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For Professional Use Only

AmpliSens® *HCV*-1/2/3-FEP

PCR kit

Instruction Manual

AmpliSens®

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TABLE OF CONTENTS

1. INTENDED USE

AmpliSens® *HCV*-1/2/3-FEP PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection and discrimination of *hepatitis C virus* (*HCV*) genotype 1, 2, and 3 RNA in *HCV*-positive clinical material (blood plasma) by using end-point hybridization-fluorescence detection of amplified products.

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

2. PRINCIPLE OF PCR DETECTION

Hepatitis C virus detection includes total RNA isolation from blood plasma and reverse transcription of RNA into cDNA combined with end-point PCR amplification of cDNA. In end-point PCR the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product. Multichannel rotor-type fluorometer is specially designed to detect fluorescence emission from the fluorophores in the reaction mixture after PCR. **F**luorescent **E**nd-**P**oint PCR (FEP-PCR) allows the detection of accumulating product without re-opening the reaction tubes after the PCR run. **AmpliSens® *HCV*-1/2/3-FEP** PCR kit uses “hot-start”, which greatly reduces frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase by using chemically modified polymerase (TaqF). Chemically modified polymerase (TaqF) is activated by heating at 95ºC for 15 min.

The *HCV* genotype 1 cDNA is detected in the FAM/Green channel.

The *HCV* genotype 2 cDNA is detected in the JOE/HEX/Yellow channel.

The *HCV* genotype 3 cDNA is detected in the Rox/Texas Red/Orange channel.

The Positive Control of Extraction, Positive Control-1-*HCV,* is detected in the FAM/Green (genotype 1) channel.

The Positive Control of Amplification, Positive Control cDNA *HCV*-123, contains an *HCV* cDNA fragment common for all genotypes and is detected in FAM/Green (genotype 1), JOE/HEX/Yellow (genotype 2), and ROX/Texas Red/Orange (genotype 3) channels.

To optimize the laboratory analysis procedure, the same RNA isolation procedure can be used for *HCV* detection, quantitation, and genotyping.

3. CONTENT

AmpliSens® *HCV*-1/2/3-FEP PCR kit is produced in one form:

AmpliSens® *HCV*-1/2/3-FEP PCR kit variant FEP, REF V1-G-FEP-CE.

AmpliSens® *HCV*-1/2/3-FEP PCR kit variant FEP includes:

Reagent	*Description*	*Volume (ml)*	*Amount*
RT-G-mix-2	colorless clear liquid	0.015	1 tube
RT-PCR-mix-1-FEP/FRT *HCV*-1/2/3	colorless clear liquid	0.6	1 tube
RT-PCR-mix-2-FEP/FRT	colorless clear liquid	0.3	1 tube
Mineral oil for PCR	colorless viscous liquid	4.0	1 bottle
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
TM-Revertase (MMlv)	colorless clear liquid	0.015	1 tube
Positive Control cDNA *HCV*-123 (C+)	colorless clear liquid	0.1	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	2 tubes
Positive Control-*1-HCV***	colorless clear liquid	0.1	1 tube

* must be used in the RNA/DNA isolation procedure as Negative Control of Extraction.

** must be used in the RNA/DNA isolation procedure as Positive Control of Extraction.

AmpliSens® *HCV*-1/2/3-FEP PCR kit variant FEP is intended for 55 reactions, including controls.

4. ADDITIONAL REQUIREMENTS

- RNA/DNA isolation kit
- Disposable powder-free gloves and laboratory coat
- Pipettes (adjustable)
- Sterile RNase/DNase-free pipette tips with aerosol barriers (up to 200 µl)
- Tube racks
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes
- PCR box
- Personal thermal cyclers (for example, Gradient Palm Cycler (Corbett Research, Australia), GeneAmp PCR System 2700 (Applied Biosystems, USA), Terzik (DNA-Technology, Russia).
- Fluorometer ALA-1/4 (Biosan, Latvia) or equivalent instrument.
- Disposable polypropylene microtubes for PCR with 0.5 (0.2) ml capacity (for example, Axygen, USA).
- Refrigerator with temperature between 2 and 8 °C
- Deep-freezer with temperature at or below minus16°C.

- Waste bin for used tips.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. 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 specimens and unused reagents in accordance with local regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes and mucose membranes. If any of these solutions come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- Workflow in the laboratory must proceed in a one directional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents to the area in which the previous step was performed.

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

6. SAMPLING AND HANDLING

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

AmpliSens® *HCV*-1/2/3-FEP PCR kit is intended to analyze RNA extracted with RNA/DNA

isolation kits from:

– *Peripheral blood plasma*

Blood samples are taken after overnight fasting into tubes with 3% EDTA solution (1 : 20). Closed tubes with blood are turned several times upside down and back again. Blood plasma should be taken and transferred to new tubes within 6 h after taking blood. For this purpose, tubes with blood are centrifuged at 800–1600 g for 20 min. Blood plasma can be stored unfrozen (at 2–8°C) for at most 3 days or frozen (at or below 68°C) for a long time.

– In some cases, blood serum can be used. In this case, the analytical sensitivity of the reagent kit is retained; however, the clinical sensitivity may be significantly decreased as a result of precipitation of viral particles during blood clot retraction. Blood serum can be stored unfrozen (at 2–8°C) for at most 3 days or frozen (at or below 68°C) for a long time.

7. WORKING CONDITIONS

AmpliSens® *HCV*-1/2/3-FEP PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. RNA/DNA isolation

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

- "RIBO-sorb", REF K2-1-Et-100-CE
- "RIBO-prep", REF K2-9-Et-100-CE
- "MAGNO-sorb", REF K2-16-1000
- "NucliSENS easyMAG" automated system (BioMerieux) can also be used.

Isolate RNA/DNA according to the manual provided by the manufacturer.

To prepare Positive Control of Extraction (PCE), mix **10 µl** of **Positive Control-1-*HCV*** and **90 µl of Negative Control.**

If RNA/DNA is isolated using the "RIBO-sorb" REF K2-1-Et-100-CE extraction kit, after the addition of clinical and control samples to lysis solution warm the mixture at 60º for 10 min prior to sorbent addition.

If RNA/DNA is isolated using the "NucliSENS easyMAG" automated system:

- "EM-plus" kit REF K2-15-96-CE (manufactured by CRIE) must be used;

- Add 30 ml (the whole content of the bottle) of the **RT-G component from the EM-Plus kit** to the bottle with the NucliSens lysis buffer, close tightly the cap and **carefully** mix by turning upside down 7-10 times (this procedure is performed once for each reagent kit).
- Set the sample volume as 0.1–1 ml;
- Set the eluate volume as 55 µl.

- Both *On-board* and *Off-board* Lysis Buffer Dispensing and Lysis Incubation modes can be used.
- Carry out RT-PCR not later than 30 min after RNA isolation.

For details, see the Guidelines and the manual to "NucliSENS easyMAG" Automated System provided by the manufacturer.

The purified RNA can be stored at 2–8 °C for at most 4 h, at temperatures not higher than minus 16 °C for 1 month, and at temperatures not higher than minus 68 °C for one year.

8.2. Preparing the reverse transcription and PCR

Total reaction volume is **25 µl**, the volume of RNA sample is **10 µl**.

All components of the reaction mix should be mixed just before use. See Appendix 1 for reaction mixture preparation tips.

1. Before starting work, thaw and thoroughly vortex all reagents of the kit. Make sure that there are no drops on the caps of the tubes.
2. Take the required number of 0.2- or 0.5-ml tubes for amplification for the clinical and control samples (two controls of extraction and one control of amplification) and the **Background** samples. The type of tubes depends on the PCR instrument used for analysis.
3. To prepare the reaction mix, if **Background** samples are prepared, mix reagents in the following proportion (per **one reaction**):
   - **10 µl of RT-PCR-mix-1-FEP/FRT-*HCV-1/2/3***
   - **5 µl of RT-PCR-mix-2-FEP/FRT**
   - **0.25 µl of RT-G-mix-2.**

Thoroughly vortex the mixture; make sure that there are no drops on the walls of the tubes. See also Appendix 1, part A.

4. Mark two tubes as **Background** and add **15 µl of the prepared mixture** (without Polymerase (TaqF) and TM-Revertase (MMlv)) and **10 µl of RNA-buffer** per each tube. Mix by pipetting. Add above **1** drop of **mineral oil for PCR.**

Background samples can be stored at 2–20ºC for 1 month and used many times provided that the PCR kit of the same lot, the same type of isolation reagents, and the same type of amplification tubes are used.
Keep away from light.

5. Add the following reagents to the tube with the remaining reaction mixture (per **one reaction**):
   - **0.5 µl of Polymerase (TaqF)**
   - **0.25 µl of TM-Revertase (MMlv)**

Carefully vortex the prepared mixture; make sure that there are no drops on the walls of the tubes. See also Appendix 1.

The volumes of Polymerase (TaqF) and TM-Revertase (MMlv) listed in Appendix 1, are calculated after deduction of 30 µl of reaction mixture withdrawn to Background samples

6. If **Background** samples are repeatedly used, prepare the reaction mixture as follows (per **one reaction**):
   - **10 µl of RT-PCR-mix-1-FEP/FRT *HCV-1/2/3***
   - **5 µl of RT-PCR-mix-2-FEP/FRT**
   - **0.25 µl of RT-G-mix-2**
   - **0.5 µl of Polymerase (TaqF)**
   - **0.25 µl of TM-Revertase (MMlv)**

   Carefully vortex the prepared mixture. Make sure that there are no drops on the walls of the tubes. See also Appendix 1.
7. Transfer **15 µl** of the prepared reaction mixture per each PCR tube. Add above **1** drop of **mineral oil for PCR.**
8. Add **10 µl** of **RNA samples** obtained from clinical samples.

Avoid transferring sorbent beads together with the RNA sample if RNA was extracted using "RIBO-sorb" and "MAGNO-sorb" kits or the "NucliSENS® easyMAG™" automated system.

9. Carry out **control reactions:**

PCE - Add **10 µl of the RNA sample** extracted from Positive Control-1-*HCV* to the tube labeled PCE (Positive Control of Extraction);

C- - add **10 µl** of **RNA sample** extracted from Negative Control to the tube labeled C- (Negative Control of Extraction);

C+ - add **10 µl** of **Positive Control cDNA *HCV*-123** to the tube labeled C+ (Positive Control of Amplification)

Make sure that there are no drops on the walls of tubes, otherwise vortex tubes briefly.

8.2.2 Amplification

Run the following program on the thermal cycler (see Table 1).

Table 1

Amplification program

Thermal cyclers with active temperature adjustment[1]				Thermal cyclers with block temperature adjustment[2]			
Step	Temperature, °C	Time	Cycles	Step	Temperature, °C	Time	Cycles
1	**50**	30 min	1	1	**50**	30 min	1
2	**95**	15 min	1	2	**95**	15 min	1
3	**95**	2 s	45	3	**95**	10 sec	45
	60	10 s			**60**	15 sec	
					72	15 sec	
4	**10**	Storage		4	**10**	Storage	

9. DATA ANALYSIS

Detection is conducted on ALA-1/4 florescence detector (Biosan, Latvia).

Please read ALA-1/4 Operating Manual before using this kit.

Program the detector according to the manufacturer's manual and the Guidelines.

The intensity of fluorescent signal is detected in three channels

Fluorescence channel	Type	Interpretation
FAM/Green	*HCV*	Genotype 1
HEX/Yellow	*HCV*	Genotype 2
ROX/Orange	*HCV*	Genotype 3

The **Internal Control** is absent.

Prior to detection, all settings should be entered and saved. Refer to **Guidelines** and **Important product information bulletin** for settings.

Results interpretation

1. When the analysis is complete, the results are automatically displayed in the table as follows:

   pos – positive result;

   neg – negative result;

   eq – equivocal result (signal is in grey zone);

   nd – invalid result (specific signal and IC signal are absent in the sample).

2. Principle of result interpretation:

[1] For example "Terzik" (DNA-Technology), "Gradient Palm Cycler" (Corbett Research), "MAXYGENE" (Axygen), "GeneAmp PCR System 2400" (Perkin Elmer) etc.

[2] For example "GeneAmp PCR System 2700" (Applied Biosystems) "PTC-100" (MJ Research), "T-personal" (Biometra) etc.

A value is considered **negative** if it is less than the specified threshold of negative result; **positive** if it is higher than the defined threshold of positive result; and **equivocal** if it falls between the thresholds.

– Positive result in the FAM/Green channel indicates the presence of *HCV* genotype 1 RNA; in the HEX/Yellow channel, the presence of *HCV* genotype 2 RNA; and in the ROX/Orange channel, the presence of *HCV* genotype 3 in the sample.

– If an equivocal result is obtained, the sample should be analyzed once again.

3. The result of the analysis is considered reliable only if both Positive and Negative Controls are passed (Table 2).

Table 2

Results for controls

Control	Stage for control	Result of automatic interpretation			Interpretation
		FAM/Green channel (*HCV*1)	HEX/JOE/ Yellow channel (*HCV*2)	ROX/Orange channel (*HCV*3)	
C-	RNA isolation,	neg	neg	neg	OK
PCE	RNA isolation	pos	neg	neg	OK
C+	Amplification	pos	pos	pos	OK

10. TROUBLESHOOTING

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

- The absence of positive signal in the sample with positive controls (PCE and C+) may indicate improper amplification program.
- If positive signal is detected in C-, the results of the analysis for all samples are considered invalid due to contamination. It is necessary to repeat the analysis of all tests once again and to take measures for detecting and eliminating the source of contamination.

11. TRANSPORTATION

AmpliSens® *HCV*-1/2/3-FEP PCR kit should be transported at 2–8 ºC for no longer than 5 days. Once received, the PCR kit should be dekitted according to the indicated storage conditions.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *HCV*-1/2/3-FEP** PCR kit (except for RT-G-mix-2, RT-PCR-mix-1-FEP/FRT *HCV*-1/2/3, RT-PCR-mix-2-FEP/FRT, polymerase (TaqF) and TM-Revertase (MMlv)) are to be stored at 2–8 °C when not in use. All components of the **AmpliSens® *HCV*-1/2/3-FEP** PCR kit are stable until the labeled expiration date. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

RT-G-mix-2, RT-PCR-mix-1-FEP/FRT *HCV*-1/2/3, RT-PCR-mix-2-FEP/FRT, polymerase (TaqF) and TM-Revertase (MMlv) are to be stored at temperature from minus 24 to minus 16 °C when not in use.

RT-PCR-mix-1-FEP/FRT *HCV*-1/2/3 is to kept away from light

13. SPECIFICATIONS

13.1. Sensitivity

The analytical sensitivity of **AmpliSens® *HCV*-1/2/3-FEP** PCR kit is specified in the table below.

Volume of sample for isolation, µl	RNA/DNA isolation method	Analytical sensitivity, IU/ml
100	"RIBO-sorb" "RIBO-prep" "NucliSENS easyMAG"	2000
1000	"NucliSENS easyMAG"	200

The claimed analytical features of **AmpliSens® *HCV*-1/2/3-FEP** PCR kit are guaranteed only when additional reagents kits, "MAGNO-sorb", RIBO-sorb", or "RIBO-prep" (manufactured by FBIS CRIE) are used.

13.2. Specificity

The analytical specificity of **AmpliSens® *HCV*-1/2/3-FEP** PCR kit is ensured by selection of specific primers and probes as well as by selection of strict reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequence comparison analysis as well as with genomic DNA/RNA of the following organisms and viruses: *hepatitis A virus; hepatitis B virus; hepatitis D virus; human immunodeficiency virus; cytomegalovirus; Epstein-Barr virus; herpes simplex virus* types 1 and 2; *chicken pox virus; human herpes virus* types 6 and 8; *parvovirus* B19; *tick-borne encephalitis virus*; *West Nile encephalitis; adenovirus* types 2, 3, and 7; *Escherichia coli*; *Staphylococcus aureus; Streptococcus pyogenes; Streptococcus agalactiae;* and *Homo sapiens*. The clinical specificity of **AmpliSens® *HCV*-1/2/3-FEP** PCR kit was confirmed in laboratory clinical trials.

14. REFERENCES

1. Handbook "Sampling, transportation, storage of clinical material for PCR diagnostics", developed by Federal Budget Institute of Science "Central Research Institute for

Epidemiology" of Federal Service for Surveillance on Consumers' Rights Protection and Human Well-Being, Moscow, 2008.
2. Guidelines to AmpliSens® *HCV*-1/2/3-FEP PCR kit.

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® *HCV*-1/2/3-FEP** PCR kit has been 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		Keep away from sunlight
	Manufacturer	**NCA**	Negative control of amplification
	Date of manufacture	**C–**	Negative control of extraction
FBIS CRIE	Federal Budget Institute of Science "Central Research Institute for Epidemiology"	**C+**	Positive control of amplification
		IC	Internal control

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
01.06.10	Page footer	Reference number is changed from V1-G-FEP to V1-G-FEP-CE
	Contents	(C+) is added after the Positive Control in the table of content
05.07.11 LA	Cover page	The phrase “For Professional Use Only” was added
	Content	New sections “Working Conditions” and “Transportation” were added
		The “Explanation of Symbols” section was renamed to “Key to Symbols Used”
	Stability and Storage	The information about the shelf life of reagents before and after the first use was added
		Information that RT-PCR-mix-1-FEP/FRT *HCV*-1/2/3 is to be kept away from light was added
	Key to Symbols Used	The explanation of symbols was corrected
	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
14.07.11 VV	Stability and Storage	Storage conditions of the AmpliSens® *HCV*-1/2/3-FEP PCR kit (except for RT-G-mix-2, RT-PCR-mix-1-FEP/FRT *HCV*-1/2/3, RT-PCR-mix-2-FEP/FRT, polymerase (TaqF) and TM-Revertase (MMlv)) are changed from ≤ –16 °C to 2–8 °C.
	Transportation	The phrase “Once received, the PCR kit should be dekitted according to the indicated storage conditions” was added
14.08.12 Ivl	Title page, Key to symbols used	Symbol IVD *in vitro* diagnostic medical device was changed to RUO research use only
10.10.12 Ivl	Content	Quality of reactions was changed from 112 to 55