Instructions for Use

RealLine HBV quantitative - Uni-Format

QUANTITATIVE ASSAY KIT FOR THE EXTRACTION AND REAL TIME PCR DETECTION OF THE HEPATITIS B VIRUS DNA

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

Research Use Only (RUO)

RealLine HBV quantitative (Uni-format) incl. Extraction	**VBD0599**	**48 Tests**
valid from:	**November 2019**	

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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

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Germany

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

1. INTENDED USE

The assay kit **RealLine HBV quantitative** is intended for detection of hepatitis B virus (HBV) DNA in plasma and serum samples. The method is based on the amplification of viral DNA by Polymerase Chain Reaction (PCR) with fluorescent detection of amplified DNA in the real-time mode.

The assay kit is designed for real-time PCR detection systems like iQ iCycler, iQ5 iCycler, CFX96 (Bio-Rad, USA), DT-96 (DNA-Technology, Russia); Rotor-Gene 3000, 6000 and Rotor-Gene Q (Qiagen, Germany), RealLine Cycler (BIORON Diagnostics GmbH) or their analogues.

The assay kit contains reagents sufficient for 4 x 12-test runs, which may be performed separately or simultaneously. It is strongly recommended to use one Positive Control sample and one Negative Control sample in each test run.

The kit can be used with either of two specimen preparation procedures, the Standard procedure or the UltraSensitive procedure. In the Standard specimen preparation procedure, HBV DNA is isolated from 100 µl of serum *(plasma)*. In the UltraSensitive specimen preparation procedure, HBV viral particles in serum *(plasma)* are concentrated by Concentrating Solution of 1 ml of serum *(plasma)*.

2. KIT CONTENTS

Specimen Preparation Reagents:	
Concentrating Solution	4 vials, 14 ml each
Lysis Reagent № 1	4 vials, 4 ml each
Lysis Reagent № 2	4 vials, 7 ml each
Sorbent (suspension of magnetic particles)	1 vial, 1 ml
Solution for DNA/RNA Precipitation	4 vials, 12 ml each
Wash Solution № 1	4 vials, 8 ml each
Wash Solution № 2	4 vials, 5 ml each
Specimen Diluent	4 vials, 3 ml each
Control samples:	
Recovery Solution for Control samples (RSC)	2 vials, 4 ml each
Positive Control (PC), lyophilized	2 vials
Negative Control (NC) (Negative serum from human)	2 vials, 12 ml each
Internal Control (IC), lyophilized	2 vials
CS1 and CS2: Samples for calibration, lyophilized - are used when the adequacy of analytical system has to be checked, see the Attachment 2	1 vial of each
Amplification reagents	
Ready Master Mix for PCR, lyophilized (RMM)	48 test tubes
Additonal	
The kit package also contains plastic caps for vials with control and calibration samples	6
Passport with the specific amounts for the controls in the upper lid of the box	

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3. PRINCIPLE OF THE METHOD

The kit package contains all the required components to perform the whole range of procedures to concentrate and isolate hepatitis B virus DNA from blood serum (*plasma*), perform PCR with hybridization fluorescent detection in real time, and calculate the amount of hepatitis B virus DNA taking into account the calibration and control samples.

The method is based on recording the process of selected specific DNA fragment amplification which consists in repeated cycles, such as temperature denaturation, annealing of primers with complementary sequences, extension of polynucleotide sequences from these primers with Taq polymerase.

The employed detecting method is based upon measuring fluorescence signals in each PCR cycle. The enhancement of fluorescence signals occurs due to the use of a hybridization DNA probe that is specific for this DNA, which binds to one of the DNA strands during the reaction, thus ensuring additional specificity of the method. The DNA probe contains fluorescent dye at the 5' end and fluorescent quencher, which considerably decreases the fluorescence intensity, at the 3' end. During the complementary strand polymerase synthesis, due to 5'–3' nuclease activity of Taq DNA polymerase, the probe splits at the 5' end, and disintegration of dye and quencher takes place, thus leading to increasing the fluorescence signal as the reaction product is accumulated. As this takes place, the measured fluorescence intensity depends on the amount of generated specific amplicons, and the dynamics of fluorescence increase is determined by the initial amount of DNA in the sample.

Quantitative evaluation of the efficiency of DNA isolation from the samples is ensured by the isolation of hepatitis B virus DNA from blood serum (*plasma*) samples along with the preliminarily added **internal control sample (IC).**

4. SPECIFICATIONS

4.1. **Specificity** of HBV DNA detection on Standard Reference Panel of negative sera – 100%.

4.2. Minimum guaranteed detectable HBV DNA concentration - 5 IU/ml.

4.3. The linear range - 100 to 1×10^8 IU/ml.

4.4. The amplification efficiency - (100 ± 10)%.

4.5. The coefficient of variation calculated for the values of the logarithm of concentration of HBV DNA - no more than 10%.

4.6. The linearity test - characterizes the coincidence of the measured value and a prescribed value (calculated taking into account the dilution factor) of the logarithm of the concentration of HBV DNA in the sample, prepared by diluting the sample with known concentration of HBV DNA - (100 ± 10)%.

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

WARNING! Failure to comply with the following requirements can lead to distortion of PCR results.

- For in vitro use only.
- 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.
- Once the work is completed, expose all working surfaces and equipment to UV bactericidal lamps for 1 hour to disinfect and prevent contamination. Then, treat them with disinfecting agents prescribed by the sanitary rules (*such as 0.2% DP-2T solution; 1–3% Diabak solution; 0.5% Lizafin solution; 0.5–1% Mistral solution, 0.5–2% Veltolen solution*).
- Dispose unused reagents and waste in accordance with country, federal, state and local regulations.
- Do not use the kit after the expiration date.

7. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED

- Real time PCR device
- Laminar safety box;
- Microcentrifuge (min RCF 13000 rpm)
- Vortex mixer;
- Thermo Shaker
- Disposable gloves, powder-free;
- Half-automatic variable-volume single-channel pipettes with disposable tips;
- Disposable DNAse/RNase-free tips with filters
- 2.0 ml polypropylene tubes, sterile, non-siliconised
- Vacuum aspirator;
- Racks for 2.0 ml and 0.2 ml tubes;
- Magnetic Rack for nucleic acids isolation.

8. SPECIMENS TRANSPORT AND STORAGE

Attention! *Specimens anticoagulated with heparin are unsuitable for this test.*

Blood should be collected in sterile tubes *(or tubes, using EDTA or ACD as the anticoagulant)*. Separate serum *(plasma)* from whole blood within 6 hours of collection. Transfer serum *(plasma)* to a sterile polypropylene tube.

Specimens may be transported and stored:

- At (18 – 25) °C – up to 2 hours;
- At (2 - 8) °C – up to 24 hours;
- Frozen at – 20°C – up to 2 weeks.

Do not freeze - thaw samples repeatedly!

Before use, centrifuge specimens of serum *(plasma)* at 13000 rpm for 5 minutes at room temperature.

9. REAGENT PREPARATION

9.1. Prior to work, take the assay kit from the refrigerator, open the package, and keep the kit components at room temperature (18 - 25) °C for 30 minutes.

9.2. Open the vial with **PC** by removing plastic cap and rubber stopper. Put the removed caps and stoppers into the container with disinfecting solution.

Add **1 ml of RSC**, tightly close the vial with new plastic cap provided with the kit. Carefully mix, keep at room temperature for 15 minutes, and then thoroughly mix again.
After diluting store at (2–8)°C for no longer than 1 month.

9.3. Open the vials with **CS1** and **CS2** by removing plastic caps and rubber stoppers. Put the removed caps and stoppers into the container with disinfecting solution.

Add **1 ml of RSC**, tightly close the vials with new plastic caps provided with the kit. Carefully mix, keep at room temperature for 15 minutes, and then thoroughly mix again
After diluting store at (2 – 8) °C for no longer than 1 month.

9.4. Open the vial with **IC** by removing plastic cap and rubber stopper. Put the removed caps and stoppers into the container with disinfecting solution.

Add **1 ml of RSC**, tightly close the vial with new plastic cap provided with the kit. Carefully mix, keep at room temperature for 15 minutes, and then thoroughly mix again.
After diluting store at (2 – 8) °C for no longer than 1 month.

9.5. Negative Control (NC) sample is ready to use.
Attention! *Once opened, NC should be stored at (2 - 8) °C and used within 1 month of preparation.*

9.6. Prior to use, heat **Lysis Reagent № 1 and № 2** at (50 - 60) °C and mix thoroughly to dissolve the precipitated material. Vortex Sorbent to a condition of homogeneous suspension. Add **140 µl** of Sorbent suspension into a vial with Lysis Reagent № 2. Mix carefully.
Attention! *Once opened, any unused portion of Lysis Reagent № 2 should be discarded.*

For the quantitative determination, the NA extraction is conducted from 100 µl of serum (plasma) or whole blood using this kit. The Internal Control must be used with the Extraction, if another RNA Extraction kit I used, please add RealLine Internal Control (REF VBC8881) to the extraction procedure

Each set of samples must contain 3 PC and 1 NC sample.
The elution volume have to be 200 µl.

Please use for the NC samples the Negative Control Sample provided with the kit and do not use water or buffer.

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10. PROCEDURE PROTOCOL

It is recommended to use three replicas of Positive Control sample and one Negative Control sample in each test run and to proceed the controls to the extraction procedure.

10.1. UltraSensitive procedure for DNA isolation: from 1 ml of serum *(plasma)*

10.1.1 When choosing the number of test samples for DNA isolation ensure that each group of isolated samples and each individual PCR run contain 3 CS1, 3 CS2, 1 NC, and 1 PC.

Attention! *Calibration samples 1 and 2 (CS1 and CS2) undergo the procedure of DNA isolation and PCR when the first reaction with the kits of this series is performed and this instrument is used. Further work with the kits of this series can be done using only two control points — NC and PC, with the calibration plot imported from the first test.*

10.1.2 Label each 2.0 ml tube for each specimen and control sample.

10.1.3 Pipette **30 µl of IC** to each tube.

10.1.4 For NC, add to the tube, marked NC, **1 ml of Negative Control NC** sample.

10.1.5 For PC, add to the tube, marked PC, **970 µl of NC**, and **30 µl of PC sample**

10.1.6 For CS1 and CS2, pipette **970 µl of NC** and **30 µl** of the corresponding **calibration sample**

10.1.7 Add **1 ml** of each specimen to the appropriately labeled tube.

10.1.8 For each specimen or control tube, add **1 ml of Concentrating Solution**.

10.1.9 Close the tubes with caps, thoroughly mix the contents by rolling over the tubes 5 times, and keep for 10–15 minutes at room temperature. Then spin at 3,000 rpm at room temperature for 5 min*

* The spinning parameters depend on the model of the centrifuge in use:
Eppendorf MiniSpin — 3,000 rpm;
Eppendorf 5415D — 2,000 rpm;
Eppendorf 5417C — 2,000 rpm; and
Heraeus BiofugePico — 2,000 rpm.

10.1.10 Using a new tip for each sample, carefully remove the supernatant without disturbing the pellet. The pellet should be clearly visible at this step.

10.1.11 Add **200 µl of Lysis Reagent № 1** to each tube. Vortex vigorously for 10-15 seconds. Some insoluble material may remain. Leave tubes for 5 minutes at room temperature. Briefly spin to collect drops from the tube walls..

10.1.12 Add **500 µl of Lysis Reagent No. 2** with sorbent to each tube (with Lysis reagent No. 1). Vortex the contents of the tubes for 10–15 seconds. Keep in thermal shaker at 56°C for 10 min at 1,300 rpm. Briefly spin to dump collect from the tube walls.

10.1.13 Add **750 µl of NA precipitator** into each tube with the test samples.

10.1.14 Vortex the contents of the tubes for 10–15 seconds. Keep for 3–5 minutes at room temperature (18–25)°C. Spin for 5 minutes at 13,000 rpm.

10.1.15 Without shaking up the pellet place the tubes in the magnetic stand. Using pipette (*or aspirator*) with individual tip, remove supernatant from each tube without touching the pellet.

10.1.16 Add **500 µl of Wash Solution No. 1** to the pellet in each tube. Vortex the contents of the tubes for 10–15 seconds. Spin at 13,000 rpm for 5 min.

10.1.17 Without shaking up the pellet place the tubes in the magnetic stand. Using pipette (*or aspirator*) with individual tip, remove supernatant from each tube without touching the pellet.

10.1.18 Add **300 µl of Wash Solution No. 2** to the pellet in each tube. Vortex the contents of the tubes for 10–15 seconds. Spin at 13,000 rpm for 5 min.

10.1.19 Without shaking up the pellet place the tubes in the magnetic stand. Using pipette (*or aspirator*) with individual tip, remove supernatant from each tube without touching the pellet.

10.1.20 Dry the pellets in open tubes at room temperature (18 – 25) °C for 2–3 min.

10.1.21 Add **200 µl of Specimen Diluent** to the pellet in each tube. Thoroughly resuspend the pellet by vortexing. Incubate in thermal shaker at 56°C for 10 min at 1,300 rpm. Centrifuge at 13,000 rpm for 1 min. Samples are now prepared for a PCR test run.

Warning! *The isolated DNA can be stored at (2 – 8) °C for no longer than 24 hrs.*

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10.2. Standard protocol for DNA isolation: from 100 µl of serum (plasma):

10.2.1 With small volume of test serum *(plasma)*, the kit can be used to detect HBV DNA in **100 µl sample**. *Be aware that, in this case, detection of HBV DNA at concentrations lower than 50 IU/ml is not guaranteed.*

10.2.2 Pick out and sign the required number of **2 ml** tubes (*according to the number of test samples, including the required controls* — 3 CS1, 3 CS2, 1 NC, and 1 PC).

10.2.3 Pipette **30 µl of IC solution** into each tube.

10.2.4 For the negative control, pipette **100 µl of NC** into the tube (*labelled as NC*).

10.2.5 For the positive control, pipette **70 µl of NC** and **30 µl of PC** into the tube (*labelled as PC*).

10.2.6 Pipette **70 µl of NC** and **30 µl** of the corresponding **calibration sample** into the tubes labelled as **CS1** and **CS2**.

10.2.7 In other tubes, pipette **100 µl of the test blood serum or plasma samples** according to the tube labels. Use individual pipette tip for each sample.

10.2.8 Add **500 µl of Lysis Reagent No. 2 with sorbent** to each tube. Vortex the contents of the tubes for 10–15 seconds. Keep in thermal shaker at 56°C for 10 min at 1,300 rpm. Briefly spin to dump drops from the tube walls.

10.2.9 Add **600 µl of NA precipitator** into each tube with the test samples.

10.2.10 Vortex the contents of the tubes for 10–15 seconds. Keep for 3–5 minutes at room temperature (18 – 25) °C. Spin for 5 minutes at room temperature at 13,000 rpm.

10.2.11 Without shaking up the pellet place the tubes in the magnetic stand. Using pipette (*or aspirator*) with individual tip, remove supernatant from each tube without touching the pellet.

10.2.12 Add **500 µl of Wash Solution No. 1** to the pellet in each tube. Vortex the contents of the tubes for 10–15 seconds. Spin at 13,000 rpm for 5 min.

10.2.13 Without shaking up the pellet place the tubes in the magnetic stand. Using pipette (*or aspirator*) with individual tip, remove supernatant from each tube without touching the pellet.

10.2.14 Add **300 µl of Wash Solution No. 2** to the pellet in each tube. Vortex the contents of the tubes for 10–15 seconds. Spin at 13,000 rpm for 5 min.

10.2.15 Without shaking up the pellet place the tubes in the magnetic stand. Using pipette (*or aspirator*) with individual tips, remove supernatant from each tube without touching the pellet.

10.2.16 Dry the pellets in open tubes at room temperature (18 – 25) °C for 2–3 minutes.

10.2.17 Add **200 µl of Specimen Diluent** to the pellet in each tube. Thoroughly resuspend the pellet by vortexing. Incubate in thermal shaker at 56°C for 10 min at 1,300 rpm. Centrifuge at 13,000 rpm for 1 min. Samples are now prepared for a PCR test run.

Warning! *The isolated DNA can be stored at (2 – 8) °C for no longer than 24 hrs.*

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10.3. PCR

10.3.1 Place the tubes with prepared samples and controls to the magnetic Rack.

10.3.2 Prepare an appropriate number of reaction tubes with Ready Master Mix (RMM). Label each reaction tube for each specimen and control.

Attention! *For blockcycler mark tubes on the lateral part, for Rotor-Gene mark on the cap.*

10.3.3 Add **50 µl** of each processed specimen and control to the appropriately labeled reaction tube using a new RNase-free tip with aerosol barrier for each sample. **Do not grasp sorbent particles!**

10.3.4 Place reaction tubes into the thermal block of real time PCR device and program real time PCR device as follows:

For iQ/iQ5 iCycler, CFX96, DT-96, RealLine Cycler:

Step 1:	50°C	2 min	
Step 2:	94°C	2 min	
Step 3:	94°C	10 sec	50 Cycles
	60°C*	20 sec	
* measurement of fluorescent at 60 °C in FAM and ROX			

For Rotor-Gene 3000/6000/Q:

Step 1:	50°C	2 min	
Step 2:	94°C	2 min	
Step 3:	94°C	10 sec	50 Cycles
	60°C*	40 sec	
* measurement of fluorescent at 60 °C in FAM and ROX			

10.3.5 Select the amplification detection channels:

- collect data through the **FAM** channel (iQ5 iCycler, CFX96, DT-96, Rotor-Gene 3000) or the ***Green*** channel (Rotor-Gene 6000, Rotor-Gene Q) for the detection of amplification signal of **IC DNA**;
- collect data through the **ROX** channel (iQ5 iCycler, CFX96, DT-96, Rotor-Gene 3000) or the ***Orange*** channel (Rotor-Gene 6000, Rotor-Gene Q) for the detection of amplification signal of **HBV DNA**

10.3.6 Program the position of tubes with samples, calibration samples, positive and negative control samples according to the instruction manual to the used device.

10.3.7 Run the program and perform PCR with fluorescent detection in real time.

11. DATA ANALYSIS AND INTERPRETATION

Perform analysis, evaluation of results, and calculation of HBV DNA concentrations in the samples according to the instruction manual to the used device.

11.1. General conditions of analysis and evaluation of results

11.1.1. In **PC**, the following should be registered:

- an increasing signal of the specific amplification product for **HBV DNA** (the **ROX *(Orange)*** channel) and determined the threshold cycle value, **Ct**, for **HBV**;

- an increasing amplification signal for **IC DNA** (the **FAM *(Green)*** channel) and determined the threshold cycle value, **Ct**, for **IC**.

11.1.2. The results of an individual PCR run are to be analysed and evaluated if, for PC, the **HBV Ct** value is within the range indicated in the insert label for the kits of this lot.

11.1.3. For **NC**, an increasing amplification signal for **IC DNA** should be registered and IC **Ct** should be determined with no significant increase in the signal for the specific amplification product of **HBV DNA**. No **ROX *(Orange)*** fluorescence increase should appear.

11.1.4. For each **test sample**, an increasing amplification signal for IC DNA (*the **FAM (Green)** channel*) should be detected and IC **Ct** should be determined. The test of the sample is considered valid if IC **Ct** of this sample in the **FAM (*Green*)** channel is less than or equal to 40.

11.2. Evaluation of results

11.2.1. Calculate (IC **Ct**)$_{av}$ as an average value for IC **Ct** for all test samples (*including controls*). The IC **Ct** values differing by more than 2 from the (IC **Ct**)$_{av}$ value should be discarded. After discarding, recalculate (**Ct** IC)$_{av}$ for the remaining values.

11.2.2. The test sample is evaluated as **negative** (*not containing HBV DNA*) if, for this sample, the **Ct** value in the **ROX (*Orange*)** channel is **more than 40 or is not determined**.

If, for such sample, the IC **Ct** value exceeds the (IC **Ct**)$_{av}$ value by more than 2, the result for this sample is not to be analysed and is evaluated as **negative**. A repeated test for this sample starting from the isolation step is required.

In case of repetitive result take another blood sample and repeat the testing once again.

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11.2.3. The test sample is evaluated as **positive**, i.e. containing HBV DNA, if, for this sample, the **Ct** value in the **ROX** (*Orange*) channel is **less than or equal to 40**.

If, for such test sample, the IC **Ct** value differs from the (IC **Ct**)$_{av}$ value by more than 2, such sample is evaluated as **positive** without indication of DNA concentration. For quantification, perform a repeated test for this sample starting from the isolation step.

11.2.4. In case of contamination **all positive** results for this individual PCR run are **considered invalid**. Take measures to detect and eliminate the contamination source and repeat the testing for all samples of this run, for which the positive result has been obtained. The samples of this run, for which the test has yielded negative results, should be considered as negative.

11.3. Quantitative analysis of results

For quantification, calculate HBV DNA concentration in the test samples according to **Annex II**.

Attention! *If DNA was isolated from 100 µl of sample, multiply the result by 10.*

11.3.1. If the calculated value of HBV DNA concentration in the sample is within the range of 100 IU/ml to 10^8 IU/ml, the result is considered as **positive**, with indication of the obtained HBV DNA concentration in the sample (*in IU/ml*).

11.3.2. If the calculated value of HBV DNA concentration in the sample is higher than 10^8 IU/ml, the result is recommended to be interpreted as **positive**, with HBV DNA concentration **"greater than 10^8 IU/ml".**

11.3.3. If the calculated value of HBV DNA concentration is less than 100 IU/ml, the result is recommended to be interpreted as **positive**, with HBV DNA concentration **"less than 100 IU/ml".**

11.3.4. The test sample is considered **negative** (*not containing HBV DNA*) if, for this sample, the **Ct** value in the **ROX (*Orange*)** channel is **above 40 or not determined**.

11.3.5. If the obtained concentration of PC is not within the range indicated in the insert label for the kit, repeat the tests for all samples from this run.

12. STORAGE AND TRANSPORTATION

- Store the assay kit at (2 - 8) °C in the manufacturer's packing.
- Transport at (2 – 8) °C. Transportation for up to 25 °C for up to 10 days is allowed.
- Do not freeze the kit!
- 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.

ANNEX I: CALCULATING THE CONCENTRATION OF SPECIFIC VIRAL NA

Calculation of HBV DNA concentration

Quantitative analysis of HCV RNA is performed using the formulas below.

1) Calculate coefficient **β**:

$$\beta = [\lg(C_{CS1}) - \lg(C_{CS2})] / [\Delta Ct_{CS2} - \Delta Ct_{CS1}],$$

where C_{CS1} and C_{CS2} are the concentrations of specific viral NA in the calibration samples (*indicated in the insert label for the kit*);

$$\Delta Ct_{CS1} = Ct_{CS1av} - IC\ Ct_{CS1av};$$

$$\Delta Ct_{CS2} = Ct_{CS2av} - IC\ Ct_{CS2av};$$

Ct_{CS1av}, Ct_{CS2av} are the average **Ct** values for **CS1** and **CS2** samples through the "**ROX**" channel; **IC Ct** $_{CS1av}$, **IC Ct** $_{CS2av}$ are the average **Ct** values for **CS1** and **CS2** samples through the "**FAM**" channel.

2) Calculate the concentration of specific viral NA in the test sample (C_k):

$$C_k = C_{CS2} \times 10^{\beta(\Delta CtCS2 - \Delta Ct\ k)},$$

where **k** is the sample number;

$$\Delta Ct_k = Ct_k - IC\ Ct_k$$

where Ct_k and **IC** Ct_k are the **Ct** values for the sample number **k** in the "**ROX**" and "**FAM**" channels, respectively.

If extraction and PCR test run for **CS1** and **CS2** are not performed, use the **β** and ΔCt_{CS2} values obtained in the first test run with the kit of this lot for calculation.

For an easier calculation we can provide you with an Excel sheet. Ask us:
techsupport@bioron.de

***Note**: For precise calculation of viral NA concentration in analyzed samples, the validation of the analytical system should be done by comparison of **Positive Control PC** concentration considering Internal Control concentrations. The PC concentration is specified in the passport of the assay kit. For this purpose three independent Positive Control samples are needed.*

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ANNEX II: Programming the device and analysis of results using Rotor-Gene cyclers: Rotor-Gene 3000, Rotor-Gene 6000 (Corbett research, Australia), Rotor-Gene Q (Qiagen, Germany)

Hereinafter, detection channels and terms corresponding to different versions of devices and software are listed in the following order: Rotor-Gene 3000 (Rotor-Gene 6000, Rotor-Gene Q).

- Click **New** button.
- Select an **Advanced** template from the tab of the New Run wizard. Click **New** button.
- Select **36-Well Rotor** type, check that No Domed 0.2 ml Tubes are used. Click **Next** button.
- In the new window, determine Reaction volume as **50 µl**. Click **Next** button.
- The temperature profile of real time PCR should be set. Click **Edit Profile** button.

Step 1:	50°C	2min	
Step 2:	95°C	2min	
Step 3:	94°C	10 sec	50 cycles
	60°C*	40 sec	

  - * Measure the fluorescence at 60°C
- Then temperature profile is set, click **OK** button.
- In the **New Run Wizard** window click **Calibrate** *(**Gain optimization**)* button. The window **Auto Gain Calibration Setup** opens. In the line **Channel Settings** choose **ROX** *(Orange)*, click **Add**. Set **Tube Position** 1, **Min Reading** 5, **Max Reading** 10, click **OK**. In the line **Channel Settings** choose **FAM** *(Green)*, click **Add**. Set **Tube Position** 1, **Min Reading** 5, **Max Reading** 10, click **OK**.
- Tick off **Perform Calibration Before 1st Acquisition**. Click **Close** button.
- Click **Next** button, start the amplification process by clicking **Start Run** button.
- Save a file in the Rotor-Gene/templates folder, named RealLine with **.ret* extension. In subsequent work RealLine template would be presented in New run wizard.
- Save reaction result file with Rotor-Gene Run File **.rex* extension.
- Record the positions of the controls and specimens according to the instruction manual of the operating device. Click **Start run** button.

Results for Internal Control DNA amplification

- Click **Analysis** button, choose **Quantitation** from the list, choose **Cycling A. FAM**, click **Show** button.
- Click **OK** button, and cancel automatic **Threshold** determination**.**
- Click **Linear scale** button.
- In the ***Quantitation analysis*** menu buttons **Dynamic tube** and **Slope Correct** should be pressed.
- Click **More Settings** ***(Outlier removal)*** button, determine **NTC threshold** value as **5%**.
- In the column **CT Calculation** *(right part of the window)* determine **Threshold** value as **0.04**.
- In the result table *(**Quant. Results** window)* **Ct** will be displayed.

Results for HBV DNA amplification

- Click **Analysis** button, choose **Quantitation** from the list, choose **Cycling A. ROX** click, **Show** button.
- Click **OK** button, and cancel automatic **Threshold** determination.
- Click **Linear scale** button.
- In the ***Quantitation analysis*** menu buttons **Dynamic tube** and **Slope Correct** should be pressed.
- Click **More Settings** *(**Outlier removal**)* button, determine **NTC threshold** value as **5%**.
- In the column **CT Calculation** *(right part of the window)* determine **Threshold** value as **0.04**.
- In the result table *(**Quant. Results** window)* **Ct** will be displayed.

ANNEX III: PROGRAMMING OF DT-96 CYCLER AND REALLINE CYCLER:

The measurement exposure must be adjusted. Choose the **Operation with the device** mode in the **Settings** menu, select the item **Measurement exposition:**
FAM to **500**;
HEX and **ROX** to **1000**;
Cy5 to **500**.
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, should be changed for other purposes.*

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