

AmpliSens® HBV-genotype-FRT PCR kit



For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

	Catalogue number		Caution
	Batch code		Contains sufficient for <n> tests
	In vitro diagnostic medical device		Use-by Date
	Version		Consult instructions for use
	Temperature limit		Keep away from sunlight
	Manufacturer	NCA	Negative control of amplification
	Date of manufacture	C-	Negative control of extraction
	Authorized representative in the European Community	C+	Positive control of amplification

1. INTENDED USE

AmpliSens® HBV-genotype-FRT PCR kit is an *in vitro* nucleic acid amplification test for differentiation of hepatitis B virus (HBV) genotypes A, B, C and D in the clinical material (blood plasma) using real-time hybridization-fluorescence detection of amplified products. AmpliSens® HBV-genotype-FRT PCR kit is recommended to use after detection of hepatitis B virus DNA by AmpliSens® HBV-Monitor-L PCR kit.

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

2. PRINCIPLE OF PCR DETECTION

The principle of analysis is based on the DNA extraction from blood plasma and DNA amplification with real-time hybridization-fluorescence detection. The samples of DNA extracted from the clinical material, in which the positive results were obtained at the stage of qualitative and/or quantitative detection, can be used.

Detection of HBV genotypes A, B, C and D by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific primers. In the real-time PCR, the amplified product is detected with the use of 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® HBV-genotype-FRT PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by the separation of nucleotides and Taq-polymerase by using chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min. The PCR kit contains the system for prevention of contamination by amplicons using the enzyme uracil-DNA-glycosylase (UDG) and deoxyuridine triphosphate (dUTP).

Detection of HBV genotypes A, B, C and D is carried out in a single tube. The PCR kit is designed for the PCR instruments with four and more fluorescence detection channels.

The results of amplification are registered in the following fluorescence channels:

Table 1

Channel for fluorophore	FAM	JOE	ROX	Cy5
DNA-target	HBV Genotype C	HBV Genotype D	HBV Genotype B	HBV Genotype A
Target gene	HBV S-gene	HBV S-gene	HBV S-gene	HBV S-gene

3. CONTENT

AmpliSens® HBV-genotype-FRT PCR kit is produced in 1 form:

variant FRT-50 F, H-1982-1-3-CE.

Variant FRT-50 F includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-1-FRT HBV genotypes C/D/B/A	clear liquid from colorless to light lilac colour	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
Positive Control DNA HBV genotypes B/A (C+B/A)	colorless clear liquid	0.2	1 tube
Positive Control DNA HBV genotypes C/D (C+C/D)	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.07	1 tube
Negative Control (C-)	colorless clear liquid	1.2	1 tube

Variant FRT-50 F is intended for 55 amplification reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Sterile RNase-free pipette tips with aerosol filters (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia), Rotor-Gene Q (QIAGEN, Germany), CFX 96 (Bio-Rad, USA)), DTprime ("DNA-Technology", Russia).
- Disposable polypropylene PCR tubes (0.1- or 0.2-ml):
 - a) 0.2-ml PCR tubes with optical transparent domed or flat caps if a plate-type instrument is used;
 - b) 0.2-ml PCR tubes with flat caps or strips of four 0.1-ml Rotor-Gene PCR tubes if a rotor-type instrument is used.
- Pipettes (adjustable).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.
- Disposable powder-free gloves and a laboratory coat.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol filters and use a new tip for every procedure.
- Store all extracted positive material (specimens, controls and amplicons) away from all other reagents and add it to the reaction mix in a distinctly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, 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 specimens and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid samples and reagents contact with the skin, eyes, and mucous membranes. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice immediately.
- Safety Data Sheets (SDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where 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 the manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens® HBV-genotype-FRT PCR kit is intended for analysis of the DNA extracted with DNA extraction kits from the clinical material (peripheral blood plasma).

Collect a blood sample in a tube with 3 % EDTA solution in the ratio 20:1 (20 parts of blood for 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 – 1,600 g for 20 min. Remove plasma and transfer in a new disposable tube. Plasma can be stored at 2–8 °C for up to 3 days and at the temperature not more than minus 68 °C for a long time.

7. WORKING CONDITIONS

AmpliSens® HBV-genotype-FRT PCR kit should be used at the temperature from 20 to 28 °C and relative humidity from 15 to 75 %.

8. PROTOCOL

8.1. DNA extraction

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

- RIBO-prep.

In the extraction procedure it is necessary to carry out the control reactions as follows:

- C-** – Add **Negative Control (C-)** to tube labeled **C-** (Negative Control of Extraction).

Extract DNA according to the manufacturer's protocol taking into account next additions and improvements:

- NOTE:**
- The volume of Negative Control (C-) is 100 µl.
 - The volume of the test sample is 100 µl.
 - The elution volume should be **100 µl**.

8.2. Preparing PCR

8.2.1 Preparing tubes for PCR

The total reaction volume is 25 µl, the volume of the DNA sample is 10 µl. The type of tubes depends on the PCR instrument used for analysis. Use disposable filter tips for adding reagents, DNA and control samples into tubes.

NOTE: All components of the reaction mix should be mixed immediately before use.

- Before starting work, thaw the all reagents of the kit, sediment the drops by short centrifugation (1-2 s).
- Take the required number of tubes/strips for amplification of the DNA obtained from clinical and control samples (one control of extraction, three controls of amplification).
- For reaction mixture preparation, add to a new tube the following reagents (calculating per one reaction): 10 µl of PCR-mix-1-FRT *HBV* genotypes C/D/B/A, 5 µl of RT-PCR-mix-2-FEP/FRT and 0.5 µl of polymerase (TaqF) (see also Table 2).

Table 2

Scheme of reaction mixture preparation

Reagent volume per one reaction, µl		Reagent volume for specified number of reactions		
		10.0	5.0	0.5
Number of clinical samples	Number of reactions ¹	PCR-mix-1-FRT ²	RT-PCR-mix-2-FEP/FRT ²	Polymerase (TaqF) ²
4	8	90	45	4.5
5	9	100	50	5.0
6	10	110	55	5.5
7	11	120	60	6.0
8	12	130	65	6.5
9	13	140	70	7.0
10	14	150	75	7.5
11	15	160	80	8.0
12	16	170	85	8.5

- Mix the prepared reaction mixture thoroughly by vortexing and sediment the drops by short centrifugation.
- Add into each tube for amplification 15 µl of prepared mixture.
- Add 10 µl of DNA samples obtained at the DNA extraction stage to prepared tubes.
- Carry out the control reactions:
 - C-** – Add 10 µl of the sample extracted from the Negative Control (C-) reagent to the tube labeled C- (Negative control of Extraction).
 - NCA** – Add 10 µl of TE-buffer to the tube labeled NCA (Negative Control of Amplification)
 - C+B/A** – Add 10 µl of Positive Control DNA *HBV* genotypes B/A (C+B/A) to the tube labeled C+B/A (Positive Control of Amplification).
 - C+C/D** – Add 10 µl of Positive Control DNA *HBV* genotypes C/D (C+C/D) to the tube labeled C+C/D (Positive Control of Amplification).

8.2.2. Amplification

- Create a temperature profile on your instrument as follows:

Table 3

AmpliSens 1 amplification program

Step	Rotor-type instruments ³			Plate-type instruments ⁴		
	Temperature, °C	Time	Cycles	Temperature, °C	Time	Cycles
1	95	15 min	1	95	15 min	1
2	95	5 s	5	95	5 s	5
	60	20 s		60	20 s	
	72	15 s		72	15 s	
3	95	5 s	40	95	5 s	40
	60	20 s		60	30 s	
		Fluorescence acquiring			72	
72	15 s	72	15 s			

Fluorescent signal is detected in the channels for the FAM, JOE, ROX and Cy5 fluorophores.

- Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin* and Guidelines [2].
- Insert tubes into the reaction module of the device.
- Run the amplification program with fluorescence detection.
- Analyze results after the amplification program is completed.

9. DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in four channels. The channels for detection of *HBV* genotypes are specified in the Table 4.

Table 4

Channel for the fluorophore	<i>HBV</i> genotype
FAM	C
JOE	D
ROX	B
Cy5	A

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a Ct value of the DNA sample in the corresponding column of the results grid.

The result of amplification in the channel is considered *positive* if the fluorescence curve crosses the threshold line in the area of reliable growth of fluorescence. The result is considered *negative* if the fluorescence curve does not cross the threshold line (Ct or Cp value is absent). The result is considered *equivocal* in all other cases.

Interpretation of results for control samples

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see Table 5).

¹ Number of clinical samples + 1 control of extraction + 3 controls of amplification (N+4, N is number of clinical samples).

² The reagent volumes are specified with a reserve for 1 extra reaction.

³ For example, Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia), Rotor-Gene Q (QIAGEN, Germany).

⁴ For example, CFX96 (Bio-Rad, USA), DTprime ("DNA-Technology", Russia).

Table 5

Control	Stage for control	Results for controls			
		Ct value in the channel for fluorophore			
		FAM	JOE	ROX	Cy5
C-	DNA extraction	Absent	Absent	Absent	Absent
NCA	PCR	Absent	Absent	Absent	Absent
C+B/A	PCR	Absent	Absent	< boundary value	< boundary value
C+C/D	PCR	< boundary value	< boundary value	Absent	Absent

NOTE: Boundary Ct values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit. See also Guidelines [2].

Interpretation of results for clinical samples

- If the Ct value is detected for the sample only in the channel for the FAM fluorophore, then the result is "*HBV* genotype C".
- If the Ct value is detected for the sample only in the channel for the JOE fluorophore, then the result is "*HBV* genotype D".
- If the Ct value is detected for the sample only in the channel for the ROX fluorophore, then the result is "*HBV* genotype B".
- If the Ct value is detected for the sample only in the channel for the Cy5 fluorophore, then the result is "*HBV* genotype A".
- If two or more Ct values are detected for the sample, then the result with double, triple and etc genotype is given.
- If the Ct value is not detected for the sample, then the result is "*HBV* genotype is not detected". If it is known that the *HBV* DNA concentration in this sample is in the range of reagent kit analytical sensitivity, then the result is "*HBV* genotype is not detected due to the low viral load".

10. TROUBLESHOOTING

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

- If the Ct value even for one of the Positive Control of Amplification (C+B/A or C+C/D) exceeds the boundary Ct value specified in the *Important Product Information Bulletin* or is absent, the PCR analysis should be repeated for all negative samples (beginning with the amplification stage).
 - If the positive signal is detected for the Negative Control of Extraction (C-) and/or Negative Control of Amplification (NCA) in any channel, the PCR analysis should be repeated for all positive samples (beginning with the amplification stage).
- If you have any further questions or if you encounter problems, please contact our Authorized representative in the European Community.

11. TRANSPORTATION

AmpliSens® *HBV*-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® *HBV*-genotype-FRT PCR kit are to be stored at the temperature from minus 24 to minus 16 °C when not in use. All components of the AmpliSens® *HBV*-genotype-FRT PCR kit are stable until the expiry date stated on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: PCR-mix-1-FRT *HBV* genotypes C/D/B/A is to be kept away from light.

13. SPECIFICATIONS

13.1. Analytical sensitivity

Clinical material	Extraction volume, µl	Nucleic acid extraction kit	Analytical sensitivity, IU/ml
Blood plasma	100	RIBO-prep	500

13.2. Analytical specificity

The analytical specificity of AmpliSens® *HBV*-genotype-FRT PCR kit is ensured by the selection of specific primers and probes as well as stringent reaction conditions. The primers and probes have been checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

The analytical sensitivity assessment has shown the absence of cross-reactions between *HBV* genotypes A, B, C, D, E, F, G and H with the use of highly concentrated recombinant positive control samples and blood plasma samples of respective *HBV* genotypes.

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

13.3. Diagnostic characteristics

103 positive and 106 negative samples of biological material (blood plasma) were used for diagnostic sensitivity and specificity determination. Positive samples of biological material were obtained from Pavol Jozef Šafárik University, Košice, Slovakia. Negative samples of biological material were obtained from SynLab, Czech Republic.

Negative samples were previously tested by HBV NAT method (HBV Real-TM Quant Dx kit, Sacace biotechnologies Srl). All samples were evaluated as negative for *HBV*.

Positive samples were previously confirmed by HBV NAT method (HBV Real-TM Quant Dx kit, Sacace biotechnologies Srl). All samples were evaluated as positive for *HBV*.

INNO-LiPA *HBV* Genotyping (Fujirebio, Japan) was used as reference assay for positive samples. *HBV* Genotype A, B, C, D Real-TM (Sacace Biotechnologies Srl, Italy) was used as reference assay for negative samples.

Table 6

Results of testing of AmpliSens® *HBV*-genotype-FRT PCR kit in comparison with the reference assay

Samples type	The results of application of AmpliSens® <i>HBV</i> -genotype-FRT PCR kit	Results of using the reference assay	
		Positive	Negative
Blood plasma	209 samples were tested	Positive	95
		Negative	8
			0
			106

Ratio of detected genotypes

Table 7

Total 103 positive samples	Detected genotype					Total genotypes detected	Combined genotypes	Genotype not detected
	C	D	B	A	E			
Reference assay	1	54	2	45	1	103	0	0
AmpliSens® <i>HBV</i> -genotype-FRT PCR kit	3	51	3	45	N/A*	102	7	8

* not applicable.

Table 8

Diagnostic characteristics of AmpliSens® *HBV*-genotype-FRT PCR kit

Test material	Diagnostic sensitivity (with a confidence level of 95 %)	Diagnostic specificity (with a confidence level of 95 %)
Blood plasma	92.2 (85.3-96.6) %	100 (96.6-100)%

14. REFERENCES

- Handbook "Sampling, Transportation, and 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.
- Guidelines to the AmpliSens® *HBV*-genotype-FRT PCR kit for differentiation of *hepatitis B virus (HBV)* genotypes A, B, C and D in the clinical material by the polymerase chain reaction (PCR) with 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 the AmpliSens® *HBV*-genotype-FRT PCR kit has been tested against predetermined specifications to ensure consistent product quality.

AmpliSens®



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