RUO

For Professional Use Only

AmpliSens® *HCV*-1/2/3-FRT

PCR kit

Instruction Manual

AmpliSens®

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

TABLE OF CONTENTS

1. INTENDED USE

AmpliSens® *HCV*-1/2/3-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection and discrimination between *hepatitis C virus* (*HCV*) genotype 1, 2, and 3 RNA in *HCV*-positive clinical material (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

Hepatitis C virus detection includes total RNA extraction from blood plasma and reverse transcription of RNA into cDNA combined with real-time PCR amplification of cDNA. *HCV* detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific *HCV* 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. The real-time monitoring of fluorescence intensities during the real-time PCR allows detection of the amplified product without re-opening the reaction tubes after the PCR run. **AmpliSens® *HCV*-1/2/3-FRT** PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. The “hot-start” is guaranteed by separation of nucleotides and Taq polymerase by using a chemically modified polymerase (TaqF). The latter 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 amplification product of an *HCV* cDNA fragment common for all three genotypes, which is used in this test as an internal control (IC) confirming the presence of *HCV* cDNA in tested samples, is detected in the Cy5/Red channel.

The Positive Control of Extraction, Positive Control-1-*HCV,* is detected in the FAM/Green (genotype 1) and Cy5/Red (all genotypes) channels.

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), ROX/Texas Red/Orange (genotype 3), and Cy5/Red (genotypes 1, 2, and 3) channels.

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

3. CONTENT

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

AmpliSens® *HCV*-1/2/3-FRT PCR kit variant FRT (for use with RG, iQ, and Mx)

REF R-V1-G-4x(RG,iQ,Mx)-CE.

AmpliSens® *HCV*-1/2/3-FRT PCR kit 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
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 extraction procedure as Negative Control of Extraction.

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

AmpliSens® *HCV*-1/2/3-FRT PCR kit is intended for 55 reverse transcription and amplification reactions including controls.

4. ADDITIONAL REQUIREMENTS

- RNA/DNA extraction 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
- Rotor-Gene 3000 or Rotor-Gene 6000 (Corbett Research, Australia) instrument; or iQ5 (Bio-Rad, USA) instrument; or Mx3000P (Stratagene, USA) instrument, or equivalent .
- Disposable 0.2-ml polypropylene PCR microtubes (with flat and domed caps for rotor-type and plate-type PCR instruments, respectively) (for example, Axygen, USA)
- Refrigerator for 2–8 °C

- Deep-freezer with temperature below or at minus 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 positive extracted 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 an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats, 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 all samples and unused reagents in compliance with local authorities' requirements.
- Samples should be considered potentially infectious and handled in a biological cabinet in compliance 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 mucose membranes. If skin, eyes and mucose membranes 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 unidirectional; it should begin in the Extraction Area 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

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

AmpliSens® *HCV*-1/2/3-FRT PCR kit is intended for analysis of RNA extracted with an RNA/DNA extraction 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-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. RNA/DNA extraction

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 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 (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 a sample volume as 0.1–1 ml
- Set the eluate volume as 50–60 µl (up to 100 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 extraction.
  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

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

All components of the reaction mixture should be mixed immediately before use. Mix reagents for the required number of reactions for experimental and control samples according to Appendix 1.

8.2.1 Preparing tubes for PCR

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 PCR tubes for amplification of clinical and control samples (including two controls of extraction and one control of amplification).
3. **To prepare the reaction mixture**, mix the reagents (**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), and 0.25 µl of TM-Revertase (MMlv)** per one reaction) in a new sterile tube (see also Appendix 1). Thoroughly vortex the mixture, make sure that there are no drops on the caps of the tubes.
4. Add **15 µl** of the prepared reaction mixture to each PCR tube.
5. Add **10 µl** of **RNA samples** isolated from the clinical samples to each PCR tube.

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.

6. Run the **control reactions**:

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

C– - Add **10 µl** of the **RNA sample** extracted from the 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 tube walls, otherwise vortex the tubes briefly.

8.2.2 Amplification

8.2.2.1. RG

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

AmpliSens-3 RG program for rotor-type instruments[1]

Step	***Temperature*, °C**	***Time***	***Fluorescence detection***	**Cycle repeats**
1 (Hold)	50	30 min	–	1
2 (Hold)	95	15 min	–	1
3 (Cycling 1)	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4 (Cycling 2)	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s	–	

3. Adjust the fluorescence channel sensitivity as described in the Guidelines.

	This program makes it possible to simultaneously carry out any combination of tests in the same instrument using the same amplification program.

8.2.2.2. iQ

1. Program the iCycler iQ or iQ5 instrument according to manufacturer's manual and the Guidelines.
2. Create a temperature profile on your iQ5 instrument as follows:

AmpliSens-3 iQ program for plate-type instruments[2]

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

3. Adjust the fluorescence channel sensitivity as described in the Guidelines.

[1] For example, Rotor-Gene 3000 or 6000 (Corbett Research, Australia)
[2] For example, iQ5 (Bio-Rad, USA), Mx3000P (Stratagene, USA), DT-96 (DNA-Technology, Russia) , or equivalent

This program makes it possible to simultaneously carry out any combination of tests in the same instrument using the same amplification program.

9. DATA ANALYSIS

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 amplification product of an *HCV* cDNA fragment common for all three *HCV* genotypes, which confirms the presence of the *HCV* cDNA in analyzed samples, is detected in the Cy5/Red channel.

The results are interpreted by the real-time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve (in the middle of the linear section of the fluorescence curve for the positive control (C+) in logarithmic coordinates).

The result of amplification is considered **positive** if the fluorescence curve is characteristic of real-time PCR (S-shaped) and crosses the threshold line once in the significant fluorescence increase section and if the Ct value detected in the channel is below the threshold value specified in the Important product information bulletin, .

The result of amplification is considered **negative** if the fluorescence curve is not S-shaped and if it does not cross the threshold line (the Ct value is absent).

In all other cases, the result is considered **equivocal**.

For data analysis settings for Rotor-Gene 3000/6000, iQ5, Mx3000, and DT-96 real-time PCR instruments, see the Guidelines.

Results interpretation

The results are interpreted by the real-time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve.

Results for controls

Control	Stage for control	Ct in channel				Inter-preta-tion
		FAM/Green	JOE/HEX/ Yellow	ROX/ Orange	Cy5/Red	
C–	RNA extraction	Neg	Neg	Neg	Neg	OK
PCE	RNA extraction	Pos (≤ Ct*)	Neg	Neg	Pos (≤ Ct*)	OK
C+	Amplification	Pos (≤ Ct*)	Pos (≤ Ct*)	Pos (≤Ct*)	Pos (≤ Ct*)	OK

*The boundary Ct values are summarized in the Important Product Information Bulletin.

Interpretation of results in clinical samples

1. The **clinical sample** contains ***HCV* genotype 1 RNA** if a positive result is detected in the FAM/Green channel.

2. The **clinical sample** contains ***HCV* genotype 2 RNA** if a positive result is detected in the Joe/Hex/Yellow channel.

3. The **clinical sample** contains ***HCV* genotype 3 RNA** if a positive result is detected in the ROX/Orange channel.

4. The **clinical sample** contains **RNA of *HCV* of another (rare) genotype** if Ct value in channels FAM/Green, Joe/Hex/Yellow, and ROX/Orange is absent and a positive result is detected in the Cy5/Red channel.

5. The **clinical sample** is regarded **equivocal** is an equivocal result is detected in any of the channels. Such sample should be analyzed once again.

6. The **clinical sample** is regarded as **nontypeable due to a low viral load** if its analysis repeated twice starting from the extraction stage yielded a negative amplification result in all the four channels (FAM/Green, JOE/HEX/Yellow, ROX/Orange, and Cy5/Red) or a combination of equivocal and negative results was obtained.

For details, see the Guidelines.

Results are accepted as significant only if both positive and negative controls of RNA extraction and the negative controls of amplification passed correctly (see above the table for controls).

10. TROUBLESHOOTING

- The absence of positive signal in PCE in FAM/Green and Cy5-Red channels or the absence of positive signal in C+ in all four channels may indicate incorrect amplification program or other errors made during RNA/DNA extraction or PCR amplification. In this case, PCR should be carried out once again.
- Detection of any Ct value in C– in the results grid for FAM/Green, JOE/HEX/Yellow, ROX/Orange, and Cy5/Red channels suggests contamination of reagents or samples. In this case, it is necessary to repeat the analysis of all tests starting from the extraction stage and to take measures for detecting and eliminating the source of contamination.

11. TRANSPORTATION

AmpliSens® *HCV*-1/2/3-FRT 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-FRT** 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. They are stable until the expiration date indicated on the label. 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 ≤ –16 °C.

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-FRT** PCR kit is specified in the table below.

Sample volume for extraction, µl	RNA/DNA extraction method	Analytical sensitivity, IU/ml
100	"RIBO-sorb" "RIBO-prep" "NucliSENS easyMAG"	500
1000	"NucliSENS easyMAG"	50

The claimed analytical features of **AmpliSens® *HCV*-1/2/3-FRT** 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-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 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-FRT** PCR kit was confirmed in laboratory clinical trials.

Cross-reactions for the above-mentioned organisms and viruses have not been detected.

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-FRT PCR kit.

15. QUALITY CONTROL

In compliance with the Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Quality Management System, each lot of **AmpliSens® *HCV*-1/2/3-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	Σ	Sufficient for
RUO	Research use only		Expiration Date
VER	Version		Consult instructions for use
	Temperature limitation		Keep away from sunlight
	Upper limit of temperature	**NCA**	Negative control of amplification
	Manufacturer	**C–**	Negative control of extraction
	Date of manufacture	**C+**	Positive control of amplification
FBIS CRIE	Federal Budget Institute of Science "Central Research Institute for Epidemiology"	**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 R-V1-G-4x(RG,iQ,Mx) to R-V1-G-4x(RG,iQ,Mx)-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-FRT 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 IvI	Title page, Key to symbols used	Symbol IVD *in vitro* diagnostic medical device was changed to RUO research use only
03.10.12 LA	13.2. Specificity	The name of the PCR kit was corrected