RUO

For Professional Use Only

AmpliSens® *HDV*-Monitor-FRT

PCR kit

Instruction Manual

AmpliSens®

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1. INTENDED USE

AmpliSens® ***HDV*****-Monitor-FRT** PCR kit is an *in vitro* nucleic acid amplification test for quantitative detection of *hepatitis D virus* (*HDV)* RNA in clinical materials (blood plasma) by using real-time hybridization-fluorescence detection.

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

2. PRINCIPLE OF PCR DETECTION

HDV detection by the reverse transcription polymerase chain reaction (RT-PCR) is based on the amplification of cDNA corresponding to a specific region of the pathogen genome, which is obtained using reverse transcription of the viral genomic RNA. Amplification is performed using specific *HDV* primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are usually linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

AmpliSens® *HDV*-Monitor-FRT 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 a chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

3. CONTENT

AmpliSens® *HDV*-Monitor-FRT PCR kit is produced in 1 form:

Form 1 includes AmpliSens® *HDV*-Monitor-FRT PCR kit variant FRT and *HDV-Q* calibration kit **REF** R-V3-MC(RG,iQ,Mx,Dt)-CE.

AmpliSens® *HDV*-Monitor-FRT PCR kit variant FRT includes:

Reagent		*Description*	*Volume (ml)*	*Quantity*
DTT frozen-dried		white powder	–	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
Polymerase (TaqF)		colorless clear liquid	0.02	4 tubes
TM-Revertase (MMlv)		colorless clear liquid	0.01	4 tubes
DNA calibrators	**PIC1 *HDV****	colorless clear liquid	0.1	4 tubes
	PIC2 *HDV***	colorless clear liquid	0.1	4 tubes
Buffer for elution		colorless clear liquid	1.2	4 tubes
Negative Control (C–)***		colorless clear liquid	1.2	4 tubes
Positive Control-1-*HDV*****		colorless clear liquid	0.06	4 tubes
Positive Control-2-*HDV*****		colorless clear liquid	0.06	4 tubes
Internal Control *ICZ*-rec (IC)*****		colorless clear liquid	0.28	4 tubes

* serves as Positive Control of Amplification (C+1).

** serves as Positive Control of Amplification (C+2).

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

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

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

AmpliSens® *HDV*-Monitor-FRT PCR kit variant FRT is intended for 80 reactions, including controls and calibrators.

HDV-Q calibration kit includes:

Reagent	*Description*	*Volume (ml)*	*Quantity*
Calibrator *HDV*-Q	yellow powder	–	1 tube
Solvent Q	colorless clear liquid	1.2	3 tubes

4. ADDITIONAL REQUIREMENTS

- DNA/RNA extraction kit.
- Disposable powder-free gloves.
- Pipettes (adjustable).
- Sterile pipette tips with filters up to 200 µl.

- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 2 ml reaction tubes.
- PCR box.
- Rotor-Gene 3000 or Rotor-Gene 6000 (Corbett Research, Australia); iQ5 (Bio-Rad, USA); Mx3000 (Stratagene, USA) instrument.
- Disposable polypropylene microtubes for PCR (0.1- or 0.2-ml; 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 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 disposable gloves, laboratory coats, protect eyes while samples and reagents handling. Thoroughly wash hands afterward.
- 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 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 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 the techniques of DNA amplification.
- 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 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 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*-Monitor-FRT PCR kit is intended for the analysis of RNA extracted from:

- *Peripheral blood plasma*

Blood samples are taken after overnight fasting into the tube with EDTA solution as anticoagulant. Invert closed tubes to ensure proper mixing. To collect plasma, centrifuge the tubes with blood at 800–1600 g for 20 min within 6 h after blood taking. Remove obtained plasma and transfer to new tubes.

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.

Storage of plasma and serum samples:

– at 2–8 °C for up to 3 days;

– at ≤ – 68 °C for a long time

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7. WORKING CONDITIONS

AmpliSens® *HDV*-Monitor-FRT PCR kit should be used at 18–25 °C

8. PROTOCOL

8.1. RNA extraction

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

- RIBO-prep, REF K2-9-Et-100-CE
- MAGNO-sorb, REF K2-16-1000-CE
- NucliSENS easyMAG, automated system can be used as well.

Extract RNA according to the manual provided by the manufacturer.

Volume of **Internal Control *ICZ*-rec** added during RNA/DNA extraction is **10 µl** per one sample.

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 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 NucliSENS easyMAG automated system:

- use of EM-plus kit REF K2-15-96-CE (manufactured by CRIE) is obligatory
- set a sample volume as 0.1 ml or 1 ml;
- set an eluate volume as 55 µl.
- both *On-board* and *Off-board* Lysis Buffer Dispensing and Lysis Incubation are possible.

See Guidelines [2] for details.

8.2. Preparing the PCR

Total reaction volume is **50 µl**, the volume of RNA/DNA sample is **25 µl**.

8.2.1 Preparing tubes for PCR

All components of the reaction mix should be mixed just before use.

See Appendix 1 for the reaction mixture preparation scheme.

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 and control samples (including 3 controls of extraction and 4 calibrators).
3. To prepare the **reaction mixture:**
   - add the entire contents of the tube with **RT-PCR-mix-2-FEP/FRT** to the tube with **DTT dried-frozen**. Thoroughly vortex and make sure there are no drops on the walls of the tube. Store the prepared mixture at 2–8 °C.
   - take a new tube and mix the following reagents calculating per one reaction: **15 µl of RT-PCR-mix-1-FL *HDV***, **10 µl of the mixture of RT-PCR-mix-2-FEP/FRT and DTT frozen-dried, 1.0 µl of polymerase (TaqF) and 0.5 µl of TM-Revertase**

(MMlv). Vortex thoroughly and make sure that there are no drops on the walls of the tubes.

It is recommended that the reaction mixture for 20 reactions is prepared in case of extraction from 12 to 16 samples (two NucliSENS easyMAG arrays). To do this, into the tube with **DTT frozen-dried** transfer the entire contents of the tube with **RT-PCR-mix-2-FEP/FRT, RT-PCR-mix-1-FL *HDV*, polymerase (TaqF), and TM-Revertase (MMlv)**. Do not store the prepared mixture!

4. Transfer 25 µl of the prepared mixture per each PCR tube. Discard unused reaction mixture.
5. Using tips with filter add **25 µl** of clinical **RNA samples**. Thoroughly mix by pipetting. Avoid air bubbling.

Avoid transferring of sorbent together with the RNA sample in case of extraction with NucliSENS easyMAG automated system.

6. Carry out the control amplification reactions:

PCE 1 -Add **25 µl of RNA sample** isolated from Positive Control-1-*HDV* to the tube for positive control of extraction 1;

PCE 2 -Add **25 µl of RNA sample** isolated from Positive Control-2-*HDV* to the tube for positive control of extraction 2;

C– -Add **25 µl of RNA sample** isolated from Negative Control to the tube for negative control of extraction;

C+$_1$ -Add **PIC1 *HDV*** to the two tubes for positive control of amplification 1 (**25 µl per each tube)**;

C+$_2$ -Add **PIC2 *HDV*** to the two tubes for positive control of amplification 2 (**25 µl per each tube)**.

Thoroughly mix by pipetting. Avoid air bubbling.

To rule out possible contamination, run an additional control reaction:

NCA -Add **25 µl** of **Buffer for elution** to the tube for negative control of amplification.

8.2. 2 Amplification

1. Program the instrument according to manufacturer's manual and the Guidelines.
2. Create a temperature profile on your instrument as follows:

AmpliSens-2 RG program (for rotor-type instruments)

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycle repeats*
1	50	15 min	–	1
2	95	15 min		1
3	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s	–	

AmpliSens-2 iQ program (for plate-type instruments)

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycle repeats*
1	50	15 min	–	1
2	95	15 min	–	1
3	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4	95	5 s	–	40
	60	30 s	FAM, HEX, ROX, Cy5	
	72	15 s	–	

It is possible to carry out any combination of tests that apply AmpliSens-2 RG or AmpliSens-2 iQ program (for example, *HDV* test, *HCV*-genotyping, etc.) within the same run.

ROX/Orange and Cy5/Red channels are activated as may be required for multiprime-format tests.

4. See the Guidelines for the settings.

9. DATA ANALYSIS

Results interpretation

The signal from the Internal Control DNA amplification product is detected in the FAM/Green channel, the signal from the *HDV* cDNA amplification product is detected in the JOE/Yellow/HEX channel.

The results are interpreted by the crossing (or not crossing) of the fluorescence curve with the threshold line set at a certain level (in the middle of the linear fragment of the positive

control fluorescence growth in the log scale), which determines the presence (or absence) of Ct values for this sample in corresponding cell in the results table.

The calibration curve is constructed and the concentrations of Positive Control (in the JOE/Yellow/HEX channel) and Internal Control (in the FAM/Green channel) in the PCR sample are determined automatically on the basis of Ct values (by the crossing of the fluorescence curve with the threshold line set at a certain level) and the specified values for DNA calibrators (PIC1 and PIC2). *HDV* RNA concentration is calculated by the following formula:

$$\frac{\text{*HDV* cDNA copies per PCR-sample}}{\text{IC cDNA copies per PCR-sample}} \times \text{coefficient A} \times \text{coefficient B} = \text{copies } \textit{HDV} \text{ RNA/ml of plasma}$$

$$\text{Coefficient A} = \frac{100}{\text{extraction volume, µl}}$$

Coefficient A = 1 in calculating PCE 1 and PCE 2 concentrations

Coefficient B (IC copies/ml of plasma) is specified in the Important Product Information Bulletin enclosed in the PCR kit and specific for each lot. Coefficient B should be calculated as the result of calibration during the first PCR run (see Appendix 2 for details).

If the result is more than 100,000,000 copies/ml, then it will be displayed as the **result more than 100,000,000 copies *HDV*/ml.** If the result is more than the linear measurement range, the sample can be analyzed after 10x dilution and the obtained result should be multiplied by 10.

If the result is less than 300 copies/ml in case of extraction from 100 µl, less than 150 copies/ml in case of extraction from 200 µl, or less than 30 copies/ml in case of extraction from 1 ml, then it will be displayed as the **result less than 300, less than 150, or less than 30 copies *HDV*/ml**, respectively.

Boundary Ct values are specified in the Important Product Information Bulletin enclosed in the PCR kit.

Results are accepted as relevant if both positive and negative controls of amplification as well as negative and positive controls of extraction are passed (see the table below).

Results for controls

Control	Stage for control	Ct in channel		Interpretation
		FAM	HEX/JOE	
C–	RNA extraction	Pos (Ct ≤ value specified in the Bulletin)	Neg	OK
PCE 1	RNA extraction	Pos (Ct ≤ value specified in the Bulletin)	**Pos** (should fall in the range specified in the Bulletin as a result of calculation with IC copies/ml)	OK
PCE 2	RNA extraction	Pos (Ct ≤ value specified in the Bulletin)	**Pos** (should fall in the range specified in the Bulletin as a result of calculation with IC copies/ml)	OK
C+$_1$	Amplification	Pos	Pos	OK
C+$_2$	Amplification	Pos	Pos	OK
NCA	Amplification	Neg	Neg	OK

10. TROUBLESHOOTING

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

1. If the Ct value for the Positive Control of Extraction (PCE) or the Positive Control of Amplification (C+) in the HEX/Yellow channel is absent, PCR should be repeated from the DNA extraction stage for all samples in which *HDV* RNA is found.
2. If the Ct value is present for the Negative Control of Extraction (C–) in HEX/Yellow channel and/or for the Negative Control of Amplification (NCA) in FAM/Green, HEX/Yellow channels, PCR should be repeated from the DNA extraction stage for all samples in which *HDV* RNA is found.
3. If the correlation coefficient, R^2, is less than 0.98 when the calibration line is plotted. Repeat PCR for all samples.
4. If the calculated concentrations of Positive Control-1 *HDV* and Positive Control-2 *HDV* do not fall in the range specified in the Important Product Information Bulletin. Repeat the test (from the RNA extraction) for all samples.

11. TRANSPORTATION

AmpliSens® *HDV*-Monitor-FRT PCR kit should be transported at 2–8 °C for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *HDV*-Monitor-FRT** PCR kit and ***HDV-Q*** calibration kit are to be stored at temperature from minus 24 to minus 16 °C when not in use. All components of the **AmpliSens® *HDV*-Monitor-FRT** PCR kit and ***HDV-Q*** calibration kit 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.

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

Do not repeat freeze-thaw cycles more than twice for Positive Control-1-*HDV,* Positive Control-2-*HDV,* PIC1 *HDV,* PIC2 *HDV,* Internal Control ICZ-rec. Store the above-mentioned reagents at 2–8 °C for up to 6 month after thawing.

13. SPECIFICATIONS

13.1. Sensitivity

The linear measurement range of AmpliSens® *HDV*-Monitor-FRT PCR kit is specified in the table below.

Volume of sample for extraction, µl	DNA/RNA extraction kit	Linear measurement range, copies/ml
100	RIBO-sorb RIBO-prep NucliSENS easyMAG	300 – 100,000,000
200	MAGNO-sorb	150 – 100,000,000
1000	MAGNO-sorb NucliSENS easyMAG	30 – 100,000,000

13.2. Specificity

The analytical specificity of **AmpliSens® *HDV*-Monitor-FRT** 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 in sequences published 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 C 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*. No cross-reaction was observed for the abovementioned organisms and viruses.

The clinical specificity of **AmpliSens® *HDV*-Monitor-FRT PCR kit** was confirmed in laboratory clinical trials.

14. REFERENCES

1. Handbook “Sampling, Transportation, and Storage of Clinical Material for PCR Diagnostics”, developed by Federal Budget Institution 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*-Monitor-FRT and AmpliSens® *HBV*-Monitor-FRT PCR kits, *HDV*-Monitor-FRT.

15. QUALITY CONTROL

In compliance with Federal Budget Institution of Science “Central Research Institute for Epidemiology” ISO 13485-Certified Quality Management System, each lot of **AmpliSens® *HDV*-Monitor-FRT** 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 Institution of Science “Central Research Institute for Epidemiology”	**C+**	Positive control of amplification
PCE	Positive control of extraction	**IC**	Internal control

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
22.06.12 BO	Cover Page	IVD-symbol was changed to RUO
	Text	“tips with aerosol barriers” was changed to “tips with filters”
	Sensitivity	Sensitivity for sample 200 µl was added
	8.1. RNA Extraction	Information about MAGNO-sorb was added
30.10.12 Ivl	Through the text	Line range unit was changed from IU/ml to copies/ml