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For Professional Use Only

AmpliSens® *CMV*-EPh

PCR kit

Instruction Manual

AmpliSens®

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1. INTENDED USE

AmpliSens® ***CMV*-EPh** PCR kit is an in vitro nucleic acid amplification test for qualitative detection of *Cytomegalovirus* (*CMV*) DNA in the clinical material by using electrophoretic detection of the amplified products in agarose gel.

2. PRINCIPLE OF PCR DETECTION

Cytomegalovirus detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen DNA specific region using specific *CMV* primers. After PCR, the amplified product is detected in agarose gel. AmpliSens® *CMV*-EPh PCR kit is a qualitative test that contains the Internal Control (IC). It must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition. AmpliSens® *CMV*-EPh PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions. Hot-start is guaranteed by separation of nucleotides and Taq-polymerase by using a wax layer. Wax melts and the reaction components mix only at 95 °C.

3. CONTENT

AmpliSens® ***CMV*-EPh** PCR kit is produced in 2 forms

AmpliSens® *CMV*-EPh PCR kit variant 100 R (0.5-ml tubes), **REF** V7-100-R0,5-CE.

AmpliSens® *CMV*-EPh PCR kit variant 100 R (0.2-ml tubes), **REF** V7-100-R0,2-CE.

AmpliSens® *CMV*-EPh PCR kit variant 100 R includes:

Reagent	***Description***	***Volume, ml***	***Quantity***
PCR-mix-1-R *CMV* ready-to-use single-dose test tubes (*under wax)*	colorless clear liquid	0.005	110 tubes of 0.5 or 0.2 ml
PCR-mix-2 blue	blue clear liquid	1.2	1 tube
Mineral oil for PCR	colorless viscous liquid	4.0	1 vial
Positive Control DNA *CMV* and human DNA (C+$_{CMV}$)	colorless clear liquid	0.2	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	1 tube

* must be used in the extraction procedure as Negative Control of Extraction.

AmpliSens® *CMV*-EPh PCR kit variant 100 R is intended for 110 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.

- Agarose gel detection kit.
- Disposable powder-free gloves.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers (up to 200 µl).
- Vortex mixer.
- Desktop microcentrifuge with a rotor for 2-ml reaction tubes (RCF max. 16,000 g).
- PCR box or biological cabinet.
- Vacuum aspirator with a flask for removing supernatant.
- Tube racks.
- 1.5-ml sterile polypropylene tubes.
- Refrigerator for 2–8 °C.
- Deep-freezer with temperature ≤ –16 °C.
- Waste bin for used tips.
- Permanent pen for labeling.
- Thermostat with controlled temperature for 25–100 °C.
- Personal thermocyclers (for example, Gradient Palm Cycler (Corbett Research, Australia) or MaxyGene (Axygen Scientific, USA)).

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use a new tip for every procedure.
- Store and handle amplicons away from all other reagents.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.
- Use protective gloves, laboratory coats, 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 a kit after its expiration date.
- Dispose of all samples and unused reagents in compliance with local authorities requirements.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills with 0.5% sodium hypochlorite solutions or another suitable disinfectant.
- Avoid contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes contact, immediately flush with water and seek medical attention
- Material Safety Data Sheets (MSDS) are available on request.

• Use of this product should be limited to personnel trained in DNA amplification techniques.

• The laboratory process must be one-directional; it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment, and reagents to the area where you carried out the previous step.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6. SAMPLING AND HANDLING

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

AmpliSens® *CMV*-EPh PCR kit is intended for analysis of DNA extracted with DNA extraction kits from:

– Whole peripheral and umbilical cord blood

Collect whole peripheral blood in the morning after overnight fasting in a tube with 6 % EDTA. Umbilical cord blood is taken during cordocentesis. Close the tube with blood and invert it several times to ensure proper mixing. Store the whole peripheral and umbilical cord blood at 20–25 °C for 12 h and at 2–8 °C for 1 day.

Do not freeze blood samples.

– White cells (packed white cells) are obtained from peripheral and/or umbilical cord blood. Briefly, add 1.0 ml of Hemolytic (manufactured by Central Research Institute of Epidemiology) and 0.25 ml of whole blood to a 1.5-ml Eppendorf tube using individual tips. Vortex the content of tubes thoroughly. Centrifuge the tubes at 8,000 rpm for 2 min. Discard the supernatant using a vacuum aspirator leaving 100 µl of the liquid above the pellet. After washing, the cell pellet should be white-colored, a pink tint above the pellet (erythrocyte debris) is allowed.

Thus obtained leukocyte pellet should be lysed immediately.

– Biopsy and autopsy material

Take material from the zone of presumable location of the microorganism, injured tissue, or the area adjoining the injury. Place the biopsy material into 2-ml sterile disposable Eppendorf tubes containing 0.3 ml of transport medium.

Storage conditions: at room temperature for 6 h, at 2–8° C for 3 days, and at ≤ –16° C for more than 3 days.

Place the sample to the sterile porcelain mortar, add an equal volume of saline or PBS. Grind the content of the porcelain mortar with a porcelain pestle to obtain a homogenous cell suspension. Transfer a 100-µl aliquot to a sterile tube for DNA extraction. The suspension should be stored at ≤ – 16° C.

– Cerebrospinal fluid

Collect the cerebrospinal fluid to a sterile Eppendorf tube by performing cerebrospinal lumbar, occipital, or ventricular puncture by the standard procedure.

Storage conditions: at room temperature for 6 h, at 2--8° C for 1 day, at ≤ –16° C for 1 month, and at ≤ – 68° C for a long time.

– Cervical swabs

Cervical swabs are taken using a sampling kit containing a combined gynecological probe for simultaneous sampling of epithelium from endocervix and ectocervix and a 5-ml tube with 0.2 ml of Transport Medium for Clinical Material from Female Urogenital Tract.

Place the epithelial swab from the cervical channel and the cervix uteri surface (ectocervix) to the tube with transport medium. Break the working part of the probe and leave it in the tube with transport medium. Close the tube tightly and mark it.

– Urethral swabs

Place the urethral swab taken with a universal probe to 2-ml tube with 0.5 ml Transport Medium for Clinical Material from Male Urogenital Tract».

Storage conditions: at 18–25° C for no longer than 5 days, at 2–8° C for no longer than 20 days, and at ≤ – 16° C for 1 year.

Only one freeze–thaw cycle of clinical material is allowed.

– First-void urine precipitate

Collect first void urine to special disposable sterile containers provided by the laboratory after thoroughly washing external genitals without antiseptic.

Storage conditions: at room temperature (18–20° C) for 1–2 h, at 2–8° C for 5–6 h.

– Sputum

Collect 0.2–1.0 ml of sputum to a sterile 1.5-ml Eppendorf tube. Before collecting sputum, make the patient to gargle his throat three times.

Storage conditions: at 2–8° C for no longer than 1 day, and at ≤ – 68° C for 1 year.

– Oropharyngeal washings and swabs

Oropharyngeal swabs are taken with a sterile dry probe with a cotton tampon by rotating motions from the surface of tonsilspalatine archs, and posterior wall of the oropharynx after preliminary gargling with water.

After swabbing, place the cotton tampon (the working part of the probe with the cotton tampon) to a sterile tube type Eppendorf with 500 µl of transport medium. Break the end of the probe so

that the tube cap be tightly sealed. Close the tube with the solution and the working part of the probe.

Storage conditions: at 2–8° C for no longer than 1 day, at ≤ –16° C for 1 month, at ≤ – 68° C for 1 year.

– Human breast milk

Breast milk is collected to a disposable sterile container after wiping the breast with a tampon with a sterile physiological solution.

The collected material should be pretreated. Take 1 ml of material using a tip with aerosol barrier, place it to the 1.5-ml screwed tube or latched in tube, mark it, and centrifuge at 7-12,000 g (10–13,000 rpm in a Minispin centrifuge, Eppendorf, Germany) for 5 min. Discard the supernatant with a vacuum aspirator and use the pellet for DNA extraction.

Storage conditions before analysis: at 2–8° C for no 3 days, at ≤ –18° C for 1 week, at ≤ – 68° C for a long time.

Multiple freeze–thaw cycle of clinical material is not allowed.

7. WORKING CONDITIONS

AmpliSens® *CMV*-EPh PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. DNA extraction

It is recommended that the following nucleic acid extraction kits are used:

- DNA-sorb-AM, **REF** K1-12-100-CE; RIBO-prep, **REF** K2-9-Et-100-CE or DNA-sorb-B, **REF** K1-2-100-CE – for sputum, oropharyngeal swabs, breast milk, first-void urine precipitate, urogenital swabs, and cerebrospinal fluid.
- DNA-sorb-B, **REF** K1-2-100-CE or DNA-sorb-C, **REF** K1-6-50-CE – for biopsy material of internals.
- DNA-sorb-B, **REF** K1-2-100-CE or RIBO-prep, **REF** K2-9-Et-100-CE – for white cells (packed white cells), whole peripheral and/or umbilical cord blood, and cerebrospinal fluid.
- DNA-sorb-B, **REF** K1-2-100-CE – for the whole peripheral and umbilical cord blood.

Extract DNA according to the manufacturer’s protocol.

8.2. Preparing PCR

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

8.2.1 Preparing tubes for PCR

1. Take the required number of tubes with **PCR-mix-1-R *CMV*** with wax for DNA amplification from clinical and control samples.
2. Add **10 µl of PCR-mix-2 blue** to the surface of the wax layer ensuring that it does not fall under the wax and mix with the reagents in the tube.
3. Add above 1 drop of **mineral oil for PCR** (~25 µl).

8.2.2 Amplification

1. Use the prepared tubes for PCR. Add **10 µl** of **DNA samples** extracted from clinical or control samples under or directly above the level of oil using tips with aerosol barrier.
2. Carry out the **control amplification reactions:**

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

C+ - Add 10 µl of **Positive Control DNA *CMV* and human DNA (C+$_{CMV}$)** to the tube for Positive Control of Amplification.

3. Run the following program in the thermocycler (see Table 1). When the temperature reaches 95 °C (pause mode), insert tubes into the cells of the thermocycler and press the button to continue.

   It is recommended to sediment drops from the walls of tubes by short centrifugation (1–3 s) before placing them into the thermocycler.

Table 1

CMV DNA amplification program

	Thermocyclers with active temperature adjustment:						Thermocyclers with block temperature adjustment **Uno II (Biometra), MiniCycler, PTC-100 (MJ Research)**		
	Terzik (DNA-Technology)			**GeneAmp PCR System 2700 (Applied Biosystems), Gradient Palm Cycler (Corbett Research)**					
Step	Temperature	Time	Cycles	Temperature	Time	Cycles	Temperature	Time	Cycles
0	**95 °C**	pause		**95 °C**	pause		**95 °C**	pause	
1	**95 °C**	5 min	1	**95 °C**	5 min	1	**95 °C**	5 min	1
2	**95 °C**	10 s	42	**95 °C**	15 s	42	**95 °C**	1 min	42
	65 °C	10 s		**65 °C**	25 s		**65 °C**	1 min	
	72 °C	10 s		**72 °C**	25 s		**72 °C**	1 min	
3	**72 °C**	1 min	1	**72 °C**	1 min	1	**72 °C**	1 min	1
4	**4 °C**	storage		**4 °C**	storage		**10 °C**	storage	

4. Amplification in thermocyclers with block and active temperature adjustment continues for 2 h and 1 h 30 min, respectively.
5. After the reaction is completed, PCR tubes should be collected and transferred to the room for PCR products analysis.

   Analysis of amplification products is performed by separation of DNA fragments in agarose gel.

   The amplified samples can be stored for 16 h at room temperature, for 1 week at 2–8 °C, (warm up samples to the room temperature before electrophoretic run).

9. DATA ANALYSIS

It is recommended to use the following detection agarose kit:

- EPh variant 200, **REF** K5-200-CE.

Analysis of results is based on the presence or absence of specific bands of amplified DNA in agarose gel (1.7 %). The length of specific amplified DNA fragments is as follows:

- *CMV,* 500 bp;
- Internal Control, 723 bp.

8.1. Interpretation of results

Put on a protective mask or use a glass filter while visualizing and photographing the gel.

Table 2

Results for controls

Control	Which step of test is controlled	Specific bands in the agarose gel		Interpretation
		500 bp	723 bp	
C–	DNA extraction	No	No	OK
NCA	Amplification	No	No	OK
C+	Amplification	**Yes**	**Yes**	OK

- The lane corresponding to **Negative Control of Extraction (C-)** must not contain any bands except for possible primer dimers which may appear in lanes below the 100-bp level.
- The lane corresponding to **Negative Control of Amplification (NCA)** must not contain any bands except for possible primer dimers which may appear in lanes below the 100-bp level.
- The lane corresponding to **Positive Control of Amplification (C+)** must contain a band at 500-bp level and the Internal Control band at 723-bp level.
- The sample is considered to be **positive** if the 500-bp band and the band of Internal Control are present in the gel.

IC band may be absent if cerebrospinal fluid was used because it contains a small number of cells.

- The sample is considered to be **negative** if the 723-bp band is present and the 500-bp band is absent in agarose gel.
- Besides 500-bp and 723-bp bands, fuzzy bands corresponding to primer dimers may appear in lanes below the 100-bp level. They are not taken into account in assessment of results.

10. TROUBLESHOOTING

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

1. If the results obtained for the control samples results do not correspond to the results for

controls listed in Table 2, the appropriate stage of the test should be repeated. Discard any reagents that may be suspect.

2. If neither the 500-kb nor the 723-kb band is detected in lanes, the result of analysis of this sample is invalid. In this case, analysis of this sample should be repeated starting from the DNA extraction stage. This may be caused by clinical processing errors that led to the loss of DNA or inhibition of PCR.
3. The appearance of nonspecific bands of different molecular weight in lanes may be caused by the lack of “hot-start” or an inappropriate temperature regimen in the thermocycler.
4. The appearance of the specific 500-bp specific band in lanes corresponding to negative controls (NCA, C–) suggests contamination of reagents or samples. In such cases, the results of analysis are considered to be invalid. Analysis of all samples must be repeated and measures to detect and eliminate the source of contamination must be taken.

11. TRANSPORTATION

AmpliSens® *CMV*-EPh PCR kit should be transported at 2–8 ºC for no longer than 5 days.

12. STABILITY AND STORAGE

All components of AmpliSens® *CMV*-EPh PCR kit are to be stored at 2–8 °C when not in use. They are stable until the expiration date stated on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

13. SPECIFICATIONS

13.1. Sensitivity

The analytical sensitivity of AmpliSens® *CMV*-EPh PCR kit is not less than $1x10^3$ GE/ml (genome equivalents per ml of a sample).

The claimed analytical characteristics of AmpliSens® *CMV*-EPh PCR kit are guaranteed only when additional reagent kits DNA-sorb-A, DNA-sorb-AM, DNA-sorb-B, DNA-sorb-C, RIBO-prep, and EPh manufactured by FBIS CRIE, are used.

13.2. Specificity

The analytical specificity of AmpliSens® *CMV*-EPh PCR kit is ensured by selection of specific primers and stringent reaction conditions. The clinical specificity of the kit was confirmed in laboratory clinical trials.

14. REFERENCES

1. Manual “Sampling, Transportation, and Storage of Clinical Material for PCR Diagnostics”, developed by Federal Budget Institute of Science “Central Research Institute of Epidemiology”, Moscow, 2008.

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Quality Management System, each lot of AmpliSens® *CMV*-EPh PCR kit is tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code	Σ	Sufficient for
RUO	Research use only		Expiration Date
VER	Version		Consult instructions for use
	Temperature limitation	**NCA**	Negative control of amplification
	Upper limit of temperature	**C–**	Negative control of extraction
	Manufacturer	**C+**	Positive control of amplification
	Date of manufacture		
FBIS CRIE	Federal Budget Institute of Science "Central Research Institute for Epidemiology"		

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
29.06.11 VV	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
	Cover page	The phrase “For Professional Use Only” was added
11.04.12 IvI	Title page, Key to symbols used	Symbol IVD *in vitro* diagnostic medical device was changed to RUO research use only