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IVD

For Professional Use Only

AmpliSens® *HAV*-FEP

PCR kit

Instruction Manual

AmpliSens®

EC REP

Ecoli s.r.o., Studenohorska 12
841 03 Bratislava 47
Slovak Republic
Tel.: +421 2 6478 9336
Fax: +421 2 6478 9040

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

TABLE OF CONTENTS

1. INTENDED USE

AmpliSens® ***HAV*****-FEP** PCR kit is an *in vitro* nucleic acid amplification test for detection of *Hepatitis A* virus *(HAV)* RNA in clinical materials (blood plasma, feces) and environmental objects (concentrated water samples) 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

PCR analysis includes the following stages: (1) RNA extraction, (2) RNA reverse transcription and cDNA/DNA amplification in the same reaction medium, and (3) end-point hybridization-fluorescence detection of amplification products.

HAV RNA detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific *HAV* primers. In **F**luorescent **E**nd-**P**oint PCR, the amplified product is detected by using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product during thermocycling. A multichannel rotor-type fluorometer is specially designed to detect fluorescence emission from the fluorophores in a reaction mixture after PCR. It allows detection of the accumulating product without re-opening the reaction tubes after the PCR run. **AmpliSens®** ***HAV*****-FEP** 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®** ***HAV*****-FEP** 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 chemically modified polymerase (TaqF). Chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

3. CONTENT

AmpliSens® ***HAV*****-FEP** PCR kit is produced in 1 form:

AmpliSens® *HAV*-FEP PCR kit variant FEP-50 F, **REF** V4-FEP-CE.

AmpliSens® *HAV*-FEP PCR kit variant FEP-50 F includes:

Reagent	*Description*	*Volume (ml)*	*Quantity*
RT-G-mix-2	colorless clear liquid	0.015	1 tube
RT-PCR-mix-1-FEP/FRT *HAV*	colorless clear liquid	0.6	1 tube
RT-PCR-mix-2-FEP/FRT	colorless clear liquid	0.3	1 tube
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
TM-Revertase (MMlv)	colorless clear liquid	0.015	1 tube
Mineral oil for PCR	colorless viscous liquid	4.0	1 dropper bottle
Positive Control cDNA *HAV*-FL / IC (C+ $_{HAV\,/\,IC}$)*	colorless clear liquid	0.1	1 tube
Negative Control (C–)**	colorless clear liquid	0.5	2 tubes
Positive Control *HAV*-FL-rec***	colorless clear liquid	0.1	1 tube
Internal Control STI-248-rec (IC)****	colorless clear liquid	0.5	1 tube
RNA-buffer	colorless clear liquid	0.6	1 tube

* This is a complex control for IC and *HAV*.

** 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).

**** add 10 µl of Internal Control STI-248-rec (IC) during the RNA extraction procedure directly to the sample/lysis mixture (RIBO-prep, **REF** K2-9-Et-50-CE).

AmpliSens® *HAV*-FEP PCR kit is intended for 55 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- RNA extraction kit.
- Transport medium.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml tubes.
- PCR box.
- Personal thermocyclers (for example, Gradient Palm Cycler (Corbett Research, Australia), GeneAmp PCR System 2700 (Applied Biosystems, USA), MaxyGene (Axygen, USA), or equivalent).
- Fluorometer (for example, ALA-1/4 (Biosan, Latvia) or equivalent).
- Personal computer.

- Disposable polypropylene microtubes for PCR (0.5- 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 aerosol barriers 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, 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 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 other suitable disinfectant.
- Avoid contact with the skin, eyes and mucosa. If skin, eyes and mucosa contact, immediately flush with water, 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

6.1. Material sampling

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

AmpliSens® ***HAV*-FEP** PCR kit is intended for the analysis of RNA extracted with RNA extraction kits from:

- peripheral blood plasma (serum);
- feces;
- water samples: wastewater concentrates (eluates), drinking water concentrates (eluates).

Container with material must be delivered to laboratory in a tank with ice within 24 h.

6.2. Preparation of the samples

Peripheral blood plasma (serum)

Blood sampling must be carrying out in the morning on an empty stomach. To obtain plasma, mix the blood with 3 % EDTA in a tube (20:1, v/v). Close the tube and turn it upside down and back several times. Centrifuge the tube at 800-1600 g for 20 min and transfer the plasma to a new tube within 6 h after taking blood. To obtain serum, tubes with blood should be kept at room temperature until a clot forms completely. Centrifuge the tube at 800-1600 g for 10 min at room temperature and then transfer the serum to a new tube. Material can be stored at 2–8 °C for 3 days and at ≤ – 68 °C for a long time.

Feces

Prepare a clarified fecal extract. For preparation use liquid stool consistency, fresh fecal suspension, or frozen fecal suspension with glycerol. Homogenize fecal suspension on the vortex. Centrifuge the suspension at 10000 g for 5 min at room temperature. Use the supernatant for RNA extraction. If necessary, store the supernatant in a new tube. The material can be stored at 2–8 °C for 1 day and at ≤ – 68 °C for a long time.

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

For preparation of fecal suspension: 1. Add 0.8 ml of PBS (or sterile isotonic NaCl solution) to 1.5-ml microcentrifuge tubes. 2. Using tips with aerosol barrier, add 0.1 g of feces and thoroughly resuspend on vortex until a homogeneous suspension forms. If fecal consistency is liquid, steps 1 and 2 are not required.

For a long storage of suspension, add glycerol to 15 % final concentration, mix thoroughly, incubate the suspension at room temperature for 1 h, and then freeze.

Concentrated water samples (eluates)

Material is used for RNA extraction without pretreatment. If the sample contains visible admixtures or has a visible color, vortex tubes with sample and then centrifuge at 10000 g for 1 min at room temperature. Use the supernatant for RNA extraction. The material can be stored at 2–8 °C for 1 day and at ≤ – 68 °C for a long time.

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

7. WORKING CONDITIONS

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

8. PROTOCOL

8.1. RNA extraction

It is recommended to use the following nucleic acid extraction kit:

- RIBO-prep, **REF** K2-9-Et-50-CE.
- NucliSENS easyMAG automated system can be used as well.

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

For Positive Control of Extraction (PCE), mix **10 µl** of **Positive Control-*HAV*-FL-rec** and **90 µl of Negative Control.**

The volume of plasma (serum) or water samples concentrates (eluates) should be 100 µl.

Volume of **Internal Control STI-248-rec (IC)** that must be added at the extraction stage depends on the RNA/DNA isolation kit used:

- **10 µl** in the case of RIBO-prep, REF K2-9-Et-100-CE.

Add **50 µl** of **Negative Control** to each tube if using fecal samples.

Purified RNA can be stored at 2–8 °C for 8 h and at ≤ –68 °C for a long time.

If RNA is isolated using NucliSENS easyMAG automated system

- "EM-plus" kit REF K2-15-96-CE (manufactured by FBIS CRIE) must be used
- set a sample volume as 100 µl
- set the eluate volume as 55 µl
- both *On-board* and *Off-board* Lysis Buffer Dispensing and Lysis Incubation modes can be used
  For details, see the Guidelines.

For RNA extraction, use only disposable sterile plastic materials with “RNase-free” and ”DNase-free” mark.

8.2. Preparing PCR

It is recommended to carry out reverse transcription combined with PCR amplification (RT-PCR) within 30 min after RNA extraction.

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

8.2.1. Preparing tubes for PCR

Carry out all control amplification reactions (positive, negative, and two Background) for testing even one RNA sample.

1. Thaw the reagents and vortex the tubes thoroughly and sediment drops from walls of tubes.
2. Prepare the required number of tubes including controls and Background tubes.
3. To prepare the reaction mix, if **Background** samples are to be prepared, mix reagents in a tube per one reaction as follows:

– **10 µl** of **RT-PCR-mix-1-FEP/FRT *HAV***

– **5 µl** of **RT-PCR-mix-2-FEP/FRT**

– **0.25 µl** of **RT-G-mix-2**.

See also Appendix 1, part A. Vortex the tubes thoroughly and sediment drops from walls of tubes.

4. Transfer **15 µl** of the prepared reaction mixture and **10 µl** of **RNA-buffer** into **2 Background** tubes. Mix by pipetting. Add above **1** drop of **mineral oil for PCR.**

Background samples can be stored at 2–20 °C for 1 month and used many times provided that the PCR kit of the same lot, the same type of extraction reagents, and the same type of amplification tubes are used.

5. Add **0.5 µl of polymerase (TaqF)** and **0.25 µl of TM-Revertase (MMlv)** (per **one reaction**) to the tube with the remaining reaction mixture according to **Appendix 1**. Carefully vortex the prepared mixture and make sure that there are no drops on the walls of the tubes.

The volumes of polymerase (TaqF) and TM-Revertase (MMlv) listed in Appendix 1 are calculated after deduction of 30 µl of reaction mixture withdrawn for the Background samples.

6. To prepare the reaction mixture, if **Background** samples are repeatedly used, mix reagents in a tube per one reaction as follows:

– **10 µl** of **RT-PCR-mix-1-FEP/FRT *HAV***

– **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)**

See also Appendix 1, part B. Vortex the tubes thoroughly and sediment drops from the walls of tubes.

7. Transfer **15 µl** of the prepared reaction mixture to other tubes. Add above **1** drop of **mineral oil for PCR**.
8. Add **10 µl** of **RNA samples** obtained from control or clinical samples on the surface or under the mineral oil for PCR.
9. Carry out control the control amplification reactions:

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

C+*HAV* / IC - Add **10 µl** of **Positive Control cDNA *HAV*-FL / IC** to the tube labeled C+*HAV* / IC (Positive Control of Amplification).

8.2.2. Reverse transcription and amplification

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

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

Table 1

***HAV* cDNA 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	Hold	**50**	30 min	1
1	**95**	15 min	1	Hold	**95**	15 min	1
3	**95**	10 s	42	Cyc-ling1	**95**	20 s	42
	60	10*s or 15**s			**60**	25 s	
	72	10*s or 15**s			**72**	25 s	
4	**10**	Storage		Cyc-ling 2	**10**	Storage	

[1] For example,
* GeneAmp PCR System 2400 (Perkin Elmer);
** Gradient Palm Cycler (Corbett Research), MaxyGene (AXYGEN Scientific), GeneAmp PCR System 2700 (Applied Biosystems).
[2] For example, PTC-100 (MJ Research), Uno-2 (Biometra), etc.

9. DATA ANALYSIS

Detection is performed using a fluorescence detector.

Please read the fluorescence detector Operating Manual before using this kit.

Detection can be conducted within 1 day after completion of amplification only if the tubes with the amplified product have been stored at 2-8 °C in a light-free area.

Program the detector according to the manufacturer's manual, Important Product Information Bulletin, and Guidelines [2].

The fluorescent signal intensity is detected in two channels:

– the signal from the **IC** amplification product is detected in the FAM channel (or analogous, depending on the detector model);

– the signal from the ***HAV*** RNA amplification product is detected in the HEX channel (or analogous, depending on the detector model).

Prior to detection, all settings should be entered and saved. Refer to the **Guidelines** and the **Important Product Information Bulletin** for settings.

9.1. Interpretation of results for clinical samples

Principle of interpretation:

- *HAV* RNA **is detected** in a sample if its signal in the HEX channel is greater than the defined threshold value of the positive result.
- *HAV* RNA **is not detected** in a sample if the signal in the HEX channel is less than the defined threshold value of the negative result whereas the signal in the FAM channel is greater than the defined threshold value.
- The result is **invalid** in a sample if the signal in the HEX channel is less than the defined threshold value of the negative result and the signal in the FAM channel is less than the defined threshold value.
- The result is **equivocal** if the signal of a sample in the HEX channel is greater than the defined threshold value of the negative result but less than the threshold value of the positive result (the signal is between thresholds).

If the result is invalid or equivocal, the PCR should be repeated once again. If the result is equivocal again, the sample is considered to be positive. If the result of the repeated test is negative, the sample is considered to be equivocal.

The result of the analysis is considered reliable only if the results obtained for both

Positive and Negative controls of amplification as well as for the Negative control of extraction are correct (Table 2).

9.2. Interpretation of results for control samples

Table 2

Results for controls

Control	Stage for control	Result of automatic interpretation		Interpretation
		FAM channel (IC)	**HEX channel** (samples)	
C–	RNA extraction	**>** threshold	**<** threshold of negative result	**"–" or OK**
PCE	RNA extraction	**>** threshold	**>** threshold of positive result	**"+" or OK**
NCA	Amplification	**<** threshold	**<** threshold of negative result	**nd or OK**
C+$_{HAV\ /\ IC}$	Amplification	**>** threshold	**>** threshold of positive result	**"+" or OK**

10. TROUBLESHOOTING

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

- Negative signal in C+$_{HAV\ /\ IC}$ may indicate incorrect programming of the temperature profile of the thermocycler, incorrect configuration of PCR, noncompliance of the storage conditions for kit components with the manufacturer's instruction, or the expiration of the reagent kit. Check programming of the thermocycler (see 8.2.2.), storage conditions, and the expiration date of the reagents and repeat PCR once again for all samples.
- If no signal was detected either in the channel for detection of the pathogen DNA or in the channel for detection of IC, the sample should be examined once again (PCR and detection). The same applies to the samples with equivocal results, because the fact that the specific signal does not exceed the threshold value is not sufficient to consider a sample as positive. If equivocal results are obtained in the second run, the analysis should be repeated starting from the DNA extraction stage.
- Positive signal for C– in the HEX channel and NCA in all channels indicates reagent or sample contamination. In this case, the results of analysis must be considered as invalid. The analyses must be repeated and measures for detecting and eliminating the contamination source must be taken.
- If the signal for PCE is less than the threshold of positive result, repeat RNA extraction stage.

If you have any further questions or if you encounter problems, please contact our

Authorized representative in the European Community.

11. TRANSPORTATION

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

12. STABILITY AND STORAGE

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

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

RT-PCR-mix-1-FEP/ FRT *HAV* is to be kept away from light.

13. SPECIFICATIONS

13.1. Sensitivity

Variant	Volume, µl	Nucleic extraction kit	Material	Sensitivity, cop/ml
Variant FEP-50 F	100	RIBO-prep	Blood plasma (serum), clarified fecal extracts, concentrated water samples (eluates)	500
	100	NucliSENS easyMAG	Blood plasma (serum), concentrated water samples (eluates)	500

13.2. Specificity

The analytical specificity of **AmpliSens® *HAV*-FEP** PCR kit is ensured by selection of specific primers and probes as well as strict reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis. The analytical specificity of **AmpliSens® *HAV*-FEP** PCR kit was checked by testing RNA/DNA of the following organisms and viruses: *HBV, HCV, HDV, HEV, HGV*, *HIV*, *CMV*, *EBV*, *HSV* I and II types, *HSV* VI and VIII types, *Enterovirus* (Coxsakie B1, B2, B3, B4, B5, B6, Polio I, II, III), human *Rotavirus* WA, *Astrovirus*, *Norovirus* I and II types, *Adenovirus* (types II, III, VII), *Shigella, Salmonella, Yersinia,*

Campylobacter, Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus agalactiae, Homo sapiens.

Cross-reactions for the listed organisms were not detected.

The clinical specificity of **AmpliSens® *HAV*-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 instruction manual AmpliSens® *HAV*-FEP, 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.

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® *HAV*-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
IVD	*In vitro* diagnostic medical device		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
EC REP	Authorised representative in the European Community	**C+$_{HAV\ /\ IC}$**	Positive control of amplification
PCE	Positive control of extraction	**IC**	Internal control
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
19.06.11 RT	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”