Instructions for Use

RealLine HBV quantitative Str-Format

REAL TIME PCR DETECTION AND QUANTITATION KIT OF HEPATITIS B VIRUS DNA

**Attention!
Please read the information about quantification process carefully!**

For research use only. Not for use in diagnostic procedures.

RealLine HBV quantitative (Str-format)	**VBD0595**	**96 Tests**
valid from	**December 2019**	

RealLine HBV quantitative Str-Format

Explanation of symbols used in labeling

RUO	For research use only!
LOT	Batch code
REF	Catalogue number
	Contains sufficient for <n> tests
	Use-by-date
	Temperature limit
	Consult instructions for use
	Keep away from sunlight
	Manufacturer

BIORON Diagnostics GmbH

In den Rauhweiden 20
67354 Römerberg
Germany

Phone +49 6232 298 44 0
Fax +49 6232 298 44 29
info@bioron.de

RealLine HBV quantitative Str-Format

TABLE OF CONTENT:

RealLine HBV quantitative Str-Format

HEPATITIS B VIRUS DNA REAL TIME PCR DETECTION AND QUANTITATION KIT
Research Use Only

1. INTRODUCTION

The assay kit **RealLine HBV quantitative** is intended for the detection and quantitative determination of *Hepatitis B virus* (HBV) DNA in blood serum (plasma), using the method of real-time polymerase chain reaction (PCR) with fluorescence detection of amplified product

The kit **RealLine HBV quantitative** is designed to detect HBV DNA isolated from serum (plasma) using the NA extraction kits:

- **RealLine Extraction 100**
- **RealLine Extraction 1000**.

The assay kit is intended for use with real-time PCR detection systems like RealLine Cyclers (BIORON Diagnostics GmbH), iQ iCycler, iQ5 iCycler, CFX96 (Bio-Rad, USA), DT-96 (DNA-Technology, Russia) or their analogues.

The kit (VBD0595) contains reagents sufficient for 96 test runs, including control samples.

For the quantification process use three replicas of Positive Control sample and one Negative Control sample in each test run.

2. KIT CONTENTS

Ready Master Mix (RMM), lyophilized	96 test tubes.
Recovery Solution for Control samples (RSC)	2 vials, 4 ml each
Weak Positive Control Sample (WPC) lyophilized	1 vial;
Positive Control (PC) sample, lyophilized	2 vials;
Samples for calibration, lyophilized (CS1 and CS2) – are used when the adequacy of analytical system has to be checked, see the Attachment 2	1 vial each.

The kit includes also:

Insert with the information of the concentration of HBV DNA in CS1, CS2, PC	1; sticked in the box inside
PCR optical film	1.5 sheets
Screw caps for the vials with control and calibration samples	5

3. PRINCIPLES OF THE PROCEDURE

Real-time PCR is based on the detection of the fluorescence produced by a reporter molecule, which increases as the reaction proceeds. Reporter molecule is a dual-labeled DNA-probe that specifically binds to the target region of pathogens DNA. Fluorescence signal increases due to the separation of fluorescence dye and quencher by Taq DNA-polymerase exonuclease activity during amplification. PCR consists of repeated cycles: temperature denaturation of DNA, primer annealing and complementary chain synthesis.

Threshold cycle value (Ct) is a cycle number at which the fluorescence generated within a reaction crosses the threshold and the fluorescence signal rises significantly above the background. Increased signal is due to the use of a DNA hybridization probe that is specific for the given DNA sequence: it binds to one of the DNA strands in the course of reaction and provides additional specificity of the method. A DNA probe consists of a fluorescence dye at the 5'-end and a fluorescence quencher at the 3'-end that significantly reduces the fluorescence intensity. During the polymerase synthesis of the complementary strand, the probe is cleaved from the 5'-end due to the 5'-3' nuclease activity of Taq DNA polymerase, the quencher and the dye become separated, thus increasing the fluorescence signal due to accumulation of the reaction product. The detected fluorescence intensity depends on initial quantity of pathogens DNA template in the sample.
Reliability of the assay is controlled by the presence of the positive result in a weakly positive control sample, which is subjected to the DNA isolation procedure along with the specimens.

The use of Internal Control sample (IC) prevents generation of false negative results associated with possible loss of DNA template during specimen preparation. IC indicates if PCR inhibitors occur in the reaction mixture. IC should be added to each sample (including control samples) prior to the DNA extraction procedure. Amplification and detection of IC influence neither sensitivity nor specificity of the target DNA PCR

RealLine HBV quantitative Str-Format

4. SPECIFICATIONS

The range of detectable concentrations (linearity region): from 100 IU to 10^8 IU of HBV DNA in the volume of the specimen.

4.1. Specificity of the detection of HBV DNA is determined by standard reference panel of negative sera, SRP NCS, as a percentage of samples identified as negative by the kit and equals 100%.

4.2. Sensitivity is determined by four samples with HBV DNA concentration of 5 IU in the volume of the sample prepared from a standard reference sample SRS HBV DNA, as a percentage of samples, identified as positive by the kit and equals 100 %.

4.3. Coefficient of variation (CV in %) is calculated for the logarithm of the HBV DNA concentration in the SRP samples. The coefficient of variation is not more than 10 %.

4.4. Linearity test - characterizes the match (in %) of the measured value and a prescribed value (calculated taking into account the dilution factor) of the logarithm of the HBV DNA concentration in the sample, prepared by diluting the CS1 calibration sample. The linearity is in the range of 90 -110 %.

4.5. Diagnostic sensitivity of the HBV DNA detection: clinical tests carried out on 206 samples of positive blood serum and plasma from 181 patients with hepatitis B in the acute and chronic forms, showed sensitivity of 100 % (range 98.4 % to 100 %, with a confidence level 90 %).

4.6. Diagnostic specificity of the HBV DNA detection: clinical trials, conducted on 210 negative samples of serum and plasma from 185 nominally healthy blood donors, patients with other infectious diseases (hepatitis C, hepatitis A, HIV-infected patients) showed 100 % specificity (range of 98.4 % -100 %, with a confidence level 90 %).

5. PRODUCT USE LIMITATIONS

For Research Use Only.
Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

6. WARNING AND PRECAUTIONS

- The kits must be used by skilled personnel only.
- When handling the kit, follow the national safety requirements for working with pathogens.
- To prevent contamination, the stages of DNA isolation and PCR test run must be spatially separated.
- Avoid microbial and nuclease contamination of reagents when removing aliquots from reagent vials.
- Wear protective disposable gloves, laboratory coats and eye protection when handling specimens and kit reagents.
- Every workplace must be provided with its own set of variable-volume pipettes, necessary auxiliary materials and equipment. It is prohibited to relocate them to other workplaces.
- The use of sterile disposable pipette tips is recommended.
- Never use the same tips for different samples.
- Do not pool reagents from different lots or from different vials of the same lot.
- Dispose unused reagents and waste in accordance with country, federal, state and local regulations.
- Do not use the kit after the expiration date at the side label of the kit.

7. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED

- Real time PCR system, like described in paragraph1
- DNA-Extraction Kits: **RealLine Extraction 100** or **RealLine Extraction 1000**
- Internal Control reagent (VBC8881) and Negative Control Sample if the kit is used with the extraction kits of other supplier;
- Safety laminar box;
- Refrigerator;
- Half-automatic variable-volume single-channel pipettes;
- Disposable medical non-sterile powder-free gloves;
- Disposable pipette tips with aerosol barrier;
- Biohazard waste container;
- Razor or scalpel.

RealLine HBV quantitative Str-Format

8. PREPARATION OF SPECIMENS

*Each group of samples undergoing the procedure of DNA isolation must include a **Positive Control sample (PC)** from this kit and a **Negative Control sample (NC)** which is a component of the DNA extraction kit.*
For quantification purposes the implementation of the Internal Control IC must be done

When using a kit of another supplier for the extraction of nucleic acids as recommended in p1, add **20 µl** of **IC (VBC8881)** to each tube.

- For NC use **100 µl** of the Negative Control Sample
- For the PC use **70 µl** of Negative Control Sample and **30 µl** of Positive Control to the tube marked PC.
-

The assay is performed on extracted DNA obtained from the sample material using one of the DNA extraction kits listed in p.4, according to the Instruction Manual to the kit. Blood serum (plasma) specimens should be prepared using EDTA or sodium citrate as an anticoagulant.

Note: For **quantification** process it is **needed to perform three replicas of Positive Control sample** and one Negative Control sample in each test run.

Attention! *In case of necessity to check the adequacy of analytical system, three replicas of both Calibration sample 1 and 2 (CS1 and CS2) should be used (see the Attachment 2). This procedure is needed for the **first time using this kit with a respective real-time cycler**.*

9. PROCEDURE PROTOCOL

9.1. Preparation of Kit Components:

Prior to the test, take the kit out of the refrigerator and keep the Ready Master Mix (RMM), PC, WPC (CS1 and CS2 if necessary) closed in the package at (18 – 25) °C for at least 30 min. Then open the package and cut off the necessary number of tubes with RMM (including the specimens and control samples: 1 NC, 1 WPC, 3 PC, and, if necessary, 3 CS1 and 3 CS2) with the razor or scalpel. Cut the tubes together with the covering film.

Put the remaining tubes immediately back into the foil pouch, squeeze the air out and tightly close it with a clip.

After initial opening, store RMM at (2 – 8) °C for no more than 3 months.

9.2. Open the vials with PC, WPC (CS1, CS2). Put the removed cap and stopper into the container with disinfectant. Add 1 ml of RSC; tightly close the vial with a new plastic cap provided with the kit. Carefully mix, keep at (18 – 25) °C for 15 min, and then thoroughly mix again.

Store diluted PC, WPC, CS1 and CS2 at (2 – 8) °C for no more than 1 month or in 50 µl aliquots at minus (18 – 24) °C for no more than 3 months.

9.3. Label the tubes with RMM for each specimen and control sample.
Attention! Labels should be placed on lateral side of the tubes, leave optical film clean

9.4. Add 50 µl of the corresponding extracted DNA solution to each tube using a separate pipette tip with filter. Tightly seal the tubes with PCR optical quality film.

9.5. Place reaction tubes into the thermal block of real time PCR device.

9.6. Program real time PCR device as follows:

Stage 1:	50°C, 2 min ;	
Stage 2:	94°C, 1 min;	
Stage 3:	94°C, 10 sec	50 cycles
	60°C, 20 sec	

** measurement of fluorescence at 60 °C.*

9.7. Select the amplification detection channels

- Collect real-time PCR data through the **FAM** channel for detection of amplification of **IC DNA**.
- Collect real-time PCR data through the **ROX** channel for detection of amplification of **HBV DNA**.

9.8. Program the positions of the samples and start the run

RealLine HBV quantitative Str-Format

10. DATA ANALYSIS

10.1. In **Positive Control PC** sample and **Weak Positive Control WPC** sample *(also for CS1 and CS2)* the program should detect:

- **ROX** fluorescent signal increase and **Ct** value (HBV DNA amplification);
- **FAM** fluorescent signal increase and **Ct** value (IC DNA amplification).

10.2. In Negative Control NC sample the program should detect a **FAM** fluorescent signal increase and **Ct** value, and no significant **ROX** fluorescent increase should appear.
If **Ct** value for NC along **ROX** channel is less than 40, this indicates the presence of contamination.

10.3. The program should detect amplification signal increase for IC DNA (channel **FAM**) in each sample and define **Ct** for IC. Probe analysis is valid if **Ct** of IC for this sample is equal to or less than 40.

10.4. Calculate (IC Ct)av as an average IC Ct of all specimens (including PC, WPC and NC). IC Ct values that differ by more than 2 from $(IC\ Ct)_{av}$ should be ignored. Recalculate $(IC\ Ct)_{av}$ for the remaining values.

10.5. The specimen is considered as **positive**, i.e. containing **HBV DNA**, if the Ct through **ROX** channel **is less than or equal to 40.**

10.6. The specimen is considered as **negative**, if the Ct through **ROX** channel is **above 40** or is not determined.
If **IC Ct** value for such sample differs from $(IC\ Ct)_{av}$ value by more than 2, the result is regarded as equivocal. A repeated analysis of the sample, starting with the DNA extraction step is required.

10.7. If the Ct value for NC through the **ROX** channel is **less than or equal to 40**, it indicates the presence of contamination. In case of contamination all positive results of this individual PCR test run are considered as equivocal. Actions are required to identify and eliminate the source of contamination, and repeat the analysis of all samples of this run that were identified positive. Samples that showed negative results in this run should be considered as negative.

RealLine HBV quantitative Str-Format

Summary of results:

	FAM IC	ROX HCV	Test	Results
PC	+	+	Valid	
	-	-	Not valid	Repeat test
	+	-	Not valid	Repeat test
	-	+	Questionable	Check results, possibly repeat tests
NC	+	-	Valid	
	-	-	Not valid	Repeat test
	+	+	Not valid	Repeat test, contamination
	-	+	Not valid	Repeat test
Samples	+	Ct < 40	Valid	Positive
	+	- Or Ct >40	Valid	Negative
	-	-	Not valid	Repeat test
	-	+	Questionable	Check results, possibly repeat test

RealLine HBV quantitative Str-Format

10.8. Quantitatitative analysis of results:

Calculate CMV DNA concentration according to Annex 1.

Attention! *When DNA is extracted from 1000 µl of specimen, the resulting value corresponds to the concentration of HBV DNA in IU/ml. When DNA is extracted from a smaller volume, the resulting value should be multiplied by k, where k = 1000/specimen volume in µl. Thus, the concentration of HBV DNA is obtained expressed in IU/ml.*

10.8.1. If calculated **HBV DNA** concentration is in dynamic range from **100 to 10^8 IU/ml, the** result is determined as **positive** with indication of calculated HBV DNA concentration in the sample (in IU/ml).

10.8.2. If the calculated HBV DNA concentration is **higher than 10^8 IU/ml** should be interpreted as **positive with HBV DNA concentration "higher than 10^8 IU/specimen".**

10.8.3. If the calculated **CMV DNA** concentration is less than 100 IU/ml, the result should be interpreted as **positive with HBV DNA concentration "less than 100 IU/specimen"**

10.8.4. Specimen is considered **negative** (not containing HBV DNA) when Ct via **ROX** channel for such specimen is **above 40 or is not determined**.

Note: *Recommended setting for the Threshold is up to 20 % of the PC or the highest fluorescence signal, especially in not validated cyclers.*

For further information and help, ask us at techsupport@bioron.de. We can provide you a calculation sheet for an easy evaluation of your data.

11. STORAGE AND TRANSPORTATION

- Store assay kit at (2 - 8) °C in the manufacturer's packing.
- Transport at (2 - 8) °C. Transportation at up to 25 °C for 10 days is allowed.
- Do not freeze reagents.
- Do not pool reagents from different lots or from different vials of the same lot.
- Strictly follow the instruction manual for reliable results.
- Do not use kits with damaged inner packages and get in contact with BIORON Diagnostics GmbH.

RealLine HBV quantitative Str-Format

ANNEX I: CALCULATION OF HBVL DNA CONCENTRATION.

1. For PC with Ct value lying in the range indicated in the insert to the kits of this lot, calculate the adjusted HBV Ct value by applying a correction for extraction efficiency, controlled by IC Ct, according to the formula:

$$\mathbf{Z_{PC} = PC\ Ct + [(IC\ Ct)_{av} - IC\ Ct],}\ \text{where:}$$

- Z_{PC} is the adjusted value HBV Ct for PC sample;
- $(IC\ Ct)_{av}$ is the average IC Ct calculated over all samples (see the Instruction Manual);
- PC Ct and IC Ct are the Ct values of this PC sample through **ROX** and **FAM** channels, respectively.

2. Calculate the average value of the adjusted Ct for PC (Z_{PCav}).
Discard Z_{PC} that differs from the Z_{PCav} value by more than 2. Then calculate Z_{PCav} for the remaining samples.

3. Calculate the quantity of HBV DNA in each specimen (Q_k) according to the formula:

$$\mathbf{Q_k = 10^{Xk}\ (IU),}\ \text{where:}$$

- Xk = A+B x (ZPCav - Zk);
- A is the coefficient, determined as logarithm of HBV DNA quantity in PC, indicated in the certificate of the respective lot:

$$A = lg\ (QPC);$$

- k is the specimen's number;
- Z_{PCav} – average value of ZPC for PC samples;

- Z_k is the adjusted Ct value of the specimen calculated as follows:

$$\mathbf{Z_k = (HBV\ Ct)_k + [(IC\ Ct)_{av} - (IC\ Ct)k];}$$

B is the coefficient dependent on the amplification efficacy and calculated according the formula:

$$\mathbf{B = lg\ (2 \times Ea/100),}\ \text{where}$$

Ea is amplification efficacy in %. For quantitative estimation use B = 0.3.

4. Calculate C_k of HBV DNA (in IU/ml) as follows:

$$\mathbf{C_k = Q_k / V_k,\ where}$$

V_k is the volume of the specimen in ml.

Thus, HBV DNA concentration in the specimen is calculated and expressed in IU/ml.

RealLine HBV quantitative Str-Format

ANNEX II: Validation of the analytical system.

Determination of HBV DNA concentration in PC using the calibration curve

For a more precise determination of HBV DNA concentration, validate the analytical system. To validate compare the value of HBV DNA in PC calculated from calibration curve with the one indicated in the Certificate.

The procedure should be carried out as follows:

1. Prior to the work, take the extraction kit and **RealLine HBV quantitative** kit from the refrigerator, open the package and keep the kit components at (18 - 25) °C for at least 30 min.

2. Prepare the kit components and NC, PC, WPC, CS1, CS2 in accordance with the Instruction Manual to the kits used for the NA extraction and detection of HBV DNA.

3. Prepare 11 tubes for extraction according to the Instruction Manual for the kit and label as: 1 tube NC, 1 tube WPC, 3 tubes of PC, CS1 and CS2.

4. Add the control samples to the appropriately labeled tubes according to the Instruction Manual for the kit used for the NA extraction.

5. Run the DNA extraction procedure according to the Instruction Manual.

6. Transfer the extracted DNA to the labeled PCR tubes in accordance with the Instruction Manual to the **RealLine HBV quantitative** kit, program the cycler, set the concentration values of CS1 and CS2 specified in the kit's certificate in accordance with Instruction Manual to the cycler in use, and run PCR.

7. For each specimen, calculate the adjusted Ct using the formula:

$$\mathbf{Z = HBV\ Ct + [(IC\ Ct)_{av} - IC\ Ct].}$$

8. Calculate the average value of Z for PC, CS1, CS2 (ZPC_{av}, $ZCS1_{av}$, $ZCS2_{av}$).
   Discard the results, where Z differs from the Z_{av} value by more than 2. Then calculate Z_{av} for the remaining samples.
9. Calculate B, that is the coefficient for calculation of HBV DNA quantity, as follows:

$$\mathbf{B = (lg(Q_{CS1}) - lg(Q_{CS2})) / (Z_{CS2av} - Z_{CS1av}),\ where:}$$

- Q_{CS1} and Q_{CS2} is the quantity of HBV DNA in calibration sample 1 and 2, respectively (indicated in the Certificate to the kit);
- If the efficiency of amplification equals 100 %, use B = lg2 = 0.3.

10. Based on the results of the analysis of calibration samples, calculate the HBV DNA quantity in PC using the formula:

$$X_{PC} = lg(Q_{CS1}) + B \times (Z_{CS1av} - Z_{PCav});$$ where

- Z_{CS1av} is the average value of Z for CS1;
- Z_{PCav} is the average value of Z for PC;

If the efficiency of amplification equals 100 %, use B = lg2 = 0.3.

11. Use PC as a reference standard in following determination of HBV DNA concentration if calculated HBV DNA concentration in PC differs from the value indicated in the Certificate no more than 3 times.

12. If all these conditions are met, calculate the quantity of HBV DNA (in IU) in the specimens using coefficient B as follows:

$$Q_k = 10^{Xk}\ [IU],$$ where:

- $X_k = lg\ (Q_{PC}) + B \times (Z_{PCav} - Z_k)$.

Coefficient B is calculated from the calibration curve.

Calculate HBV DNA concentration by the following formula:

$$C_k = Q_k / V_k,$$ **where**

- V_k is the specimen volume in ml.

Thus, HBV DNA concentration in the specimen is calculated and expressed in IU/ml.

ANNEX III: Settings for RealLine Cycler and DT96:

For these cyclers the measurement exposure must be adjusted. Choose the **Operation with the device** mode in the **Settings** menu, select the item **Measurement exposition:**

- **FAM** to **250**
- **ROX** to **1000**

Confirm that the current exposure value is saved by pressing **YES**

Attention! The specified exposure values are applicable only for RealLine kits and, if necessary, must be changed for other purposes.

RealLine HBV quantitative Str-Format

BIORON Diagnostics GmbH
info@bioron.de - www.bioron.de