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For Professional Use Only

AmpliSens® *HCV*-genotype-FRT

PCR kit

Instruction Manual

AmpliSens®

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TABLE OF CONTENTS

1. INTENDED USE

AmpliSens® *HCV*-genotype-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection and differentiation of *hepatitis C virus* (*HCV*) genotypes in the clinical materials (peripheral blood plasma) by means of real-time hybridization-fluorescence detection.

PCR kit variant FRT-g1-4 is intended to detect *HCV* genotypes 1a, 1b, 2, 3a, and 4.

PCR kit variant FRT-g1-6 is intended to detect *HCV* genotypes 1a, 1b, 2, 3a, 4, 5a, and 6.

AmpliSens® *HCV*-genotype-FRT PCR kit is recommended for use after detection of *hepatitis C virus* RNA by qualitative or quantitative analysis with the use of PCR kits manufactured by FBIS CRIE (for example, **AmpliSens® *HCV*-FRT** (real-time hybridization-fluorescence detection), **AmpliSens® *HCV*-FEP** (end-point hybridization-fluorescence detection), or **AmpliSens® *HCV*-EPh** (electrophoretic detection in agarose gel).

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

2. PRINCIPLE OF PCR DETECTION

Detection of *HCV* genotypes 1a, 1b, 2, 3a, 4, 5a, and 6 by polymerase chain reaction (PCR) is based on the amplification of a pathogen genome specific region using specific primers. In real-time PCR, the amplified product is detected by using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product. 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® *HCV*-genotype-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), which is activated by heating at 95 °C for 15 min.

HCV genotype detection includes:

(a) Total RNA extraction from blood plasma simultaneously with the recombinant Internal Control (IC) sample.

(b) Reverse transcription of cDNA on RNA template.

(c) Real-time PCR of *HCV* cDNA.

To rule out possible false negative results, the Internal Control is included in the assay. This makes it possible to monitor all stages of the analysis and reveal the effect of PCR

inhibitors on the result.

Detection of *HCV* genotypes in a single clinical sample is carried out in several tubes. Either two *HCV* genotypes or *HCV* genotype and IC can be discriminated in one tube during the run.

The PCR kit is designed for the real-time PCR instruments with two and more fluorescence detection channels. The table below shows the channels for detection of *HCV* genotypes for each of the reaction mixtures used:

Reaction mixture	**1b/3a**	**1a/2**	**IC/4**	**5a/6**[1]
Channel	***HCV* genotype to be detected**			
FAM/Green	1b	1a	IC	5a
JOE/HEX/Yellow/Cy3	3a	2	4	6

3. CONTENT

AmpliSens® *HCV*-genotype-FRT PCR kit is produced in 2 forms:

AmpliSens® *HCV*-genotype-FRT PCR kit variant FRT-g1-4, **REF** R-V1-G(1-4)-2x(RG,iQ,Mx,Dt,SC)-CE.

AmpliSens® *HCV*-genotype-FRT PCR kit variant FRT-g1-6, **REF** R-V1-G(1-6)-2x(RG,iQ,Mx,Dt,SC)-CE.

AmpliSens® *HCV*-genotype-FRT PCR kit variant FRT-g1-4 includes:

Reagent	***Description***	***Volume, ml***	***Quantity***
PCR-mix-1-FRT *HCV* genotypes 1b/3a	colorless clear liquid	0.6	1 tube
PCR-mix-1-FRT *HCV* genotypes 1a/2	colorless clear liquid	0.6	1 tube
PCR-mix-1-FRT *HCV* IC/genotype 4	colorless clear liquid	0.6	1 tube
RT-PCR-mix-2-FEP/FRT	colorless clear liquid	0.3	3 tubes
Polymerase (TaqF)	colorless clear liquid	0.03	3 tubes
Positive Control cDNA *HCV* genotypes 1b/3a ($C+_{1b/3a}$)	colorless clear liquid	0.2	1 tube
Positive Control cDNA *HCV* genotypes 1a/2 ($C+_{1a/2}$)	colorless clear liquid	0.2	1 tube
Positive Control cDNA *HCV* IC/genotype 4 ($C+_{IC/4}$)	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.5	1 tube

[1] Included in the PCR kit variant FRT-g1-6.

Negative Control (C–)*	colorless clear liquid	0.5	1 tube
Internal Control STI-248-rec (IC)**	colorless clear liquid	0.5	1 tube

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

must be used in extraction as the Internal Control (see "RIBO-sorb" **REF K2-1-Et-50-CE or RIBO-prep **REF** K2-9-Et-50-CE protocols).

AmpliSens® *HCV*-genotype-FRT PCR kit variant FRT-g1-4 is intended for 55 tests (165 amplification reactions) including controls.

AmpliSens® *HCV*-genotype-FRT PCR kit variant FRT-g1-6 includes:

Reagent	***Description***	***Volume, ml***	***Quantity***
PCR-mix-1-FRT *HCV* genotypes 1b/3a	colorless clear liquid	0.6	1 tube
PCR-mix-1-FRT *HCV* genotypes 1a/2	colorless clear liquid	0.6	1 tube
PCR-mix-1-FRT *HCV* IC/genotype 4	colorless clear liquid	0.6	1 tube
PCR-mix-1-FRT *HCV* genotypes 5a/6	colorless clear liquid	0.6	1 tube
RT-PCR-mix-2-FEP/FRT	colorless clear liquid	0.3	4 tubes
Polymerase (TaqF)	colorless clear liquid	0.03	4 tubes
Positive Control cDNA *HCV* genotypes 1b/3a ($C+_{1b/3a}$)	colorless clear liquid	0.2	1 tube
Positive Control cDNA *HCV* genotypes 1a/2 ($C+_{1a/2}$)	colorless clear liquid	0.2	1 tube
Positive Control cDNA *HCV* IC/genotype 4 ($C+_{IC/4}$)	colorless clear liquid	0.2	1 tube
Positive Control cDNA *HCV* genotypes 5a/6 ($C+_{5a/6}$)	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C-)*	colorless clear liquid	0.5	1 tube
Internal Control STI-248-rec (IC)**	colorless clear liquid	0.5	1 tube

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

must be used in the extraction as the Internal Control (see "RIBO-sorb" **REF K2-1-Et-50-CE or RIBO-prep **REF** K2-9-Et-50-CE protocols).

AmpliSens® *HCV*-genotype-FRT PCR kit variant FRT-g1-6 is intended for 55 tests

(220 amplification reactions) including controls.

4. ADDITIONAL REQUIREMENTS

- Nucleic acid extraction kit
- Reverse transcription reagent 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.
- PCR box.
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.
- Rotor-Gene 3000 or Rotor-Gene 6000 (Corbett Research, Australia); Rotor-Gene Q (Qiagen, Germany), iCycler iQ5 (Bio-Rad, USA); SmartCycler II (Cepheid, USA) or Mx3000P, Mx3005P (Stratagene, USA).
- Disposable polypropylene tubes for PCR with
  - 0.2 (0.1) ml capacity suitable for rotor-type PCR instruments (flat-cap tubes or striped tubes);
  - 0.2 ml capacity suitable for plate-type PCR instruments (domed-cap tubes or striped tubes)
  - 0.025 ml capacity tubes if SmartCycler (Cepheid, USA) is used.
- Centrifuge and tube racks (Cepheid, USA) if SmartCycler (Cepheid, USA) is used.

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 an assay.
- 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 DNA amplification techniques.
- Workflow in the laboratory must proceed in a unidirectional 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 samples of biological material for PCR-analysis, transportation, and storage are described in detail in the manufacturer's handbook [1]. It is recommended that this handbook is read before starting the work.

AmpliSens® *HCV*-genotype-FRT PCR kit is intended for the analysis of RNA extracted with nucleic acid extraction kits from:

— *Peripheral blood plasma*

Collect a blood sample in a tube with 3% EDTA solution in the ratio of 20:1 (20 parts of blood to 1 part of EDTA). Invert the closed tube several times to ensure adequate mixing. Remove and transfer the plasma specimen in a new tube within 6 h from the time of blood taking. To do this, centrifuge the tube with blood at 800 – 1600 rpm for 20 min.

Storage of plasma samples:

– at 2–8 °C for up to 3 days;

– at ≤–68 °C for a long time.

7. WORKING CONDITIONS

AmpliSens® *HCV* genotype-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. RNA extraction

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

- **RIBO-sorb** REF K2-1-Et-50-CE (procedure is described below).
- **RIBO-prep**, REF K2-9-Et-50-CE (follow the instructions of the manufacturer);

RNase-free and DNase-free plastic ware should be used only.

Obtained RNA is not to be stored.
Proceed to reverse transcription immediately after obtaining RNA sample.

"RIBO-sorb" extraction instructions

1. Warm up **Lysis Solution** and **Washing Solution 1** (if stored at 2–8 ºC) at 60 °C until the ice crystals disappear.
2. Take the required number of 1.5 ml tubes including the tube for Negative Control of Extraction (**C–**). Label the tubes.
3. Add **10 µl of IC STI-248-rec** to the bottom of each tube.
4. Add **450 µl of Lysis Solution** to each tube.

If a considerable number of samples is processed, it is recommended to mix **Lysis Solution** and **IC STI-248-rec** in a new tube (450 µl of Lysis Solution and 10 µl of IC STI-248-rec per one sample) and transfer **450 µl of prepared mixture** into each 1.5-ml tube

5. Add **100 µl of plasma sample** per each tube containing Lysis Solution and IC using a tip with filters. Add **100 µl of Negative Control** sample to the tube intended for the Negative Control of Extraction (C–).
6. Close the tubes and vortex. To remove drops from tubes walls, centrifuge at 5,000 rpm for 5 s.
7. Thoroughly resuspend **Sorbent** with the vortex. Add **25 µl** of resuspended sorbent into each test tube. Use a new tip for every tube.
8. Vortex the tubes and then incubate at room temperature for 10 min stirring every 2 min.
9. Centrifuge the tubes at 7,000 g for 1 min.

10. Remove and discard the supernatant from the tubes with vacuum aspirator. Use a new tip for every tube.
11. Add **400 µl** of **Washing Solution 1** to each tube. Vortex thoroughly (until the sorbent is completely resuspended), then centrifuge at 7,000 g for 1 min. Remove and discard the supernatant with vacuum aspirator. Use a new tip for every tube.
12. Add **500 µl** of **Washing Solution 3** to each tube. Vortex thoroughly (until sorbent is fully resuspended), then centrifuge at 7,000 g for 1 min. Remove and discard the supernatant with vacuum aspirator. Use a new tip for every tube.
13. Add **400 µl** of **Washing Solution 4** to each tube. Vortex thoroughly (until sorbent is fully resuspended) then centrifuge at 7,000 g for 1 min. Remove and discard the supernatant with vacuum aspirator. Use a new tip for every tube.
14. Incubate the tubes at 60 °C for 15 min to dry the sorbent. Make sure the tubes are open while incubating.
15. Add **50 µl of RNA-buffer** to each tube. Resuspend the sorbent in RNA-buffer, incubate at 60 °C for 2 min, and then vortex. To sediment the sorbent, centrifuge the tubes at 12,000 g for 1 min.

Do not store RNA samples.
Perform reverse transcription immediately after RNA samples are collected.
Be careful when removing RNA: do not transfer the sorbent.
Centrifuge the tube before removing RNA sample if the sorbent looks resuspended.

8.2 Reverse transcription

It's recommended that the following reverse transcription reagent kit is used:

- REVERTA-L, **REF** K3-4-50-CE (procedure is describe below).

Carry out reverse transcription immediately after mixing reaction components.

The total reaction volume is **20 µl**, the volume of RNA sample is **10 µl**.

RNase-free and DNase-free plastic consumables should be used only.

A. Preparing tubes

1. Thaw the tubes with **RT-mix** and **RT-G-mix-1**and thoroughly vortex. Remove drops from the walls of the tubes.
2. Take the required number of 0.2- or 0.5-ml tubes (depends on the type of thermocycler or thermostate to be used) including a tube for Negative Control of extraction (C–). Label the tubes.
3. Reverse transcription for 10-12 samples:

a) Prepare the reaction mixture for 12 reactions. To do this, add **5 µl** of **RT-G-mix-1** to the tube with **RT-mix** and vortex. To remove drops from tubes walls, centrifuge briefly.

b) Add **6 µl** of **revertase (MMlv)** to the tube with the reaction mixture, pipette 5 times, and vortex. To remove drops from tubes walls, centrifuge briefly.

4. Reverse transcription for less than 10 samples:

In a new tube mix the reagents in the following order: **10 µl of RT-mix, 0.4 µl of RT-G-mix-1**, and **0.5 µl of revertase (MMlv)** (the quantities are calculated per one reaction; also see table 1). When adding RT-G-mix-1 and revertase (MMlv), pipette each reagent at least 5 times. Vortex the mixture and remove drops from the walls of the tubes.

Revertase (MMlv) is temperature-sensitive and should not be kept at room temperature for a long while! Place the reagent in a freezer immediately after use.

Table 1

Scheme of reaction mixture preparation

Volume of reagent per one reaction, µl	10.0	0.4	0.5
Number of clinical samples	**RT-mix**	**RT-G-mix-1**	**Revertase (MMlv)**
4	**60**[2]	**2.4**	**3.0**
5	**70**	**2.8**	**3.5**
6	**80**	**3.2**	**4.0**
7	**90**	**3.6**	**4.5**
8	**100**	**4.0**	**5.0**

5. Transfer **10 µl** of the prepared mixture into each tube.

6. Add **10 µl of RNA-sample** to each tube with the reaction mixture. Carefully vortex. To remove drops from tubes walls, centrifuge briefly.

Avoid sorbent transferring when adding RNA-samples extracted with RIBO-sorb reagent kit

B. Reverse transcription

1. Place the tubes in a thermostat (or a thermal cycler) and incubate at 37 °C for 30 min.

If reverse transcription is carried out with the use of a real-time thermocycler, assign the required program (see table 2 and Guidelines [2])

[2] The volumes of reagents are calculated for an amount of clinical samples plus 1 control of RNA extraction plus 1 extra reaction.

Table 2

Program for reverse transcription to be carried out in a real-time thermocycler[3]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycle repeats*
1	37	30 min	–	1

2. Add **20 µl of DNA-buffer** to each tube by the end of the reverse transcription. Use a new tip for each sample. Carefully vortex the tubes. Make sure there are no drops on the walls of the tubes. Obtained cDNA samples can be used for PCR.

Storage of cDNA samples:

– at ≤–16 °C for 1 week;

– at ≤–68 °C for 1 year.

8.3 Preparing the PCR

8.3.1 Preparing tubes for PCR

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

Prepare reaction mixture just before PCR analysis. See tables 3 and 4 for the scheme of reaction mixture preparation.

Variant FRT-g1-4

1. Thaw the reagents, thoroughly vortex, and centrifuge shortly to remove drops from the walls of the tubes.
2. Take the required number of PCR tubes (including 1 control of RNA extraction and 2 controls of amplification).

Each sample should be analysed with the use of 3 reaction mixtures; therefore, prepare 3 tubes for each sample. If a rotor-type instrument is used, label the tubes as follows: **sample no._1b/3a**; **sample no._1a/2**; **sample no._IC/4**.
If analysis is carried out in a plate-type instrument, use a marked plate.

3. Take 3 tubes of 0.5 ml to prepare the reaction mixtures. Label the tubes as follows: **1b/3a**, **1a/2**, and **IC/4**.
4. Per each of three labeled tubes, add the following reagents (calculating per one reaction): **5 µl of RT-PCR-mix-2-FEP/FRT, 0.5 µl of polymerase (TaqF), 10 µl** of the required **PCR-mix-1-FRT *HCV* genotype** (see table 3). Make sure that PCR-mix-1-FRT *HCV* genotype 1b/3a is added to the tube labeled 1b/3a and so on. Vortex the tubes with the prepared reaction mixtures and centrifuge shortly to remove drops from the walls of the tubes.

[3] For example, Rotor-Gene 3000 or 6000 (Corbett Research, Australia); Rotor-Gene Q (Qiagen, Germany); iCycler iQ5 (Bio-Rad, USA); Mx3000P (Stratagene, USA).

Take into account 1 extra reaction when calculating reagents for reaction mixtures (see table 3)

Table 3

Scheme of reaction mixture preparation

Volume of reagent per one reaction, µl		**10.0**	**5.0**	**0.5**
Number of clinical samples	**Number of test samples[4]**	**PCR-mix-1-FRT *HCV* genotype__***	**RT-PCR-mix-2-FEP/FRT***	**Polymerase (TaqF)***
4	**7**	**80**	**40**	**4.0**
5	**8**	**90**	**45**	**4.5**
6	**9**	**100**	**50**	**5.0**
7	**10**	**110**	**55**	**5.5**
8	**11**	**120**	**60**	**6.0**
9	**12**	**130**	**65**	**6.5**
10	**13**	**140**	**70**	**7.0**
11	**14**	**150**	**75**	**7.5**
12	**15**	**160**	**80**	**8.0**

* Volumes for one extra reaction are included.

5. Transfer **15 µl** of the **prepared mixture** to the PCR tubes according to marking. Make sure that PCR-mix-1-FRT *HCV* genotypes 1b/3a is added to the tubes labeled as "sample no._1b/3a" and so on.
6. Add **10 µl** of **cDNA samples** obtained at the stage of reverse transcription to the tubes. Make sure that each sample (including Negative Control of extraction, C–) is added to 3 tubes, containing 1b/3a, 1a/2, and IC/4 reaction mixtures.
7. Carry out control amplification reactions:

NCA – add **10 µl** of **TE-buffer** to the tubes containing **1b/3a, 1a/2, and IC/4** reaction mixtures (Negative Control of Amplification).

$C+_{1b/3a}$ – Add **10 µl** of **Positive Control cDNA *HCV* genotypes 1b/3a ($C+_{1b/3a}$)** to the tube with the **1b/3a** reaction mixture (Positive Control of Amplification)

$C+_{1a/2}$ – Add **10 µl** of **Positive Control cDNA *HCV* genotypes 1a/2 ($C+_{1a/2}$)** to the tube with the **1a/2** reaction mixture (Positive Control of Amplification)

$C+_{IC/4}$ – Add **10 µl** of **Positive Control cDNA *HCV* IC/genotype 4 ($C+_{IC/4}$)** to the tube with the **IC/4** reaction mixture (Positive Control of Amplification).

[4] Number of clinical samples + 1 control of RNA extraction + 2 controls of PCR (N+3, N – number of clinical samples).

Variant FRT-g1-6

1. Thaw the reagents, thoroughly vortex the tubes, and centrifuge shortly to remove drops from the walls of the tubes.
2. Take the required number of PCR tubes (including 1 control of RNA extraction and 2 controls of amplification).

Each sample should be analysed with the use of 4 reaction mixtures; therefore, prepare 4 tubes for each sample. If rotor-type instrument is used, label the 0.2-ml tubes as follows:
sample no._1b/3a; sample no._1a/2; sample no._IC/4; sample no._5a/6.
If striped tubes are used for rotor-type instruments or if the analysis is carried out in a plate-type instrument, use a marked plate.

3. Collect 4 tubes of 0.5 ml to prepare the reaction mixtures. Label the tubes as follows: **1b/3a**, **1a/2**, **IC/4, and 5a/6**.
4. Per each of four labeled tubes add the following reagents (calculating per one reaction): **5 µl of RT-PCR-mix-2-FEP/FRT, 0.5 µl of polymerase (TaqF), 10 µl** of the required **PCR-mix-1-FRT *HCV* genotype** (see table 4). Make sure that PCR-mix-1-FRT *HCV* genotypes 1b/3a is added to the tubes labeled 1b/3a and so on. Vortex the tubes with prepared reaction mixtures and centrifuge shortly to remove drops from the tubes walls.

Table 4

Scheme of reaction mixture preparation

Volume of reagent per one reaction, µl		10.0	5.00	0.50
Number of clinical samples	**Number of test samples[5]**	**PCR-mix-1-FRT *HCV* genotype__***	**RT-PCR-mix-2-FEP/FRT***	**Polymerase (TaqF)***
4	**7**	**80**	**40**	**4.0**
5	**8**	**90**	**45**	**4.5**
6	**9**	**100**	**50**	**5.0**
7	**10**	**110**	**55**	**5.5**
8	**11**	**120**	**60**	**6.0**
9	**12**	**130**	**65**	**6.5**
10	**13**	**140**	**70**	**7.0**
11	**14**	**150**	**75**	**7.5**
12	**15**	**160**	**80**	**8.0**

* Volumes for one extra reaction are included.

5. Transfer **15 µl** of the **prepared mixture** to the PCR tubes according to marking. Make sure that PCR-mix-1-FRT *HCV* genotypes 1b/3a is added to the tubes labeled as "sample no._1b/3a" and so on.

   If striped tubes are used, the reaction mixtures should be transferred to the tubes in accordance with the order indicated on Fig. 1.

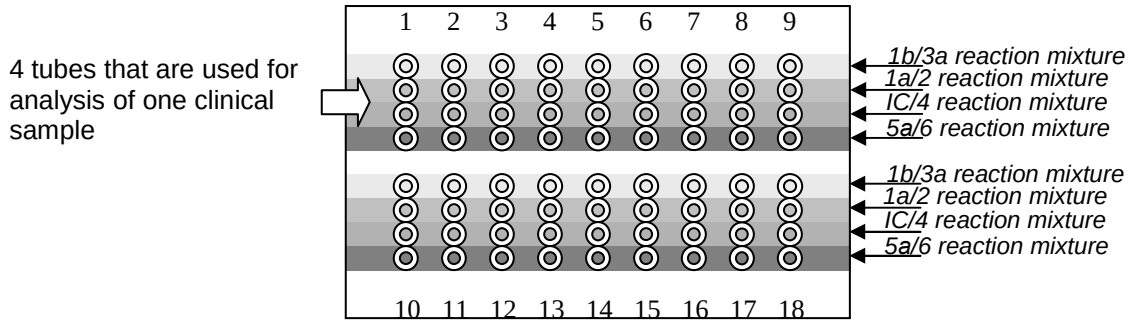

Figure 1 – Scheme of distribution of reaction mixtures and samples in case striped tubes are used.

6. Add **10 µl** of **cDNA samples** obtained at the stage of reverse transcription to the tubes. Make sure that each sample (including Negative Control of extraction, C–) is added to 4 tubes containing 1b/3a, 1a/2, IC/4, and 5a/6 reaction mixtures.

[5] Number of clinical samples + 1 control of RNA extraction + 2 controls of PCR (N+3, N – number of clinical samples).

7. Carry out control amplification reactions:

NCA – add **10 µl** of **TE-buffer** to the tubes containing **1b/3a, 1a/2, IC/4, and 5a/6** reaction mixtures (Negative Control of Amplification).

C+$_{1b/3a}$ – Add **10 µl** of **Positive Control cDNA *HCV* genotypes 1b/3a (C+$_{1b/3a}$)** to the tube with **1b/3a** reaction mixture (Positive Control of Amplification)

C+$_{1a/2}$ – Add **10 µl** of **Positive Control cDNA *HCV* genotypes 1a/2 (C+$_{1a/2}$)** to the tube with **1a/2** reaction mixture (Positive Control of Amplification)

C+$_{IC/4}$ – Add **10 µl** of **Positive Control cDNA *HCV* IC/genotype 4 (C+$_{IC/4}$)** to the tube with **IC/4** reaction mixture (Positive Control of Amplification).

C+$_{5a/6}$ – Add **10 µl** of **Positive Control cDNA *HCV* genotypes 5a/6 (C+$_{5a/6}$)** to the tube with **5a/6** reaction mixture (Positive Control of Amplification).

8.3.2. Amplification

1. Create a temperature profile on your real-time instrument as follows:

Table 5

AmpliSens-1 amplification program for rotor-type instruments[6]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycle repeats*
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow	
	72	15 s	–	

Table 6

AmpliSens-1 amplification program for plate-type instruments[7] and Smart Cycler II (Cepheid, USA)

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycle repeats*
1	95	15 min (900 s[8])	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, JOE/HEX/Cy3	
	72	15 s	–	

2. Insert the tubes into the reaction module of the device.
3. Run the amplification program with fluorescence detection.
4. Analyze results after the amplification program is completed.

[6] For example Rotor-Gene 3000/6000 (Corbett Research, Australia), Rotor-Gene Q (Qiagen, Germany).
[7] For example, iCycler iQ5 (Bio-Rad, USA); Mx3000P, Mx3005P (Stratagene, USA).
[8] For Smart Cycler II (Cepheid, USA).

9. DATA ANALYSIS

The analysis of results is carried out by the software of the real-time thermocycler used. Curves of accumulation of fluorescent signal in the FAM (or equivalent) channel and JOE (or equivalent) channel are analyzed.

Results are interpreted by the crossing (or not crossing) of the fluorescence curve with the threshold line set at a specific level. That determines presence (or absence) of *Ct* (cycle threshold) value of a sample in the appropriate cell of the result grid.

The result of amplification in the channel is considered *positive* if the fluorescence curve is S-shaped and crosses the threshold line in the area of reliable growth of fluorescence. The result of amplification in the channel is considered *negative* if the fluorescence curve does not have the typical shape and does not cross the threshold line (*Ct* or *Cp* value is undefined). The result of amplification in the channel is considered equivocal in all other cases.

Table 7 shows the channels for detection of *HCV* genotypes for each of the reaction mixtures applied in the PCR assay.

Table 7

Reaction mixture	**1b/3a**	**1a/2**	**IC/4**	**5a/6**[9]
Channel	***HCV* genotype to be detected**			
FAM/Green	1b	1a	IC	5a
JOE/HEX/Yellow/Cy3	3a	2	4	6

Interpretation of results for control samples

The results of analysis are accepted as relevant if the results obtained for both Positive and Negative Controls of amplification as well as the Negative Control of extraction are correct (see table below).

[9] Included in the PCR kit variant FRT-g1-6.

Table 8

Results for controls

Reaction mixture	1b/3a		1a/2		IC/4		5a/6[10]	
Control	Result of amplification in channel							
	FAM/Green	JOE/HEX/ Yellow/Cy3	FAM/Green	JOE/HEX/ Yellow/Cy3	FAM/Green	JOE/HEX/ Yellow/Cy3	FAM/Green	JOE/HEX/ Yellow/Cy3
C–	*Ct* is absent	*Ct* is absent	*Ct* is absent	*Ct* is absent	**< boundary *Ct* value**	*Ct* is absent	*Ct* is absent	*Ct* is absent
NCA	*Ct* is absent	*Ct* is absent	*Ct* is absent	*Ct* is absent	*Ct* is absent	*Ct* is absent	*Ct* is absent	*Ct* is absent
$C+_{1b/3a}$	**< boundary *Ct* value**	**< boundary *Ct* value**	*	*	*	*	*	*
$C+_{1a/2}$	*	*	**< boundary *Ct* value**	**< boundary *Ct* value**	*	*	*	*
$C+_{IC/4}$	*	*	*	*	**< boundary *Ct* value**	**< boundary *Ct* value**	*	*
$C+_{5a/6}$	*	*	*	*	*	*	**< boundary *Ct* value**	**< boundary *Ct* value**

* Not analyzed with the indicated reaction mixture.

Ct boundary values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

Interpretation of results for clinical samples

1. The *HCV* genotype is identified by comparison of amplification results obtained with three (variant FRT-1g-4) or four reaction tubes (variant FRT-1g-6) in accordance with table 7. Take into account the following:

   a) If the detected *Ct* value represents a single *HCV* genotype, then the result **"Genotype…"** is to be displayed;

   b) If two or more *Ct* values are detected for a sample, then dual, triple, etc. genotype is to be displayed. However, there is an exception:

   - If *Ct* value is detected in both the JOE/HEX/Yellow/Cy3 channel for the IC/4 reaction mixture (*HCV* genotype 4) and the FAM/Green channel for the 1b/3a reaction mixture (*HCV* genotype 1b) and the *Ct* value of *HCV* genotype 4 is greater than the *Ct* value of *HCV* genotype 1b by 10 cycles, then the result **"Genotype 4"** is to be displayed.

[10] Included in the PCR kit variant FRT-g1-6.

2. If only the *Ct* value of the Internal Control (IC/4 reaction mixture, FAM/Green channel) is detected in the results grid and this value is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*, then the result **"*HCV* genotype is not detected"** is to be displayed. Moreover, if it is known that the concentration of *HCV* RNA is within the limits of the analytical sensitivity of the PCR kit, then the result **"*HCV* genotype is not detected due to low viral load"** is to be displayed.
3. If the *Ct* values corresponding to all genotypes are absent while the *Ct* value for the Internal Control (IC/4 reaction mixture, FAM/Green channel) is absent or greater than the boundary *Ct* value (specified in the *Important Product Information Bulletin*), the PCR analysis should be repeated beginning with the RNA extraction stage.

10. TROUBLESHOOTING

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

1. If the *Ct* value of at least one Positive Control of Amplification ($C+_{1b/3a}$, $C+_{1a/2}$, $C+_{IC/4}$, or $C+_{5a/6}$) is greater than the boundary Ct value specified in the *Important Product Information Bulletin* or absent, the PCR analysis should be repeated for all samples beginning with the RNA extraction stage.
2. If a positive signal is detected for the Negative Control of extraction (C–) with at least one of the following reaction mixtures: 1b/3a, 1a/2, 5a/6 in any channel and/or with the IC/4 reaction mixture in the JOE/HEX/Yellow/Cy3 channel, PCR analysis should be repeated beginning with the RNA extraction stage for all samples that showed *HCV* genotype with this reaction mixture.
3. If a positive signal is detected for the Negative Control of amplification (NCA) in any of the channels with any reaction mixture, PCR analysis should be repeated for all samples that showed *HCV* genotype with this reaction mixture beginning with the RNA extraction stage.

11. TRANSPORTATION

AmpliSens® ***HCV*-genotype-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®** ***HCV*-genotype-FRT** PCR kit should be stored as specified below when not in use. All components are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise

stated.

Store at 2 – 8 °C

Negative Control,
TE-buffer,
Internal Control STI-248-rec

Store at
temperature from minus 24 to minus 16 °C

PCR kit (except for Negative Control, TE-buffer, and Internal Control STI-248-rec)

PCR-mix-1-FRT *HCV* genotypes 1b/3a,
PCR-mix-1-FRT *HCV* genotypes 1a/2,
PCR-mix-1-FRT *HCV* IC/genotype 4,
PCR-mix-1-FRT *HCV* genotypes 5a/6

are to be kept away from light

13. SPECIFICATIONS

13.1. Sensitivity

Extraction volume, µl	Nucleic acid extraction kit	PCR kit	Analytical sensitivity, IU/ml
100	RIBO-sorb	PCR kit variant FRT-g1-4 variant FRT-g1-6	$2.5x10^3$
100	RIBO-prep	PCR kit variant FRT-g1-4 variant FRT-g1-6	$5x10^3$

13.2. Specificity

The analytical specificity of **AmpliSens® *HCV*-genotype-FRT** PCR kit is assured by selection of specific primers and probes, as well as the selection of strict reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

The absence of cross-reactions between *HCV* genotypes 1a, 1b, 2, 3a, 4, 5a, and 6 was confirmed with the use of highly concentrated recombinant positive control samples and plasma samples as a part of assessment of the analytical specificity of the PCR kit.

The clinical specificity of **AmpliSens ® *HCV*-genotype-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 Institute of Science “Central Research Institute for Epidemiology”.
2. Guidelines to AmpliSens® *HCV*-genotype-FRT PCR kit for qualitative detection and differentiation of *hepatitis C virus* (*HCV*) genotypes in the clinical materials by means of real-time hybridization-fluorescence detection developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology”.

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®** ***HCV*-genotype-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
	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+**	Positive control of amplification
		IC	Internal control

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
14.06.12 BO	Title page	IVD symbol was changed to RUO
	Text	Tips with aerosol barriers were changed to tips with filters
09.10.12 LA	Page footer	TR-V1-S-G(1-4)-2x(RG,iQ,Mx,Dt,SC)-CE; TR-V1-S-G(1-6)-2x(RG,iQ,Mx,Dt,SC)-CE catalog numbers were deleted
		R-V1-G(1-4)-2x(RG,iQ,Mx,Dt,SC)-CE catalog number was added
	3. Content	Release form including “RIBO-sorb” variant 50, “REVERTA-L” variant 50 and PCR kit variant FRT-g1-4 was deleted
		Release form including “RIBO-sorb” variant 50, “REVERTA-L” variant 50 and PCR kit variant FRT-g1-6 was deleted
		Release form including PCR kit variant FRT-g1-6 was added
		Contents of the RIBO-sorb and REVERTA-L reagent kits were deleted
	5. General precautions	Hazard symbols, risk and safety phrases related to the components included in the RIBO-sorb reagent kit were deleted