-screen-software is carried out using the Microsoft® Excel program included in Microsoft® Office applications. To start work it is necessary to copy the software file from the data medium or the Manufacturer's website to the hard disc of the personal computer. The procedure of work with the software, as well as its purpose and characteristics are described in the *Instructions* tab of the AmpliSens® *EBV / CMV / HHV6A/B*-screen software.

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.

Qualitative analysis

Principle of interpretation is the following:

Table 7

Results interpretation

Ct value in the channel for the fluorophore				Result
FAM	**JOE**	**ROX**	**Cy5**	
< boundary value*	< boundary value	determined or absent	determined or absent	*EBV* DNA **is detected**
< boundary value*	determined or absent	< boundary value	determined or absent	*CMV* DNA **is detected**
< boundary value*	determined or absent	determined or absent	< boundary value	*HHV6A/B* DNA **is detected**
< boundary value*	absent	absent	absent	*EBV* DNA, *CMV* DNA, *HHV6A/B* DNA **are NOT detected**
absent or > boundary value	determined or absent	determined or absent	determined or absent	**Invalid* result**
< boundary value*	> boundary value	> boundary value	> boundary value	**Equivocal****

***** For samples of saliva, plasma of venous, umbilical cord blood, cerebrospinal fluid, amniotic fluid, transudates, bronchoalveolar lavage fluid, sputum, the absence or exceeding of the boundary *Ct* value **is ALLOWED**.

** In case of **invalid** result, it is necessary to repeat PCR-analysis of the corresponding test sample starting from the DNA extraction stage (if the *Ct* value in the channel for FAM fluorophore is greater than the boundary value) or to repeat the biological material sampling and PCR-analysis (if the *Ct* value of the channel for FAM fluorophore is absent).

*** In case of **equivocal** result, it is necessary to repeat PCR-analysis of the corresponding sample in two repeats, starting from the DNA extraction stage. If a reproducible positive *Ct* value is obtained, the result is considered **positive**.

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

The result of the PCR analysis is considered reliable only if the results obtained for controls of extraction and amplification stages are correct (according to the Table 8 and the *Important Product Information Bulletin* enclosed to the PCR kit).

Table 8

Results for controls in qualitative analysis

Control	Stage for control	*Ct* in the channel for fluorophore			
		FAM	**JOE**	**ROX**	**Cy5**
PCE	DNA extraction	<boundary value	<boundary value	<boundary value	<boundary value
C–	DNA extraction	Absent	Absent	Absent	Absent
NCA	PCR	Absent	Absent	Absent	Absent
C+	PCR	<boundary value	<boundary value	<boundary value	<boundary value

For quantitative analysis, based on the set values of DNA-calibrator concentration and the obtained *Ct* values, the calibration line is automatically drawn and the concentrations of *EBV* DNA, *CMV* DNA, *HHV6A/B* and human DNA (IC Glob) in copies/reaction are calculated. The obtained values are used to calculate the number of copies of *EBV* DNA, *CMV* DNA, *HHV6A/B* DNA in 1 ml of test samples of all types of biological material:

number of *EBV* DNA copies per reaction	**x 100**	**x A**	**=copies/ml**
number of *CMV* DNA copies per reaction	**x 100**	**x A**	**=copies/ml**
number of *HHV6A/B* DNA copies per reaction	**x 100**	**x A**	**=copies/ml**

where:

A – coefficient, taking into account the extraction volume, is calculated by the formula:

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

For samples of whole venous, umbilical cord blood; leukocytes of venous, umbilical cord blood; tissue (biopsy, surgical, autopsy) material) the obtained values of *EBV* DNA, *CMV* DNA, *HHV6A/B* DNA concentration in copies per reaction can be normalised to the standard number of human cells (number of copies of *EBV* DNA, *CMV* DNA, *HHV6A/B* DNA per 10^5 human cells). Calculation of normalised concentration values of *EBV* DNA, *CMV* DNA, *HHV6A/B* DNA is performed according to the following formulas:

$$\lg\left(\frac{\text{number of } \textit{CMV} \text{ DNA copies in PCR sample}}{\text{number of Glob DNA copies in PCR sample}} \times 2{\cdot}10^5\right) = \lg(\textit{CMV} \text{ DNA copies}/10^5 \text{ cells})$$

$$\lg\left(\frac{\text{number of } \textit{EBV} \text{ DNA copies in PCR sample}}{\text{number of Glob DNA copies in PCR sample}} \times 2{\cdot}10^5\right) = \lg(\textit{EBV} \text{ DNA copies}/10^5 \text{ cells})$$

$$\lg\left(\frac{\text{number of } \textit{HHV6A/B} \text{ DNA copies in PCR sample}}{\text{number of Glob DNA copies in PCR sample}} \times 2{\cdot}10^5\right) = \lg(\textit{HHV6A/B} \text{ DNA copies}/10^5 \text{ cells})$$

NOTE: The normalised concentration values reflect the number of pathogen cells relative to the number of human cells. In addition, the human DNA concentration value allows the quality of biological material collection to be assessed. A conversion factor is used to calculate the normalised values: $2x10^5$ human genomes = 10^5 cells.

NOTE: Concentration values of DNA-calibrators are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

Principle of interpretation is the following:

Table 9

Results Interpretation for the test samples (quantitative analysis)

Result	Interpretation
Invalid – concentration cannot be calculated (when studying whole venous, umbilical cord blood, leukocytes from venous, umbilical cord blood, tissue (biopsy, surgical, autopsy) material)	IC Glob DNA concentration is less than 2000 copies/ml. It is necessary to repeat the PCR-analysis of this sample starting from DNA extraction stage. If IC Glob DNA is absent in the test sample, biological material sampling and PCR-analysis are to be repeated
Invalid – concentration calculation is not possible (for oropharyngeal swabs)	IC Glob DNA concentration is less than 500 copies/ml. It is necessary to repeat the PCR-analysis of this sample starting from DNA extraction stage. If IC Glob DNA is absent in the test sample biological material sampling and PCR-analysis are to be repeated
EBV DNA, *CMV* DNA, *HHV6A/B* DNA are not detected	The *Ct* value for *EBV* DNA, *CMV* DNA, *HHV6A/B* DNA is absent and the IC Glob concentration in the channel for FAM fluorophore is greater than boundary value*
Less than $1x10^3$ (when extracted from 100 and 200 µl of sample) and less than 300 (when extracted from 1000 µl of sample) copies *EBV* DNA/ml and/or *CMV* DNA/ml and/or *HHV6A/B* DNA/ml	*EBV* DNA and/or *CMV* DNA and/or *HHV6A/B* DNA is detected at a concentration less than the lower limit of the measurement range of the PCR kit
X x 10^y copies *EBV* DNA/ml and/or *CMV* DNA/ml and/or *HHV6A/B* DNA/ml	*EBV* DNA and/or *CMV* DNA and/or *HHV6A/B* DNA is detected at a concentration within the measurement range of the PCR kit
More than $1x10^7$ copies *EBV* DNA/ml and/or *CMV* DNA/ml and/or *HHV6A/B* DNA/ml	*EBV* DNA and/or *CMV* DNA and/or *HHV6A/B* DNA is detected at a concentration greater than the upper limit of the measurement range of the PCR kit. If an accurate quantitative result is required, dilute the DNA sample with TE-buffer reagent (e.g. 10 times) and repeat the PCR-analysis from the amplification stage. The result obtained in the repeat test should be multiplied by the sample dilution factor

***NOTE:** For saliva, venous plasma, umbilical cord blood, cerebrospinal fluid, amniotic fluid, transudate, bronchoalveolar lavage fluid and sputum samples, the quantity of IC Glob DNA less than 500 copies/reaction **is ACCEPTABLE**.

The result of the PCR analysis is considered reliable only if the results obtained for controls of extraction and amplification stages are correct (according to the Table 10 and the *Important Product Information Bulletin* enclosed to the PCR kit).

Table 10

Results for controls in qualitative analysis

Control	Stage for control	*Ct* in the channel for fluorophore			
		FAM	**JOE**	**ROX**	**Cy5**
PCE	DNA extraction	concentration value falls in the range	concentration value falls in the range	concentration value falls in the range	concentration value falls in the range
C–	DNA extraction	*Ct* value is absent	*Ct* value is absent	*Ct* value is absent	*Ct* value is absent
NCA	PCR	*Ct* value is absent	*Ct* value is absent	*Ct* value is absent	*Ct* value is absent
KSG1, KSG2	PCR	*Ct* value and calculated concentration are determined	*Ct* value and calculated concentration are determined	*Ct* value and calculated concentration are determined	*Ct* value and calculated concentration are determined

NOTE: The *Ct* boundary values and concentration range of **Positive Control DNA *EBV / CMV / HHV*6 and human DNA** 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. While qualitative PCR-analysis the *Ct* value determined for the Positive Control of amplification (C+) in the channels for the FAM and/or JOE and/or ROX and/or Cy5 fluorophores is greater than the boundary *Ct* value or absent. The amplification and detection should be repeated for all the samples in which the specific DNA was not detected.
2. While qualitative PCR-analysis the *Ct* value determined for the Positive Control of Extraction (PCE) in the channels for the FAM and/or JOE and/or ROX and/or Cy5 fluorophores is greater than the boundary *Ct* value or absent. The PCR-analysis should be repeated for all samples beginning with the DNA extraction stage.
3. While quantitative PCR-analysis the calculated concentration of the Positive control DNA *EBV / CMV / HHV6* and human DNA does not fit in the range specified in the *Important Product Information Bulletin*. The PCR-analysis should be repeated for all samples beginning with the DNA extraction stage.
4. If the *Ct* value is determined for the Negative Control of Extraction (C–) in the channels for the FAM and/or JOE and/or ROX and/or Cy5 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 should be repeated for all samples in which specific DNA was detected beginning with the DNA extraction stage.

5. If the *Ct* value is determined for the Negative Control of amplification (NCA) in any of the channels for the FAM and/or JOE and/or ROX and/or Cy5 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.
6. While quantitative PCR-analysis the *Ct* value is absent for the DNA-calibrators KSG1 and KSG2 in any of the specified detection channels (see Table 10). The amplification and detection should be repeated for all samples.
7. While quantitative PCR-analysis the correlation coefficient R2 is less than 0.98 when plotting the calibration curve. Check the correctness of set concentrations of DNA-calibrators in accordance with the *Important Product Information Bulletin* enclosed to the PCR kit. If the improper result has been obtained again the amplification and detection for all the samples should be repeated.
8. 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® *EBV / CMV / HHV6A/B*-screen-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® *EBV / CMV / HHV6A/B*-screen-FRT** PCR kit are to be stored at 2–8 ºC when not in use (except for PCR-mix-1-FRT *EBV* / *CMV* / *HHV*6 / Glob and PCR-buffer-H).

NOTE: PCR-mix-1-FRT *EBV* / *CMV* / *HHV*6 / Glob and PCR-buffer-H are to be stored at the temperature from minus 24 to minus 16 ºC.

NOTE: PCR-mix-1-FRT *EBV* / *CMV* / *HHV*6 / Glob is to be kept away from light.

13. SPECIFICATIONS

13.1. Analytical sensitivity and linear range

Biological material	Nucleic acid extraction kit	Sample volume for extraction, µl	Analytical sensitivity, copies/ml	Linear measurement range, copies/ml
Whole venous, umbilical cord blood	RIBO-prep	100	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	200	400	$1x10^3 – 1x10^7$
Leukocytes of venous, umbilical cord blood	RIBO-prep	100	400	$1x10^3 – 1x10^7$
Plasma of venous, umbilical cord blood	RIBO-prep	100	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	200	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	1000	50	$300 – 1x10^7$
Oropharyngeal swab	RIBO-prep	100	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	200	400	$1x10^3 – 1x10^7$
Saliva	RIBO-prep	100	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	200	400	$1x10^3 – 1x10^7$
Cerebrospinal fluid	RIBO-prep	100	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	200	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	1000	50	$300 – 1x10^7$
Amniotic liquid	RIBO-prep	100	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	200	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	1000	50	$300 – 1x10^7$
Transsudates	RIBO-prep	100	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	200	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	1000	50	$300 – 1x10^7$
Bronchoalveolar lavage fluid	RIBO-prep	100	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	200	400	$1x10^3 – 1x10^7$
Sputum	RIBO-prep	100	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	200	400	$1x10^3 – 1x10^7$
Tissue (biopsy, surgical, autopsy) material	RIBO-prep	100	400	$1x10^3 – 1x10^7$
	MAGNO-sorb	200	400	$1x10^3 – 1x10^7$

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

13.2. Analytical specificity

AmpliSens® *EBV / CMV / HHV*6-screen-FRT PCR kit detects DNA fragments of *Lymphocryptovirus humangamma4*, *Cytomegalovirus humanbeta5*, *Roseolovirus humanbeta6a/Roseolovirus humanbeta6b* when studying a reference strain *Lymphocryptovirus humangamma4* B95-8, reference strain *Cytomegalovirus humanbeta5* AD 169 in concentration no less then 10^4 copies/ml, clinical isolates *Roseolovirus humanbeta6a и Roseolovirus humanbeta6b* in concentration no less then 10^5 copies/ml followed by confirmation of the specificity of the result by direct sequencing of nucleotide sequences.

The analytical specificity was confirmed on the investigating of DNA/RNA of following microorganism/strains and human genomic DNA:

- strains from ATCC® (American Type Culture Collection, США): *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 $1x10^7$ and no less than $1x10^4$ copies/ml;
- clinical isolates from a panel of strains and isolates at the disposal of FBIS CRIE: *Betapolyomavirus hominis*, *Betapolyomavirus secuhominis*, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Enterovirus* spp., *Erythroparvovirus primate1*, *Mycobacterium tuberculosis*, *Pneumocystis jirovecii*, *Rhadinovirus humangamma8*, *Roseolovirus humanbeta7*, *Simplexvirus humanalpha1*, *Simplexvirus humanalpha2*, *Streptococcus pneumoniae*, *Toxoplasma gondii*, *Varicellovirus humanalpha3* in concentration no more than $1x10^7$ and no less than $1x10^4$ copies/ml;
- human DNA in concentration 0.2 mg/ml.

The nonspecific responses were absent while testing DNA/RNA samples of the above-mentioned microorganisms and human DNA.

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

13.3. Reproducibility, repeatability and trueness

Repeatability and reproducibility were determined by testing samples of whole venous blood, oropharyngeal mucosal swab, cerebrospinal fluid artificially contaminated with the quality control samples containing *EBV*, *CMV*, and *HHV6A/B* DNA at a concentration of $1x10^5$ and $1x10^7$ copies/ml.

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 reagent kit in two independent laboratories, by different operators, on different days, on different instruments.

Table 11

Reproducibility

Extraction kit	Sample volume for extraction, µl	Micro-organism	Initial concentration value, lg copies/ml	Number of repeats	Average concentration value, lg copies/ml	Standard deviation (SD)	Coefficient of variation (CV), %
RIBO-prep	100	*EBV*	7.0	10	7.00	0.04	0.50
			5.0	10	4.99	0.07	1.48
		CMV	7.0	10	7.02	0.03	0.42
			5.0	10	5.07	0.04	0.86
		HHV6 A/B	7.0	10	6.86	0.03	0.41
			5.0	10	4.85	0.06	1.30
MAGNO-sorb	200	*EBV*	7.0	10	6.99	0.04	0.55
			5.0	10	5.04	0.04	0.73
		CMV	7.0	10	6.71	0.02	0.25
			5.0	10	4.75	0.03	0.60
		HHV6 A/B	7.0	10	7.02	0.02	0.27
			5.0	10	5.05	0.03	0.57
MAGNO-sorb	1000	*EBV*	7.0	10	7.04	0.02	0.22
			5.0	10	5.02	0.03	0.62
		CMV	7.0	10	6.70	0.06	0.93
			5.0	10	4.67	0.05	1.13
		HHV6 A/B	7.0	10	6.83	0.04	0.54
			5.0	10	4.87	0.07	1.34

Table 12

Repeatability

Extraction kit	Sample volume for extraction, µl	Micro-organism	Initial concentration value, lg copies/ml	Number of repeats	Average concentration value, lg copies/ml	Standard deviation (SD)	Coefficient of variation (CV), %
RIBO-prep	100	*EBV*	7.0	30	6.92	0.16	2.27
			5.0	30	4.92	0.16	3.23
		CMV	7.0	30	6.96	0.13	1.84
			5.0	30	4.98	0.11	2.12
		HHV6 A/B	7.0	30	6.81	0.15	2.16
			5.0	30	4.75	0.17	3.54
MAGNO-sorb	200	*EBV*	7.0	30	7.00	0.08	1.13
			5.0	30	5.02	0.08	1.54
		CMV	7.0	30	6.77	0.06	0.82
			5.0	30	4.82	0.07	1.41
		HHV6 A/B	7.0	30	6.91	0.06	0.84
			5.0	30	4.96	0.08	1.71
MAGNO-sorb	1000	*EBV*	7.0	30	7.07	0.07	1.00
			5.0	30	5.07	0.07	1.38
		CMV	7.0	30	6.71	0.11	1.71
			5.0	30	4.71	0.09	1.94
		HHV6 A/B	7.0	30	6.91	0.08	1.11
			5.0	30	4.92	0.12	2.41

The trueness was determined by testing samples of whole venous blood, oropharyngeal swab, and cerebrospinal fluid artificially contaminated with the quality control samples containing *EBV*, *CMV*, and *HHV6A/B* DNA with the concentration of at least $5x10^3$ copies/ml.

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Table 13

Trueness

Extraction kit	Sample volume for extraction, µl	Micro-organism	Number of repeats	Average value of measurement, lg	Specified value, lg	Bias (B), %
RIBO-prep	100	*EBV*	5.00	30	4.92	-1.67
		CMV	5.00	30	4.98	-0.47
		HHV6A/B	5.00	30	4.75	-5.07
MAGNO-sorb	200	*EBV*	5.00	30	5.02	0.47
		CMV	5.00	30	4.82	-3.60
		HHV6A/B	5.00	30	4.96	-0.87
MAGNO-sorb	1000	*EBV*	5.00	30	5.07	1.40
		CMV	5.00	30	4.71	-5.80
		HHV6A/B	5.00	30	4.92	-1.60

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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® *EBV / CMV / HHV6A/B*-screen-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
23.06.11 RT	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science "Central Research Institute for Epidemiology"
12.03.13 ME	Cover page	IVD symbol was replaced with RUO symbol
	Key to symbols used	
27.07.16 ME	Through the text	Corrections according to the template. Grammar corrections. The clinical material saliva and oropharyngeal swabs was deleted
	8.2.1 Preparing tubes for PCR	Appendix 1 was integrated into the text of the instruction manual as Table 1
	9. Data analysis	The section was rewritten
	10. Troubleshooting	The section has been supplemented
27.12.17 ME	3. Content	The color of the reagent was specified
03.06.21 KK	2. Principle of PCR detection	The table with targets and the information about the enzyme UDG were added
	Through the text	The text formatting was changed
	Footer	The phrase "Not for use in the Russian Federation" was added
	Through the text	Corrections according to the template Grammar corrections
	10. Troubleshooting	The information for Negative Control of Amplification (NCA) and Negative Control of Extraction (C–) was corrected
22.06.23 EM	3. Content Footer	REF R-V48(RG,iQ,Mx)-CE was added
04.09.24 HM	Through the text	The text formatting was changed
	1. Intended use	The intended use was specified. The list of biological material was expanded. The subsection *Indications and contra-indications for use of the reagent kit* was added
	3. Content	Composition was changed
	4. Additional requirements	The section was actualized and updated with materials and instruments
	6. Sampling and handling	The information about sampling and handling was expanded. The subsection *Interfering substances and limitations of using test material samples* was added
	8. Protocol	Working procedure was rewritten
	9. Data Analysis	Information on the correspondence of the amplification product and channels for the fluorophore, the principle of results interpretation for the test samples and controls are presented in tables
	10. Troubleshooting	The section was rewritten
	13. Specifications	The list of microorganisms/strains to prove the analytical specificity was expanded. The subsection *13.3. Reproducibility, repeatability and trueness* was added
	14. References	References were renewed
19.12.24 HM	3. Content Footer	REF R-V48(RG,iQ,Mx)-CE-B was deleted