

IVD

For *in Vitro* Diagnostic Use

TABLE OF CONTENTS

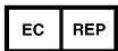
1. INTENDED USE	3
2. PRINCIPLE OF PCR DETECTION	3
3. CONTENT	3
4. ADDITIONAL REQUIREMENTS.....	4
5. GENERAL PRECAUTIONS	4
6. SAMPLING AND HANDLING	5
7. PROTOCOL	5
8. DATA ANALYSIS.....	9
9. TROUBLESHOOTING	10
10. STABILITY AND STORAGE.....	10
11. SPECIFICATIONS.....	11
12. REFERENCES.....	11
13. QUALITY CONTROL	12
14. EXPLANATION OF SYMBOLS	12

AmpliSens[®] HAV-FRT

PCR kit

Instruction Manual

AmpliSens[®]



Ecoli s.r.o., Studenohorská 12
841 03 Bratislava 47
Slovak Republic
Tel.: +421 2 6478 9336
Fax: +421 2 6478 9040
ecoli@ecoli.sk
www.ecoli.sk www.pcrdiagnostics.eu



Federal State Institution of Science
Central Research Institute of Epidemiology
3A Novogireevskaya Street
Moscow 111123 Russia

1. INTENDED USE

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

PCR analysis includes the following stages: (1) RNA extraction and (2) RNA reverse transcription and cDNA/DNA amplification in the same reaction medium with real-time fluorescence-hybridization detection.

HAV RNA detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific *HAV* primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run. **AmpliSens[®] HAV-FRT** 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-FRT** 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-FRT PCR kit is produced in 1 form:

AmpliSens[®] *HAV-FRT* PCR kit variant FRT-50 F, **REF** R-V4(RG,iQ,Mx)-CE.

AmpliSens[®] *HAV-FRT* PCR kit variant FRT-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 (MMIv)	colorless clear liquid	0.015	1 tube
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-FRT* PCR kit is intended for 55 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- RNA extraction kit.
- 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 rotor for 2 ml reaction tubes.
- PCR box.
- Personal thermocyclers (for example, Rotor-Gene 3000 or Rotor-Gene 6000 (Corbett Research, Australia); iCycler iQ5 (Bio-Rad, USA); Mx 3000P (Stratagene, USA) or equivalent).
- Personal computer.
- 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 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, 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 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 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

6.1. Material sampling



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

AmpliSens® HAV-FRT 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), and .

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 the 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. PROTOCOL

7.1. RNA extraction

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

- 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 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

7.2. 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**.

7.2.1. Preparing tubes for PCR



Carry out 2 control amplification reactions even while testing only 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.
3. Mix **RT-PCR-mix-1-FEP/FRT HAV** with **RT-PCR-mix-1-FEP/FRT**, **RT-G-mix-2**, **polymerase (TaqF)**, and **TM-Revertase (MMIv)** according to **Appendix 1**. Vortex the tubes thoroughly and sediment drops from walls of tubes.
4. Transfer **15 µl** of the prepared mixture to the tubes.
5. Add **10 µl** of **RNA** obtained from clinical or control samples into the prepared tubes

using tips with aerosol barrier. Carefully mix by pipetting.

6. 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).

7.2.2. Reverse transcription and amplification

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

Table 1

AmpliSens-3 amplification program for rotor-type instruments¹

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	50	30 min	–	1
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow/HEX	
	72	15 s	–	

Table 2

AmpliSens-3 amplification program for plate-type instruments²

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	50	30 min	–	1
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s	FAM, HEX/JOE	
	72	15 s	–	

3. Insert tubes into the instrument.
4. Adjust the fluorescence channel sensitivity and carry out data analysis according to Guidelines.

¹ For example, RotorGene 3000 and RotorGene 6000 (Corbett Research, Australia).

² For example, iCycler iQ, iQ5 (Bio-Rad, USA), Mx3000P (Stratagene, USA)

8. DATA ANALYSIS

IC is detected in the FAM/Green fluorescence channel, HAV RNA is detected in the JOE/Yellow/HEX fluorescence channel.

8.1. Results interpretation for samples

The results are interpreted by the software of instrument by the crossing (or not-crossing) of the fluorescence curve with the threshold line.

Principle of interpretation:

- HAV RNA is **detected** in a sample if Ct of a sample does not exceed the specified boundary value in the JOE/Yellow/HEX channel. Moreover, the fluorescence curve should cross the threshold line in the area of exponential fluorescence growth.
- HAV RNA is **not detected** in a sample if its Ct is not defined in the result grid in the JOE/Yellow/HEX channel (the fluorescence curve does not cross the threshold line) while Ct in the FAM/Green channel does not exceed the specified boundary value.
- The result is considered to be **invalid** if Ct of a sample in the FAM/Green channel is absent whereas Ct in the JOE/Yellow/HEX channel is either absent or greater than the specified boundary value. It is necessary to repeat RNA extraction for such a sample.
- The result is considered to be **equivocal** if Ct of a sample exceeds the specified boundary value in the JOE/Yellow/HEX channel. It is necessary to repeat RNA extraction for such a sample. If the result repeats as positive, the sample is considered to be positive. If the result repeats as negative, the sample is considered to be equivocal.

8.2. Results interpretation for control samples

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 positive and negative controls of extraction are correct.

Table 3

Results for controls

Control	Stage for control	Ct value in channel JOE/Yellow/HEX	Ct value in channel FAM/Green	Interpretation
C-	RNA extraction	Neg	Pos (\leq boundary value*)	OK
PCE	RNA extraction	Pos (\leq boundary value*)	Pos (\leq boundary value*)	OK
NCA	Amplification	Neg	Neg	OK
C+ _{HAV/IC}	Amplification	Pos (\leq boundary value*)	Pos (\leq boundary value*)	OK

* For Ct values, see the **Important Product Information Bulletin**.

9. TROUBLESHOOTING

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

- If the Ct value is absent in both JOE/Yellow/HEX and FAM/Green channels or the Ct value in the JOE/Yellow /HEX channel is higher than the specified boundary value, PCR should be repeated. If the same result is obtained, the extraction stage for the sample should be repeated. If the IC signal of this sample was detected normally in any other PCR test, it is not necessary to repeat the extraction stage (if iCycler iQ or iQ5 instruments are used).
- If the Ct value is present for C- in the JOE/Yellow/HEX channel and/or for NCA in both channels in the results grid, it indicates contamination of reagents or samples. In such cases, the results of analysis must be considered as invalid. Test analysis must be repeated and measures to detect and eliminate the source of contamination must be taken.
- If the Ct value for PCE is absent or exceeds the boundary value, repeat RNA extraction stage.
- If no signal is detected for the positive control of amplification, it may suggest that the programming of the temperature profile of the used Instrument was incorrect, or that the configuration of the PCR reaction was incorrect, or that the storage conditions for kit components did not comply with the manufacturer's instruction, or that the reagent kit expired. Programming of the used instrument, storage conditions, and the expiration date of the reagents should be checked, and then PCR should be repeated.
- If a positive result (the fluorescence curve crosses the threshold line) is detected for a sample that has a fluorescence curve without the typical exponential growth phase (the curve is linear), this may suggest incorrect setting of the threshold line or incorrect calculation of baseline parameters. Such a result should not be considered as positive. Once the threshold line has been set correctly, PCR analysis of the sample should be repeated (if iCycler iQ or iQ5 instruments are used).

10. STABILITY AND STORAGE

All components of the **AmpliSens[®] HAV-FRT** PCR kit (except for RT-G-mix-2, polymerase (TaqF), TM-Revertase (MMIv), 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-FRT** PCR kit are stable until the expiration date on the label.



RT-G-mix-2, polymerase (TaqF), TM-Revertase (MMIv), RT-PCR-mix-1-FEP/FRT HAV, and RT-PCR-mix-2-FEP/FRT should be stored at ≤ -16 °C.



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

11. SPECIFICATIONS

11.1. Sensitivity

Variant	Volume, µl	Nucleic extraction kit	Material	Sensitivity, cop/ml
Variant FRT-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

11.2. Specificity

The analytical specificity of **AmpliSens® HAV-FRT** 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-FRT** 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* (Coxsackie 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-FRT** PCR kit was confirmed in laboratory clinical trials.

12. REFERENCES

- Handbook "Sampling, Transportation, Storage of Clinical Material for PCR Diagnostics", developed by Federal State Institution of Science "Central Research Institute of Epidemiology" of Federal Service for Surveillance on Consumers' Rights Protection and Human Well-Being, Moscow, 2008.
- Guidelines to instruction manual **AmpliSens® HAV-FRT**.

13. QUALITY CONTROL

In compliance with Federal State Institution of Science "Central Research Institute of Epidemiology" ISO 13485-Certified Quality Management System, each lot of **AmpliSens® HAV-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

14. EXPLANATION OF SYMBOLS



Manufacturer



Temperature limitation



Use by



Batch code



For *in Vitro* Diagnostic Use



Version



Catalogue number



Caution, consult accompanying documents



Contains sufficient for <n> tests



Negative Control of Amplification



Consult instructions for use



Negative control of extraction



Positive control of amplification



Internal Control



Positive control of extraction



Central Research Institute of Epidemiology (Moscow, Russia)

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
19.10.10	Content	Abbreviation C ⁺ _{HAV/IC} is added for Positive Control cDNA HAV-FL / IC Footnotes are changed
	Stability and storage	The phrase about keeping RT-PCR-mix-1-FEP/ FRT HAV away from light is added