DNA-TECHNOLOGY

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RUO

For research use only

HBV Quantitative REAL-TIME PCR Kit

INSTRUCTION FOR USE

"DNA-Technology Research & Production", LLC,

142281, Russia,

Moscow Region, Protvino,

Zheleznodorozhnaya Street, 20

Phone/fax: +7(495) 640.17.71

E-mail: info@dna-technology.com

https://www.dna-technology.com

Customer service department

E-mail: hotline@dna-technology.ru

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Q1-P614-23/9ER

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1. INTENDED USE

The **HBV Quantitative REAL-TIME PCR Kit i**s an *in vitro N*ucleic Acid Test (NAT) – pathogen-detection-based product. The **HBV Quantitative REAL-TIME PCR Kit** is intended for quantitative detection of hepatitis B virus DNA in human biological material (blood plasma, blood serum) by real-time PCR.

Indications for the test: need for quantitative detection of hepatitis B virus DNA.

The **HBV Quantitative REAL-TIME PCR Kit** can be used in research practice.

The application of the kit does not depend on population and demographic aspects. There are no contradictions for use of the **HBV Quantitative REAL-TIME PCR Kit.**

Potential users: qualified personnel trained research methods and rules of work in the laboratory.

It is necessary to apply the kit only as directed in this instruction for use.

2. METHOD

Method: polymerase chain reaction (PCR) with real-time results detection; quantitative analysis.

Method principle: The implemented PCR method is based on amplification of a target DNA sequence. The process of amplification includes repeating cycles of thermal DNA denaturation, annealing of primers with complementary sequences and further elongation of polynucleotide chains by Taq-polymerase.

To increase the sensitivity and specificity of the amplification reaction, the use of a hot-start is provided. Hot-start is provided by the PCR-mix preparation consisting of two layers separated by a layer of paraffin. The polymerase chain reaction starts only when paraffin is melted. It excludes non-specific anchoring of primers to DNA target at lower temperatures. Additionally, when the reaction is finished and the tubes cool down, paraffin provides sealing of the mix and additional protection from contamination with amplification products.

The **HBV Quantitative REAL-TIME PCR Kit** includes the internal control (DNA-IC “A”), which is added into the test samples at DNA extraction stage and is intended to assess the quality of the DNA extraction and polymerase chain reaction.

DNA probes, each containing a fluorescent label and a fluorescence quencher, are introduced into the PCR-mix. When a specific product is formed, the DNA probe is destroyed and the quenching agent stops affecting the fluorescent label, which leads to an increase in the fluorescence level. The number of destroyed probes (hence the fluorescence level) increases in proportion to the number of specific amplicons formed, and the fluorescence level is measured at each amplification cycle.

The DNA probes used to detect the amplification product of the desired HBV DNA include the Rox fluorescent tag.

The DNA probes used to detect the product of the internal control include the Hex fluorescent tag.

For HBV DNA quantitative assessment, the reagent kit includes calibration samples in two concentrations: HBV-ST1 in $5.0x10^7$ IU/mL and HBV-ST2 in $5.0x10^4$ IU/mL.

Using calibration samples allows to build a calibration curve which helps to calculate the HBV DNA concentration in test samples of blood plasma/serum.

Table 1 shows the detection channels of amplification products.

Table 1. Detection channels of amplification products

Fam	Hex/Vic	Rox	Cy5	Cy5.5
-	IC*	HBV DNA	-	-

* Internal control (DNA-IC “A”)

The automatic analysis is available on “DNA-Technology” made instruments: DTlite, DTprime or DTprime II REAL-TIME Thermal Cyclers for **HBV Quantitative REAL-TIME PCR Kit** (see the catalogue at https://www.dna-technology.com to see available supply options). The current version of the software is

available for download at https://www.dna-technology.com/software.

The **HBV Quantitative REAL-TIME PCR Kit** is also approved for use with CFX96 (Bio-Rad) real-time thermal cycler and Applied Biosystems QuantStudio 5 (Life Technologies Holdings Pte. Ltd.) real-time thermal cycler.

3. CONTENT

The **HBV Quantitative REAL-TIME PCR Kit** content is represented in Table 2.

Table 2. The **HBV Quantitative REAL-TIME PCR Kit** content, package S (standard) for Q1-614-S3/9ER and Q1-614-23/9ER

Reagent	Description	Total volume	Amount
Paraffin sealed PCR-mix	Colorless or pink transparent liquid under waxy white fraction	1.44 mL (15 µL in each)	tubes, 12 strips of 8 or 96 individual tubes
Enzyme Taq/RT	Colorless transparent viscous liquid	55 µL	1 tube
RT-PCR-buffer "V"	Colorless transparent liquid	1.62 mL (810 µL in each)	2 tubes
Internal control DNA-IC "A"[1]	Colorless transparent liquid	2.0 mL (1.0 mL in each)	2 tubes
Positive control[1]	Colorless transparent liquid	130 µL	1 tube
Standard HBV-ST1 (5×10^7 IU/mL)	Colorless transparent liquid	1 tube	360 µL
Standard HBV-ST2 (5×10^4 IU/mL)	Colorless transparent liquid	1 tube	360 µL
Strip caps*	12 strips of 8		

*- for package S, strips (REF Q1-P614-S3/9ER)

All components are ready to use and do not require additional preparation for operation.

The **HBV Quantitative REAL-TIME PCR Kit** is intended for single use and designed for 96 tests (no more than 6 runs), including analysis of test samples, calibration samples, negative controls and positive controls.

4. REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

The following equipment, reagents and consumables are required for the procedure:

- UV PCR cabinet;
- Real-time detecting thermal cycler[2];
- Vortex mixer;
- Vortex rotor for 0.2 mL stripped tubes (only for package S, strips);
- Refrigerator with freezer;
- PCR tube rack for 0.2 mL tubes or strips;
- Tube rack for 1.5 mL tubes;

[1] Positive control is marked as "C+". Internal control DNA-IC "A" is marked as "DNA-IC "A"".

[2] Hereinafter – detecting thermal cycler; the required parameters for detecting thermal cyclers are listed below.

- Tube rack for 2.0 mL tubes;
- Single channel pipettes (dispensers covering 2.0-1,000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 20 µL, 200 µL, 1,000 µL);
- Pipette stand;
- RNase and DNase free 1.5 mL microcentrifuge tubes with caps;
- RNase and DNase free 2.0 mL microcentrifuge tubes with caps;
- Powder-free surgical gloves;
- Container for used pipette tips, tubes and other consumables;
- Nucleic acid extraction kit (**PREP-NA-ULTRA**; **PREP-MB-ULTRA**; **PREP-MB-LITE** are recommended).

The following detecting thermal cyclers are recommended:

- **DTprime** (**DTprime *M*** modification), "DNA-Technology R&P", LLC, Russia;
- **DTprime II** (**DTprime II *M*** modification), "DNA-Technology R&P", LLC, Russia;
- **DTlite** (**DTlite *S*** modification), "DNA-Technology R&P", LLC, Russia;
- **CFX96** (Optical Reaction Module CFX96), Bio-Rad Laboratories, Inc.; USA;
- **Applied Biosystems QuantStudio 5**, Life Technologies Holdings Pte. Ltd., Singapore.

For the use of detecting thermal cyclers other than those listed above, please consult the reagent kit manufacturer for approval.

Software:

The most recent version of the DT thermal cyclers software can be downloaded from https://www.dna-technology.com/software.

The OS supported: all versions of Windows starting from 7.

5. TRANSPORT AND STORAGE CONDITIONS

Expiry date – 12 months from the date of production.

5.1. Storage conditions

- All components of **HBV Quantitative REAL-TIME PCR Kit**, except Enzyme Taq/RT, must be stored in a refrigerator or a cooling chamber at temperatures from 2 °C to 8 °C over the storage period. The paraffin sealed PCR-mix for amplification must be stored in a refrigerator or a cooling chamber at temperatures from 2 °C to 8 °C and out of light over the storage period.
- The Enzyme Taq/RT must be stored in a freezer at temperatures from minus 22 °C to minus 18 °C over the storage period.

WARNING! The excessive temperature and light can be detrimental to product performance.

5.2. Transport conditions

Transportation of the reagent kit is carried out in thermoboxes with ice packs by all types of roofed transport at the temperature inside the container corresponding to the storage conditions of the kit components.

- It is allowed to transport the kit, except for Enzyme Taq/RT, in thermoboxes with ice packs by all types of roofed transport at the temperature inside the thermoboxes from 2 °C to 25 °C for no longer than 5 days.
- It is allowed to transport the Enzyme Taq/RT in thermoboxes with ice packs by all types of roofed transport at the temperature inside the thermoboxes up to 25 °C for no longer than 5 days.

WARNING! Reagent kits transported with violation of temperature conditions must not be used.

5.3. Shelf-life of the kit following the first opening of the primary container

- All components of the kit, except for Enzyme Taq/RT, must be stored in a refrigerator or a cooling chamber at temperatures from 2 °C to 8 °C over the storage period;
- Paraffin sealed PCR-mix must be stored in a refrigerator or a cooling chamber at temperatures from 2 °C to 8 °C and out of light over the storage period;
- Enzyme Taq/RT must be stored in a freezer at temperatures from minus 22 °C to minus 18 °C over the storage period.

WARNING! The kits stored under undue conditions must not be used.

An expired **HBV Quantitative REAL-TIME PCR Kit** must not be used.

We strongly recommend to follow the given instructions in order to obtain accurate and reliable results.

The conformity of the **HBV Quantitative REAL-TIME PCR Kit** to the prescribed technical requirements is subject to compliance of storage, transportation and handling conditions recommended by manufacturer.

6. WARNINGS AND PRECAUTIONS

Only personnel trained in the methods of molecular research are allowed to work with the kit.

Handle and dispose all biological samples, reagents and materials used to carry out the analysis as if they were able to transmit infective agents. The samples must be exclusively employed for certain type of analysis. Samples must be handled under a laminar flow hood. Tubes containing different samples must never be opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive dispensation type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA. The reagents must be handled under a laminar flow hood. The reagents required for amplification must be prepared in such a way that they can be used in a single session. Pipettes used to handle reagents must

be exclusively employed for this specific purpose. The pipettes must be of the positive dispensation type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA. Avoid direct contact with the biological samples reagents and materials used to carry out the analysis. Wear powder-free surgical gloves. Wear protective clothing (work clothes and personal protective equipment) working with microorganisms classified as particularly pathogenic. The protective clothing and personal protective equipment must comply with the work to be performed and health and safety requirements. Avoid producing spills or aerosol. Any material being exposed to biological samples must be treated for at least 30 minutes with disinfecting solution or autoclaved for 1 hour at 121 °C before disposal.

Molecular biology procedures, such as nucleic acids extraction, reverse transcription, PCR-amplification and detection require qualified staff to avoid the risk of erroneous results, especially due to the degradation of nucleic acids contained in the samples or sample contamination by amplification products.

All oligonucleotide components are produced by artificial synthesis technology according to internal quality control protocol and do not contain blood or products of blood processing.

Positive control is produced by artificial DNA synthesis technology. Positive control does not include parts of infectious agents.

All the liquid solutions are designed for single use and can not be used more than once in amplification reactions. Plastic tubes do not contain phthalates. Do not breathe gas/fumes/vapor/spray produced by the components of the kit. Do not eat/drink components of the kit. Avoid contact with eyes. Only use the reagents provided in the kit and those recommended by manufacturer. Do not mix reagents from different batches. Do not use reagents from third party manufacturers' kits. All laboratory equipment, including pipettes, test tube racks, laboratory glassware, lab coats, bouffant caps, etc., as well as reagents should be strictly stationary. It is not allowed to move them from one room to another. Equip separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions. Wear lab coats, gloves and tools, which are exclusively employed for the extraction/preparation of the amplification reaction and for the amplification/detection of the amplification products. Never transfer lab coats, gloves and tools from the area designed for amplification/detection of the amplification products to the area designed for extraction/preparation of amplification reactions. Amplification products must be handled in such a way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be exclusively employed for this specific purpose. Remove PCR waste only in a closed form. Remove waste materials (tubes, tips) only in a special closed container containing a disinfectant solution. Work surfaces, as well as rooms where NA extraction and PCR are performed, must be irradiated with bactericidal irradiators for 30 minutes before and after the work.

Do not open the tubes after amplification. Waste materials are disposed of in accordance with local and national standards. All surfaces in the laboratory (work tables, test tube racks, equipment, etc.) must be treated daily with disinfecting solution.

Emergency actions

Inhalation: Inhalation of the PCR-mix contained within this kit is unlikely, however care should be taken.

Eye Contact: If any component of this kit enters the eyes, wash eyes gently under potable running water for 15 minutes or longer, making sure that the eyelids are held open. If pain or irritation occurs, obtain medical attention.

Skin Contact: If any component of this kit contacts the skin and causes discomfort, remove any contaminated clothing. Wash affected area with plenty of soap and water. If pain or irritation occurs, obtain medical attention.

Ingestion: If any component of this kit is ingested, wash mouth out with water. If irritation or discomfort occurs, obtain medical attention.

Do not use the kit:

- When the transportation and storage conditions are breached;
- When the reagents' appearance does not respond to the kit passport;
- When the kit components packaging is breached;
- After the expiry date provided.

Significant health effects are **NOT** anticipated from routine use of this kit when adhering to the instructions listed in the current manual.

7. SAMPLES

Blood plasma/serum obtained from human peripheral whole blood is used for the analysis.

General requirements

The quality of sampling, sample storage, transport and pretreatment are of great importance for obtaining correct results.

Incorrect sampling may lead to unreliable results and, therefore, to the necessity for repeated sampling.

Interfering substances

The presence of PCR inhibitors in a sample may cause doubtful (unreliable) results. The sign of PCR inhibition is the simultaneous absence of internal control and specific product of amplification.

The maximum concentration of interfering substances, which do not affect the amplification of the laboratory control and internal control DNA-IC "A": triglycerides — up to 40 mmol/L of plasma sample, hemoglobin — up to 2.0 g/L, bilirubin — up to 340 µmol/L, total protein — 60 g/L.

Concentrations of exogenous substances in biomaterial samples (blood plasma/serum) that do not affect the PCR are as follows: acyclovir, atazanavir, ribavirin, rifampicin, isoniazid, azithromycin – up to three times the maximum therapeutic concentration.

Sample collection

Whole peripheral blood must be collected in accordance with **PREP-NA-ULTRA**, **PREP-MB-ULTRA** and **PREP-MB-LITE** extraction kits' instructions for use.

Transportation and storage of the samples

Whole peripheral blood must be collected in accordance with **PREP-NA-ULTRA**, **PREP-MB-ULTRA** and **PREP-MB-LITE** extraction kits' instructions for use.

Sample preparation (obtaining blood plasma/serum)

Whole peripheral blood must be collected in accordance with **PREP-NA-ULTRA**, **PREP-MB-ULTRA** and **PREP-MB-LITE** extraction kits' instructions for use.

8. PROCEDURE

WARNING! The risk of cross-contamination between samples at all stages of the test, especially during aliquoting and DNA extraction, is a serious concern when performing research in a laboratory. Cross-contamination with high-copy biomaterial can lead to sporadic false-positive results.

To prevent cross-contamination of the biological material in the laboratory, the following rules are recommended:

1. conduct a visual assessment of the incoming biomaterial and dispose of the test tubes with broken integrity;
2. use negative control in each protocol, starting from DNA extraction stage;
3. use tips with aerosol filters at all stages of the analysis;
4. observe the analysis methods; open Eppendorf-type tubes without touching the inner part of the tube cap with gloved hand;
5. do not touch the edge of the tube with the tip when adding reagents.

8.1 DNA extraction

To extract HBV DNA from blood plasma/serum, reagent kits for viral NA extraction from blood are used (**PREP-NA-ULTRA, PREP-MB-ULTRA and PREP-MB-LITE** manufactured by "DNA-Technology R&P", LLC).

NA extraction is carried out according to the instruction to the extraction kit being used.

WARNING! The obtained NA preparation should be used within two hours for the polymerase chain reaction.

8.2 The use of control samples at the stage of nucleic acid extraction

8.2.1 Internal control DNA-IC "A"

To exclude false negative results and to control the quality of the test, an **internal control DNA-IC "A"** must be added into the test samples during NA extraction.

8.2.2 Negative control (from PREP-NA-ULTRA, PREP-MB-ULTRA and PREP-MB-LITE extraction kits)

To exclude false positive results and to control the quality of the detection, **a negative control must be used**.

At the stage of nucleic acid extraction, a negative control (negative control from the **PREP-NA-ULTRA**, **PREP-MB-ULTRA** extraction kits or dilution solution for controls from the **PREP-MB-LITE** extraction kit) must be prepared and run through all stages of extraction simultaneously with the DNA extraction from test samples in accordance with the instructions for use of the corresponding reagent kit. Each group of extracted samples must include one negative control ("C-").

8.3 Calibration samples HBV-ST1 and HBV-ST2 (included in the reagent kit)

Calibration samples HBV-ST1 and HBV-ST2 must be run through NA extraction stage.

8.4 HBV DNA extraction using PREP-NA-ULTRA extraction kit

Introduce 10 µL of internal control DNA-IC "A" per sample.

Before extraction, dilute calibration samples with negative control from the **PREP-NA-ULTRA** extraction kit.

- For HBV DNA extraction from 1,000 µL of test sample introduce into each HBV-ST1 and HBV-ST2 tube 20 µL of the corresponding calibration sample and 980 µL of negative control.
- For HBV DNA extraction from 500 µL of test sample introduce into each HBV-ST1 and HBV-ST2 tube 20 µL of the corresponding calibration sample and 480 µL of negative control.
- For HBV DNA extraction from 250 µL of test sample introduce into each HBV-ST1 and HBV-ST2 tube 20 µL of the corresponding calibration sample and 230 µL of negative control.

In case of a mixed run (simultaneous analysis of samples extracted from 1,000 µL, 500 µL and 250 µL) extract calibration samples and negative control from 1,000 µL.

Each group of test samples must include 1 negative control, 3 calibration samples "ST1" and 3 calibration samples "ST2" (see Table 3).

8.5 HBV DNA extraction using PREP-MB-ULTRA extraction kit

Introduce 20 µL of internal control DNA-IC "A" per sample.

Before extraction, dilute calibration samples with negative control from the **PREP-MB-ULTRA** extraction kit.

- For HBV DNA extraction from 500 µL of test sample introduce into each HBV-ST1 and HBV-ST2 tube 20 µL of the corresponding calibration sample and 480 µL of negative control.

Each group of test samples must include 1 negative control, 3 calibration samples "ST1" and 3 calibration samples "ST2" (see Table 3).

8.6 HBV DNA extraction using PREP-MB-LITE extraction kit

Introduce 20 µL of internal control DNA-IC "A" per sample.

Before extraction, dilute calibration samples with dilution solution for controls from the **PREP-MB-LITE** extraction kit.

- For HBV DNA extraction from 250 µL of test sample introduce into each HBV-ST1 and HBV-ST2 tube 20 µL of the corresponding calibration sample and 230 µL of dilution solution for controls.
- If a small volume of biomaterial is provided, extraction from 100 µL of biomaterial sample is possible. In this case, introduce into each HBV-ST1 and HBV-ST2 tube 20 µL of the corresponding calibration sample and 80 µL of dilution solution for controls.

In case of a mixed run (simultaneous analysis of samples extracted from 250 µL and 100 µL) extract calibration samples and negative control from 250 µL.

Each group of test samples must include 1 negative control, 3 calibration samples "ST1" and 3 calibration samples "ST2" (see Table 3).

Table 3. Tube marking example for DNA extraction

Samples of plasma/serum Nos. 1 – 6	**"C-"**	**HBV-ST1**	**HBV-ST2**
Tube No. 1	Tube "C-"	Tube "ST1-1"	Tube "ST2-1"
Tube No. 2		Tube "ST1-2"	Tube "ST2-2"
Tube No. 3		Tube "ST1-3"	Tube "ST2-3"
Tube No. 4			
Tube No. 5			
Tube No. 6			

8.7 Preparing PCR

WARNING!

1. The reagents and tubes should be kept away from direct sun light.
2. Strictly observe the completeness of the strips and caps for them. Do not use the caps for the strips of the other kits!

8.7.1. Mark the required number of tubes/stripped tubes with the paraffin sealed PCR-mix:

- 1 tube for each test sample of blood plasma/serum;
- 3 tubes for calibration sample HBV-ST1 "ST-1";
- 3 tubes for calibration sample HBV-ST2 "ST-2";
- 1 tube for negative control "C-";
- 1 tube for positive control "C+".

WARNING! The reagents are intended for up to 6 runs, considering variable number of test samples, 3 calibration samples HBV-ST1, 3 calibration samples HBV-ST2, 1 negative control and 1 positive control per run).

Example: to test 6 samples, mark 6 tubes (one for each sample), 3 tubes for "ST1", 3 tubes for "ST2", 1 tube for "C-" and 1 tube for "C+". The total number of tubes is 14.

8.7.2. Vortex the RT-PCR-buffer "V" and Enzyme Taq/RT thoroughly for 1-3 seconds, then spin briefly for 3-5 seconds.

WARNING! Enzyme Taq/RT must be taken out from the freezer immediately prior to use.

8.7.3. Prepare the mixture of RT-PCR-buffer “V” and Enzyme Taq/RT. Add to one tube:

- 15.0 x (N+1) µL of RT-PCR-buffer “V”;
- 0.5 x (N+1) µL of Enzyme Taq/RT,

where N is the number of test samples considering “ST1”, “ST2”, “C-”, “C+”.

Mixture can be stored at temperature from 2 °C to 8 °C for no more than one hour.

Example: to test 6 samples (14 marked tubes), prepare the mixture of RT-PCR-buffer “V” and Enzyme Taq/RT for 15 (14+1) tubes, i.e. mix 225 µL of RT-PCR-buffer “V” and 7.5 µL of Enzyme Taq/RT.

WARNING! Taking the Enzyme Taq/RT, it is necessary to dip the tip no more than 1.0 mm and observe the rules for viscous liquids dispensing. Thoroughly flush the remaining Enzyme Taq/RT from the tip by pipetting at least 5 times.

8.7.4. Vortex the tube with the mixture of RT-PCR-buffer “V” and Enzyme Taq/RT thoroughly, then spin briefly for 1-3 seconds.

8.7.5. Add 15 µL of the RT-PCR-buffer “V” and Enzyme Taq/RT mixture into each tube. Avoid paraffin layer break. Close the tubes/strips.

WARNING! After adding the mixture of RT-PCR-buffer “V” and Enzyme Taq/RT into the tubes with PCR-mix, immediately proceed to 8.7.6 – 8.7.12.

8.7.6. Vortex the tubes with test samples, “C-” and “C+” for 3-5 seconds and spin on vortex for 1-3 seconds.

WARNING!

1. Before introducing DNA preparation, calibration samples and negative control into tubes with PCR-mix, fulfill the recommendations for DNA preparation use listed in the **PREP-NA-ULTRA**, **PREP-MB-ULTRA** and **PREP-MB-LITE** reagent kits instructions.
2. To prevent contamination, only open the caps of the tubes into which the DNA sample is to be added and close them before adding the next DNA sample. If strips are used, close the strip caps after adding the sample before proceeding with the next sample. Close the tubes/strips tightly. Use filter tips.

8.7.7. Add 20 µL of DNA sample into the corresponding marked tubes. Do not add DNA into “ST1”, “ST2”, “C-”, “C+” tubes. Avoid paraffin layer break.

8.7.8. Add 20 µL of the corresponding calibration sample which passed whole DNA extraction procedure into the corresponding tubes marked “ST1” and “ST2” (3 tubes for each calibration sample). Avoid paraffin layer break.

8.7.9. Add 20 µL of negative control (C-), which passed whole DNA extraction procedures, into the corresponding tube.

8.7.10. Add 20 µL of positive control (C+) into the corresponding tube. Avoid paraffin layer break.

8.7.11. Vortex the tubes for 3–5 seconds.

8.7.12. Set the tubes/strips into real-time thermal cycler.

8.7.13. **For DT detecting thermal cyclers:**

Launch the operating software for DT instrument[3]. Add corresponding test[4], specify the number and IDs of the samples, calibration samples, positive and negative control samples. Specify concentrations for calibration samples: $5.0x10^7$ for ST1 and $5.0x10^4$ for ST2. Specify the position of the tubes/strips in the thermal unit (see 8.7.12) and run RT-PCR. See Table 4.

[3] Please, apply to Operation Manual for DTprime, DTprime II and DTlite Real-Time PCR instruments PART II.
[4] Instructions for uploading "files with test parameters" can be found on "DNA-Technology's" website https://www.dna-technology.com/assaylibrary.

8.7.14. **For CFX96 and QuantStudio 5 thermal cyclers:**

Specify concentrations for calibration samples: $5.0x10^7$ for ST1 and $5.0x10^4$ for ST2 and run RT-PCR considering the PCR-mix volume of 50 µL. See Tables 5, 6.

Table 4. The RT-PCR program for DTlite, DTprime and DTprime II

Step	Temperature, °C	Min.	Sec.	Number of cycles	Optical measurement	Type of the step
1	47	15	0	1		Cycle
2	95	5	0	1		Cycle
3	95	0	10	50		Cycle
	59	0	20		√	
5	25[1]	…	…	Holding		Holding
√ - optical measurement [1] – holding at 10 °C is allowed						

Table 5. The RT-PCR program for CFX96 (Bio-Rad)

Step	Temperature, °C	Time, min:sec	Cycle repeats
1	47	15:00	1
2	95	5:00	1
3	95	0:10	50
4	59 √	0:20	
√ - optical measurement (Plate Read), set the fluorescence measurement on the Hex and Rox channels at 59 °C			

Table 6. The RT-PCR program for Applied Biosystems QuantStudio 5*

Step	No.	Temperature, °C	Time min:sec	Number of cycles (repeats)
Holding	1	47	15:00	1
	2	95	05:00	1
PCR	1	95	0:10	50
	2	59 √	0:25	
√ - data collection for fluorophores (Vic (Hex), Rox) is on * - experiment type is Standard curve. Quick Run mode is acceptable.				

9. CONTROLS

The **HBV Quantitative REAL-TIME PCR Kit** contains positive control. Positive control is a cloned part of the virus genome. It is produced with genetic engineering techniques and characterized by automatic sequencing. The kit includes the Internal control (DNA-IC "A"). DNA-IC "A" is intended to assess the quality of the DNA extraction and polymerase chain reaction. To reveal possible contamination a negative control is required.

WARNING! A negative control sample should go through all stages of DNA extraction. Negative control from an extraction kit can be used as a negative control sample in volumes indicated in supplied instructions.

The test result is considered valid when:

- the exponential growth of the fluorescence level for the specific product is present, in this case the internal control is not considered;
- the exponential growth of the fluorescence level for the specific product is absent and for internal control is present.

The test result is considered invalid when the exponential growth of the fluorescence level for the specific product and for internal control is not observed.

If positive control (C+) does **not** express growing fluorescence of the specific product or positive result, it is required to repeat the whole test. It may be caused by inhibitors, operation error or violation of storage and handling.

If negative control (C-) expresses growing fluorescence of the specific product or positive result, all tests of the current batch are considered false. Decontamination is required.

10. DATA ANALYSIS

10.1. The Real-time PCR thermal cycler software delivered with the thermal cycler analyzes the results automatically.

10.2. When using CFX96 detection thermal cyclers, use regression type analysis (Cq Determination Mode: Regression) and exclude the first 5 cycles from the analysis (Analyze Date from Cycle 5 to 50).

10.3. When using the Applied Biosystems QuantStudio 5 detecting thermal cycler, amplification data can be obtained by different methods, e.g. base threshold (Ct) or relative threshold (Crt). Therefore, these points may have different abbreviations (Ct, Crt) but are further processed in the same way. In the relative threshold (Crt) setting, the initial cycle of Crt is “5”. The interpretation of the DNA-IC "A" amplification results (Vic) in Table 7 corresponds to the relative threshold (Crt).

10.4. When using CFX96 and Applied Biosystems QuantStudio 5 detecting thermal cyclers, analyze the selected samples and exclude the wells which signal exceeds the background signal of the instrument, but has a linear, not S-shaped character.

10.5. If there is no service software, the HBV DNA concentration in blood plasma/serum (C1) is calculated by the formula

$$C1 = C2 * k \qquad (1),$$

where C1 is the DNA concentration in the original plasma/serum sample;
C2 is the DNA concentration measured by the calibration curve given by standards ST1 and ST2;
k is the conversion coefficient calculated by the formula:

$$k = \frac{V1}{V2} \qquad (2)$$

V1 is the volume of standard ST introduced into the negative control during the DNA extraction procedure (20 µL);
V2 is the volume of plasma from which DNA was extracted (1,000 µL, 500 µL or 250 µL if DNA was extracted using **PREP-NA-ULTRA** extraction kit; 500 µL if DNA was extracted using **PREP-MB-ULTRA** extraction kit; 250 µL or 100 µL if DNA was extracted using **PREP-MB-LITE** extraction kit).
WARNING! The value of the coefficient *k* for calculating the concentration:

- For HBV DNA extraction from 1000 µL of plasma/serum **k = 0.02.**
- For HBV DNA extraction from 500 µL of plasma/serum **k = 0.04.**
- For HBV DNA extraction from 250 µL of plasma/serum **k = 0.08.**
- For HBV DNA extraction from 100 µL of plasma/serum **k = 0.2**

- For positive control concentration calculation **k = 0.02**.

Calculation example:

1) Concentration measured by the CFX96 software (primary data), is **2.88E+07** – C2 value in the formula (1).

If DNA was extracted from 1,000 µL, then k = 0.02, and concentration in the original sample is **$2.88*10^7$ x 0.02 = 576000** IU/mL or **5.76E+05** IU/mL.

If DNA was extracted from 500 µL, then k = 0.04, and concentration in the original sample is **$2.88*10^7$ x 0.04 = 1152000** IU/mL or **1.15E+06** IU/mL.

If DNA was extracted from 250 µL, then k = 0.08, and concentration in the original sample is **$2.88*10^7$ x 0.08 = 2304000** IU/mL or **2,30E+06** IU/mL.

If DNA was extracted from 100 µL, then k = 0.2, and concentration in the original sample is **$2.88*10^7$ x 0.2 = 5760000** IU/mL or **5.76E+06** IU/mL.

2) Concentration measured by the DTprime software (primary data), is **36300000** – C2 value in the formula (1).

If DNA was extracted from 1,000 µL, then k = 0.02, and concentration in the original sample is **36300000 x 0.02 = 726000** IU/mL or **7.26E+05** IU/mL.

If DNA was extracted from 500 µL, then k = 0.04, and concentration in the original sample is **36300000 x 0.04 = 1452000** IU/mL or **1.45E+06** IU/mL.

If DNA was extracted from 250 µL, then k = 0.08, and concentration in the original sample is **36300000 x 0,08** = **2904000** IU/mL or **2.90E+06** IU/mL.

If DNA was extracted from 100 µL, then k = 0.2, and concentration in the original sample is **36300000 x 0,2 = 7260000** IU/mL or **7.26E+06** IU/mL.

10.6. In case of a mixed run (simultaneous analysis of NA samples extracted from 1,000 µL, 500 µL and 250 µL of blood plasma/serum) select the volume for samples which differs from the standard extraction volume (1,000 µL for HBV DNA extraction) and calculate HBV DNA concentration using the corresponding coefficient (see p. 10.5).

10.7. The interpretation should be performed in accordance with Table 7. Run results are valid if the result interpretation conditions for controls are observed.

Table 7. PCR results interpretation

<table>
<tr><td colspan="3">Detection channel</td><td rowspan="3">Result interpretation</td><td rowspan="3"></td></tr>
<tr><td colspan="2">Rox*,
in a range,
calculated concentration
IU/mL</td><td rowspan="2">Hex/Vic
Cp/Cq/Crt</td></tr>
<tr><td>Extraction from 250-1000 µL</td><td>Extraction from 100 µL</td><td>Extraction from 250-1000 µL</td><td>Extraction from 100 µL</td></tr>
<tr><td colspan="5">Test samples</td></tr>
<tr><td>2.5x10^1 – 1.0x10^9</td><td>1.0×10^2 – 2.5×10^9</td><td>Not considered</td><td colspan="2">Positive result
with specification of viral load in the sample (IU/mL)</td></tr>
<tr><td rowspan="2">< 25</td><td rowspan="2">< 100</td><td rowspan="2">Not considered</td><td colspan="2">Positive result
with specification</td></tr>
<tr><td>“< 25 IU/mL” (no specific value!)</td><td>“< 100 IU/mL” (no specific value!)</td></tr>
<tr><td rowspan="2">> 1.0x10^9</td><td rowspan="2">> 2.5x10^9</td><td rowspan="2">Not considered</td><td colspan="2">Positive result
with specification</td></tr>
<tr><td>“> 1.0x10^9 IU/mL” (no specific value!)</td><td>“> 2.5x10^9 IU/mL” (no specific value!)</td></tr>
<tr><td colspan="2">Not specified</td><td>≤35</td><td colspan="2">Negative result,
HBV DNA is not detected</td></tr>
<tr><td colspan="2">Not specified</td><td>>35 or not specified</td><td colspan="2">Unreliable result</td></tr>
<tr><td colspan="5">Negative control</td></tr>
<tr><td colspan="2">Not specified</td><td>≤35</td><td colspan="2">Negative result (concentration is not specified)
Run results are valid</td></tr>
<tr><td colspan="5">Positive control</td></tr>
<tr><td colspan="2">2.0x10^4-2,0x10^{5**}</td><td>Not considered</td><td colspan="2">Positive result
with specification of DNA concentration in the sample (IU/mL)
Run results are valid</td></tr>
<tr><td colspan="5">*- linear measurement range depends on the sample extraction volume (see p. 11.c)
**- if in a positive control the detected concentration is beyond the 2.0×10^4 – 2.0×10^5 IU/mL range, repeat the test</td></tr>
</table>

10.8. Unreliable results may be caused by the presence of inhibitors in the nucleic acid preparation obtained from the biological material, errors in the pre-analytical stage, incorrect implementation of the analysis Protocol, non-compliance with the temperature mode of amplification, etc. In this case, either repeated PCR with the DNA preparation, or repeated DNA extraction and PCR, or repeated biomaterial collection (performed sequentially) is required.

10.9. If an unreliable result is obtained for a positive control (positive control does not correspond to the declared range), the results of the whole run batch are considered invalid. In this case the repeated run for the whole batch of samples is required.

10.10. If a positive result for a negative control, the results of the entire run are considered unreliable. In this case, special measures are required to detect and eliminate possible contamination.

11. SPECIFICATIONS

a. Analytical specificity

In samples of human biological material containing hepatitis B virus DNA, the amplification software of the detection thermal cycler records a positive amplification result for the specific product on the Rox detection channel.

The reagent kit detects the following HBV genotypes: A, B, C, D, E, F, G, H. The ability to detect A, B, C, D genotypes was confirmed during clinical trials. Detection of E, F, G, H genotypes was confirmed by *in silico* ePCR.

In samples of biological material not containing HBV DNA, the amplification software of the detection thermal cycler records a negative result of specific product amplification on Rox detection channel and a positive result of internal control (IC) amplification on Hex detection channel.

The analytical specificity of the reagent kit was evaluated on a panel of nucleic acids from the following organisms: RNA from HAV, HDV, HGV, HIV, HCV, DNA from EBV, CMV, HSV I, HSV II, influenza A virus, influenza B virus, and human DNA at up to $1.0x10^8$ copies/mL of the sample. No cross-reactions for these organisms and viruses were recorded

b. Analytical sensitivity (limit of detection)

- 4 IU/mL for DNA extraction from 1,000 µL of blood plasma/serum sample using **PREP-NA-ULTRA** extraction kit;
- 5 IU/mL for DNA extraction from 500 µL of blood plasma/serum sample using **PREP-NA-ULTRA** extraction kit;
- 15 IU/mL for DNA extraction from 250 µL of blood plasma/serum sample using **PREP-NA-ULTRA** extraction kit;
- 4 IU/mL for DNA extraction from 500 µL of blood plasma/serum sample using **PREP-MB-ULTRA** extraction kit;
- 15 IU/mL for DNA extraction from 250 µL of blood plasma/serum sample using **PREP-MB-LITE** extraction kit;
- 40 IU/mL for DNA extraction from 1,000 µL of blood plasma/serum sample using **PREP-MB-LITE** extraction kit.

LOD is determined by the analysis of serial dilutions of the laboratory control sample (LCS). For the calibration of laboratory control sample (LCS) in International Units (IU) used internationally recognized calibrator 4th WHO International Standard code 10/266.

c. Linear measurement range

- $2.5×10^1$ – $1.0×10^9$ IU/mL for extraction from 250-1,000 µL of sample;
- $1.0×10^2$ – $2,.5×10^9$ IU/mL for extraction from 100 µL of sample.

d. Coefficient of result variation (%CV) is up to 10% (for Log10 concentration of HBV DNA IU/mL).

12. TROUBLESHOOTING

Table 7. Troubleshooting

	Result	Possible cause	Solution
C+	-	Operation error PCR inhibition Violation of storage and handling requirements	Repeat the whole test Dispose of the current batch
C-	+	Contamination	Dispose of the current batch Perform decontamination procedures
IC	-	PCR inhibition DNA extraction violation	Repeat DNA extraction Repeat the whole test Resample

If you face to any undescribed issues contact our customer service department:

Phone: +7(495) 640-16-93

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com/support

13. QUALITY CONTROL

The quality control procedures performed in accordance with ISO 9001:2015 and ISO 13485:2016:

- observation of quality management in manufacturing of products;
- creation of values for customers;
- maintenance of the best service quality and customer management.

Technical support:

E-mail: hotline@dna-technology.ru,

https://www.dna-technology.com

Manufacturer:

"DNA-Technology Research & Production", LLC,

142281, Russia, Moscow Region,

Protvino, Zheleznodorozhnaya Street, 20

Phone/fax: +7(495) 640.17.71

E-mail: info@dna-technology.com

https://www.dna-technology.com

Seller: "DNA-Technology" LLC,

117587, Russia, Moscow,

int. ter. Municipal District Chertanovo Severnoye,

Varshavskoye shosse, 125 Zh, building 5, floor 1, office 12

Phone/fax: +7(495) 640.17.71

E-mail: info@dna-technology.com

https://www.dna-technology.com

14. KEY TO SYMBOLS

RUO	For research use only		Date of manufacture
	Temperature limit		Consult instructions for use
Σ	Contains sufficient for<n>tests	REF	Catalogue number
	Use-by date		Manufacturer
LOT	Batch code		Keep away from sunlight
VER	Version	NON STERILE	Non-sterile
	Caution		

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