RUO

For Professional Use Only

AmpliSens® *HDV*-FEP

PCR kit

Instruction Manual

AmpliSens®

Federal Budget Institute of Science “Central Research Institute for Epidemiology”
3A Novogireevskaya Street
Moscow 111123 Russia

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1. INTENDED USE

AmpliSens® ***HDV*-FEP** PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *hepatitis D virus* (*HDV*) RNA in clinical material (blood plasma) by means of 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 D virus detection includes RNA extraction from blood plasma together with internal control sample (IC), reverse transcription of RNA, and 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 fluorescent excitation from the fluorophores in a reaction mix after PCR. Fluorescent End-Point PCR (FEP-PCR) allows the detection of accumulating product without re-opening of the reaction tubes after the PCR run. **AmpliSens® *HDV*-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) activates by heating at 95 °C for 15 min.

The IC amplification product is detected in the FAM channel. The *HDV* cDNA amplification product is detected in the JOE channel. The Positive Control of Extraction, Positive Control *HDV*-rec, is detected in FAM (IC) and JOE (*HDV*) channels. The Positive Control of Amplification, Positive Control cDNA *HDV*-FL (C+$_{HDV\text{-}FL}$), is the complex control for *HDV* and IC. It is detected in FAM (IC) and JOE (*HDV*) channels.

3. CONTENT

AmpliSens® *HDV*-FEP is produced in 1 form:

AmpliSens® *HDV*-FEP PCR kit variant FEP, REF V3-FEP-CE.

AmpliSens® *HDV*-FEP PCR kit variant FEP includes:

Reagent	***Description***	***Volume (ml)***	***Quantity***
RT-G-mix-2	colorless, clear liquid	0.015	4 tubes
RT-PCR-mix-1-FL *HDV*	colorless, clear liquid	0.3	4 tubes
RT-PCR-mix-2-FEP/FRT	colorless, clear liquid	0.2	4 tubes
Mineral oil for PCR	colorless viscous liquid	4.0	1 dropper bottle
Polymerase (TaqF)	colorless, clear liquid	0.02	4 tubes
TM-Revertase (MMlv)	colorless, clear liquid	0.01	4 tubes
Positive Control cDNA *HDV*-FL (C+$_{HDV\text{-}FL}$)	colorless, clear liquid	0.1	4 tubes
Buffer for elution	colorless, clear liquid	1.2	2 tubes
Negative Control (C-)*	colorless, clear liquid	1.2	4 tubes
Positive Control *HDV*-rec**	colorless, clear liquid	0.06	4 tubes
Internal Control *ICZ*-rec (IC)***	colorless, clear liquid	0.28	4 tubes

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

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

*** must be added during the RNA/DNA extraction procedure.

AmpliSens® *HCV*-FEP PCR kit variant FEP is intended for 112 reactions, including controls.

4. ADDITIONAL REQUIREMENTS

- RNA/DNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Automated pipettors (dosers) of variable volumes.
- Sterile RNase/DNase-free pipette tips with filters (up to 200 µl).
- Tube racks.
- Centrifuge/vortex mixer.
- PCR box.
- Personal thermocyclers (for example, Rotor-Gene 3000 or Rotor-Gene 6000 (Corbett Research, Australia); iQ5 (Bio-Rad, USA), Mx3000P (Stratagene, USA) or equivalent).
- Disposable polypropylene microtubes for PCR with 0.2 ml capacity (for example, Axygen, USA).
- Refrigerator for 2–8 °C.

- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with filters 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® *HDV*-FEP PCR kit is intended for the reverse transcription of RNA and amplification of cDNA extracted by RNA/DNA extraction kits from peripheral blood plasma.

– *Peripheral blood plasma*

Blood samples are taken after overnight fasting into the tube with EDTA solution as anticoagulant. 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 for such material is the same but the clinical sensitivity can be reduced in view of viral particles coprecipitation during 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® *HDV*-FEP PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. RNA/DNA extraction

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

- RIBO-sorb, **REF** K2-1-Et-50-CE
- RIBO-prep, **REF** K2-9-Et-50-CE
- MAGNO-sorb, **REF** K2-16-1000-CE
- NucliSENS easyMAG automated system can be used either.

Carry out the RNA/DNA extraction according to the manual provided by the manufacturer.

For Positive Control of extraction (PCE) mix **10 µl of Positive Control *HDV*-rec** and **90 µl Negative Control**

Volume of Internal Control added during RNA/DNA extraction depends on the reagents kit used:

- add **10 µl of Internal Control *ICZ*-rec** to a sample/lysis mixture (RIBO-prep or RIBO-sorb)

If using RIBO-sorb kit, it is necessary to incubate tubes with sample/lysis mixture (before sorbent adding) at 60 °C for 10 min and then centrifuge briefly.

If using RIBO-prep kit:

- after preparing the Controls, incubate tubes with them at **65 °C for 5 min** and then vortex. Make sure there are no drops on the walls of the tubes.
- after adding **Solution for Precipitation**, **Washing Solution 3**, **Washing Solution 4**, and **RNA-buffer**, centrifuge all tubes at 12,000 g.

If using the MAGNO-sorb kit:

- to extract RNA from blood plasma sample of 1000 µl, the volume of the **Internal Control *ICZ*-rec** required for extraction from 24 samples is **0.28 ml**.
- to prepare the Positive Control of extraction, **PCE**, add **90 µl** of the **Negative Control (C–)** sample and **10 µl** of the **Positive Control *HDV*-rec** sample to a tube containing **lysis solution**.
- to prepare the Negative Control of extraction, **C–**, add **100 µl** of the **Negative Control (C–)** sample to a tube containing **lysis solution**.
- the volume of **Buffer for elution** required for extraction from both 1000 and 200 µl of blood plasma samples is **70 µl**.

If NucliSENS easyMAG automated system is applied:

- use of EM-plus kit **REF** K2-15-96 (manufactured by CRIE) is obligatory
- 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).
- Mix 10 µl of the **Internal Control (IC) sample with** 10 µl of **NucliSens magnetic silica** and 10 µl of **Component A** from the **EM-plus kit** with per one sample for RNA/DNA extraction in a new sterile tube using disposable tips with filters.
- set a sample volume as 0.1 ml or 1 ml
- set an eluate volume as 50-60 µl (up to 100 µl).
- both On-board and Off-board Lysis Buffer Dispensing and Lysis Incubation are possible.

See Guidelines for details.

8.2. Preparing the reverse transcription and PCR

Total reaction volume is **25 µl**, the volume of RNA/DNA 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. Thaw all reagents, thoroughly vortex, and make sure that there are no drops on the walls of the tubes.
2. Collect the required number of the PCR tubes for amplification of clinical, control (including 2 controls of extraction and 1 control of amplification), and the Background samples.
3. In case of Background tubes preparation, make the reaction mixture as follows (calculating per **one reaction**):
   - **10 µl of RT-PCR-mix-1-FL *HDV***
   - **5 µl of RT-PCR-mix-2-FEP/FRT**
   - **0.25 µl of RT-G-mix-2**

Carefully vortex prepared mixture; make sure that there are no drops on the walls of the tubes. See Appendix 1, part A as well.

4. Mark two tubes as **Background** and add **15 µl of prepared mixture** (without

Polymerase (TaqF) and TM-Revertase (MMlv)) and **10 µl of buffer for elution** per each tube. Mix by pipeting. Add above **1** drop of **mineral oil for PCR**.

Background tubes that have once passed thermal cycling can be stored at 2-25 °C for up to 1 month and used repeatedly.
Multiple use of Background samples is permitted in case of application of the same PCR kit lot, the same extraction reagents, and the same type of PCR tubes.

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

Carefully vortex prepared mix; make sure that there are no drops on the walls of the tubes. Refer also to Appendix 1, part A.

Volumes of Polymerase (TaqF) and TM-Revertase (MMlv) listed in Appendix 1, are calculated after deduction of 30 µl reaction mixture intended for two Background samples

6. In case of multiple use of the Background samples, make the reaction mixture as follows (calculating per **one reaction**):
   - **10 µl of RT-PCR-mix-1-FL *HDV***
   - **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 prepared mixture. Make sure that there are no drops on the walls of the tubes. See Appendix 1, part B as well.
7. Transfer **15 µl** of 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 or control samples at the stage of RNA extraction.

When adding of RNA samples extracted by RIBO-sorb and NucliSENS easyMAG it is necessary to avoid transferring of the sorbent into the reaction mix.

9. Carry out **control reactions**:

PCE - Add **10 µl of RNA sample** extracted from Positive Control *HDV*-rec to the tube labeled PCE;
C- - Add **10 µl of RNA sample** extracted from Negative Control to the tube labeled C-;
C+*HDV*-FL - Add **10 µl of Positive Control cDNA *HDV*-FL** to the tube labeled C+*HDV*-FL.

To rule out possible contamination, carry out additional control reaction:

NCA -Add **10 µl of buffer for elution** to the tube labeled NCA (Negative 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 thermocycler (see Table 1).

Table 1

Amplification program

Thermocyclers with active temperature adjustment[1]				**Thermocyclers 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 s	45
	60	10 s			**60**	15 s	
					72	15 s	
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 use of this kit.

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

Intensity of fluorescent signal is detected in two channels:

– accumulation of Internal Control cDNA amplified product is detected in the FAM channel (or analogical);

– accumulation of *HDV* cDNA amplified product is detected in the HEX channel (or analogical).

Before 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 in the manner of following indications:

   pos – positive result;

   neg – negative result;

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

[1]For example, 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.

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

2. Principle of result interpretation:

   The value is considered **negative** if it is less than defined threshold of negative result; **positive** if it is more than defined threshold of positive result; **equivocal** if it fits between the thresholds.

   – Positive result in HEX channel indicates *HDV* RNA in the sample.

   – Result is invalid if the signal in FAM channel is less than defined negative threshold and the signal in HEX channel is less than defined threshold as well.

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 channel (IC)	HEX channel *(HDV)*	
C-	RNA extraction	**IC+**	neg	OK
PCE	RNA extraction	**IC+**	pos	OK
C+$_{HDV\text{-FL}}$	Amplification	**IC+**	pos	OK
NCA	Amplification	**IC-**	neg	OK

10. TROUBLESHOOTING

If analysis results are not obtained as per the following examples:

- If the signal less than the threshold of positive result is detected for PCE or C+$_{HDV\text{-FL}}$ in the HEX channel, then the analysis (starting from the extraction) should be repeated for all samples in which *HDV* RNA has not been found.
- If the signal more than the threshold of the positive result is detected for C- and/or NCA in the HEX channel, then the analysis (starting from the extraction) should be repeated for all samples in which *HDV* RNA has been found.
- If the **nd** result is obtained for samples except for NCA, the analysis should be repeated (starting from the extraction). The **nd** result is normal only for NCA sample.
- If the **eq** result is registered for samples, the analysis should be repeated (starting from the extraction). If the same result is obtained once again, the sample is considered positive.
- If positive signal is detected in C- and/or NCA, results of the analysis for all samples are considered invalid due to contamination. It is necessary to repeat the analysis of all tests, and also to take measures to detect and eliminate the source of contamination.

11. TRANSPORTATION

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

12. STABILITY AND STORAGE

All components of the **AmpliSens®** ***HDV*-FEP** PCR kit are to be stored at temperature from minus 24 to minus 16 °C when not in use. All components of the **AmpliSens®** ***HDV*-FEP** PCR kit are to be stable until labeled expiration date. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

Positive Control cDNA *HDV*-FL, Positive Control *HDV*-rec, and Internal Control *ICZ*-rec should not be frozen/thawed more than twice. After thawing, Positive Control cDNA *HDV*-FL, Positive Control-*HCDV*-rec, and Internal Control *ICZ*-rec are to be stored at 2-8 °C for up to 6 months.

RT-PCR-mix-1-FL *HDV* is to be kept away from light.

13. SPECIFICATIONS

13.1. Sensitivity

The analytical sensitivity of **AmpliSens®** ***HDV*-FEP** PCR kit is specified in the table below.

Volume of sample for extraction, µl	RNA/DNA extraction method	Analytical sensitivity, copies / ml
100	RIBO-sorb RIBO-prep NucliSENS easyMAG	500
200	MAGNO-sorb	250
1000	MAGNO-sorb NucliSENS easyMAG	50

The claimed analytical features of **AmpliSens®** ***HDV*-FEP** PCR kit are guaranteed only when additional reagents kits, RIBO-sorb, RIBO-prep or MAGNO-sorb (manufactured by FBIS CRIE) are used. NucliSENS easyMAG manufactured by bioMérieux, France can be used either.

13.2. Specificity

The analytical specificity of **AmpliSens®** ***HDV*-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® *HDV*-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.

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® *HDV*-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		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+*HDV*-FL**	Positive control of amplification
PCE	Positive control of extractior	**IC**	Internal control

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
13.07.10	Text	Reference number was changed from V3-FEP to V3-FEP-CE,
	Page footer	
04.12.10	Sampling and handling	Sentence « Blood samples are taken after overnight fasting into tubes with 3% EDTA solution (1:20)» is changed into «Blood samples are taken after overnight fasting into the tube with EDTA solution as anticoagulant».
	Through the text	Corrections through the text
		MAGNO-sorb mention was deleted
		Abbreviation C+$_{HDV\text{-FL}}$ is added for Positive Control cDNA *HDV*-FL
21.03.11 RT	Stability and storage	The phrase about keeping away from light of RT-PCR-mix-1-FL *HDV* was added
08.07.11 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-FL *HDV* 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”
15.09.11 RT	8. PROTOCOL 8.1. RNA/DNA extraction	The information about using RIBO-prep kit was added
14.06.12 BO	Title page	IVD symbol was changed to RUO
	Through the text	Tips with aerosol barriers were changed to tips with filters
		All references to isolation procedure were changed to extraction procedure
19.06.12 BO	Sensitivity	Information about MAGNO-sorb extraction kit was added
	RNA/DNA extraction	
	Sensitivity	Sensitivity for sample of 200 µl was added