ECOLI

eSens

CE

IVD

For Professional Use Only

eSens HBV genotype QL PCR kit

REF ES3112B

Instructions for Use

1 INTENDED USE

eSens HBV genotype QL 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.

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.

eSens HBV genotype QL 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

eSens HBV genotype QL PCR kit (ES3112B) 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

eSens HBV genotype QL PCR kit 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 Q (QIAGEN, Germany), CFX 96 Touch, CFX 96 Opus (Bio-Rad, USA), QuantStudio 5 (Thermo Fisher Scientific), or equivalent).
- Disposable polypropylene PCR tubes:
  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 distantly 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

eSens HBV genotype QL 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 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

eSens HBV genotype QL 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

Any commercial nucleic acid extraction kit, if IVD-CE validated for the indicated specimen types, could be used.

Ecoli Dx, s.r.o. recommends:

- For the manual extraction

- **RIBO-prep** (K2-9-Et-100-CE)

- For the automatic extraction

- **ePure Viral Nucleic Acid Extraction Kit** (E2003)

NOTE: Extract the DNA according to the manufacturer's protocol.

8.2 Preparing PCR

8.2.1 Preparing tubes for PCR

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.

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

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

1. Before starting work, thaw the all reagents of the kit, sediment the drops by short centrifugation (1-2 s).
2. 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).
3. 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 for specified number of reactions		
Reagent volume per one reaction, µl		**10.0**	**5.0**	**0.5**
Number of clinical samples	**Number of Reactions***	**PCR-mix-1-FRT****	**RT-PCR-mix-2-FEP/FRT****	**Polymerase (TaqF)****
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**

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

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

4. Mix the prepared reaction mixture thoroughly by vortexing and sediment the drops by short centrifugation.
5. Add into each tube for amplification **15 µl** of prepared mixture.
6. Add **10** of **DNA samples** obtained at the DNA extraction stage to prepared tubes.
7. 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

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

Table 3

eSens-1 amplification program

	Rotor-type instruments (e.g Rotor-Gene Q or equivalent)			**Plate-type instruments** (e.g CFX 96 Touch, CFX 96 Opus, QuantStudio 5 or equivalent.)		
Step	**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 Fluorescence acquiring**		**60**	**30 s Fluorescence acquiring**	
	72	**15 s**		**72**	**15 s**	

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

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

8.3 Instrument Settings

Test settings for rotor-type instruments

Channel	**Calibrate/Gain Optimisation**	**Threshold**	**Dynamic tube**	**Slope Correct**	**More Settings/ Outlier Removal**
FAM/Green	from 5 FI to 10 FI	0.03	on	on	20 %
JOE/Yellow	from 5 FI to 10 FI	0.03	on	on	10 %
ROX/Orange	from 5 FI to 10 FI	0.03	on	on	10 %
Cy5/Red	from 5 FI to 10 FI	0.03	on	on	15 %

Test settings for plate-type instruments

Note: Set the heating/cooling rate ***Ramp Rate* 2,5 °C/s** by clicking the ***Step Options*** button for each step of cycling.

Channel	Threshold value*
FAM	25 %
JOE/HEX	15 %
ROX	15 %
Cy5	25 %

***** *Threshold* is set manually at the level corresponding to the certain percent of maximum fluorescence obtained for the Positive Control sample in the last amplification cycle in the respective channel.

DTprime

Type of analysis	Method	Criterion of the PCR positive result	Normalization	Lower line/ threshold for the positive result	Upper line/ threshold for normalization	Fitting (smoothing) of data	Channel	Threshold* value
Ct (*Cp*) for all channels	Threshold (*Ct*)	70 %	off	10 %	30 %	off	FAM	25 %
							HEX	15 %
							ROX	15 %
							Cy5	25 %

***** *Threshold* is set manually at the level corresponding to the certain percent of maximum fluorescence obtained for the Positive Control sample in the last amplification cycle in the respective channel.

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	***HBV* 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 and Table 6).

Table 5

Results for controls

Control	Stage for control	*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

Interpretation of results for clinical samples

1. If the *Ct* value is detected for the sample only in the channel for the **FAM** fluorophore, then the result is **"*HBV* genotype C"**.
2. If the *Ct* value is detected for the sample only in the channel for the **JOE** fluorophore, then the result is **"*HBV* genotype D"**.
3. If the *Ct* value is detected for the sample only in the channel for the **ROX** fluorophore, then the result is **"*HBV* genotype B"**.
4. If the *Ct* value is detected for the sample only in the channel for the **Cy5** fluorophore, then the result is **"*HBV* genotype A"**.
5. If two or more *Ct* values are detected for the sample, then the result with double, triple and etc genotype is given.
6. 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"**.

Table 6

Boundary *Ct* values

Sample	Rotor-type instruments				Plate-type instruments			
	Channel for fluorophore							
	FAM	JOE	ROX	Cy5	FAM	JOE	ROX	Cy5
$C+_{B/A}$	-	-	22	22	-	-	25	26
$C+_{C/D}$	25	22	-	-	29	25	-	-

10 TROUBLESHOOTING

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

1. If the *Ct* value even for one of the Positive Control of Amplification ($C+_{B/A}$ or $C+_{C/D}$) exceeds the specified boundary *Ct* value or is absent, the PCR analysis should be repeated for all negative samples (beginning with the amplification stage).

2. 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).

11 TRANSPORTATION

eSens HBV genotype QL PCR kit should be transported at 2–8 °C for no longer than 5 days.

12 STABILITY AND STORAGE

All components of the **eSens HBV genotype QL PCR kit** are to be stored at the temperature from minus 24 to minus 16 °C when not in use. All components of the **eSens HBV genotype QL 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 **eSens HBV genotype QL 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 **eSens HBV genotype QL 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 7

Results of testing of eSens HBV genotype QL PCR kit in comparison with the reference assay

Samples type	The results of application of eSens HBV genotype QL PCR kit		Results of using the reference assay	
			Positive	Negative
Blood plasma	209 samples were tested	Positive	95	0
		Negative	8	106

Table 8

Ratio of detected genotypes

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
eSens HBV genotype QL PCR kit	3	51	3	45	N/A*	102	7	8

* not applicable.

Table 9

Diagnostic characteristics of eSens HBV genotype QL 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 QUALITY CONTROL

The production process, including batch release, is carried out in accordance with an established quality management system certified according to ISO 13485.

15 KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code		Contains sufficient for <n> tests
IVD	*In vitro* diagnostic medical device		Use-by Date
VER	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
EC REP	Authorized representative in the European Community	**C+**	Positive control of amplification

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
01_04/2022		

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