ROBOSCREEN DIAGNOSTICS

Instructions for Use

RoboGene HBV DNA Quantification Kit 3.0

Rev. 3 _ 01 / 2022

CE 0483

IVD

Order No.:

847-0207710032	32 reactions
847-0207710096	96 reactions
847-0207710192	192 reactions

CE
0483

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Contents

1 Introduction

1.1 Intended use

The RoboGene HBV DNA Quantification Kit 3.0 is intended for real-time PCR quantification of Hepatitis B Virus (HBV) DNA in human EDTA- or citrate plasma and serum samples. For specimen purification, the manual method (using the INSTANT Virus RNA/DNA Kit) as well as the automated method (using the INSTANT Virus RNA/DNA Kit - FX) is validated. For amplification and detection the RoboGene HBV DNA Quantification Kit 3.0 is validated in combination with the following real-time PCR devices: qTOWER 2 & 3; CFX96; LightCycler® 480; 7500 Fast, Rotor-Gene® 3000/6000/Q, RealLine Cycler 48 / 96 and QuantStudio 5 Real-Time-PCR System. The assay is purposed for the clinical management of patients with Hepatitis B in conjunction with clinical presentation and other laboratory markers for HBV infection.

This test is intended to evaluate viral response to antiviral treatment as measured by changes of HBV DNA levels in plasma and serum. Furthermore, in the course of antiviral therapy the probability of a sustained viral response can be assessed.

The RoboGene HBV DNA Quantification Kit 3.0 is not intended for use as a screening test for the detection of HBV DNA in blood or blood products or as a diagnostic test to confirm the presence of a HBV infection.

1.2 Pathogen information

The human hepatitis B virus (HBV), which was discovered by Blumberg in 1965, is a small-enveloped DNA virus with a genome of approximately 3200 nucleotides causing acute and chronic hepatitis. Despite the introduction of a vaccine for HBV in 1981, HBV infection still represents a major global health burden. Many studies highlight chronic HBV infection as the main risk factor for hepatocellular carcinoma development. HBV transmission occurs vertically and horizontally via exchange of body fluids. The chronicity rate is about 5 % in adult-acquired, but it reaches 90 % in neonatally acquired infections. [1] Due to the lack of proofreading activity of the viral polymerase, the genetic variability of HBV is high. At present 9 HBV genotypes, A-I, are phylogenetically classified, based on an intergroup divergence of ≥ 8% across the complete genome, with a possible 10th genotype J that currently has been detected in only one patient [2]. Genotypes A-D, F, H and I are further differentiated into several subgenotypes with at least 4% sequence diversity. The HBV genotypes, and certain subgenotypes, show distinct geographical predominancies, and vary in clinical manifestation of infection and response to antiviral therapy [2].

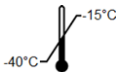
CONSULT INSTRUCTION FOR USE
This package insert must be read carefully prior to use. Given instructions must be followed accordingly. Reliability of results cannot be guaranteed if there are any deviations from the instructions in this package insert.

1.3 Technical assistance

If you have any questions or problems regarding any aspects of the RoboGene HBV DNA Quantification Kit 3.0 please do not hesitate to contact our technical support team which consists of experts with long-time experience in the field of molecular diagnostics. For technical assistance please contact us at the manufacturer site as shown inside the cover of the IFU.

1.4 Symbols and Abbreviations

For easy reference and orientation, the IFU uses the following warning and information symbols as well as the shown methodology:

Symbol	Information
	**REF** Catalogue number
	**Content** Contains sufficient reagents for <N> tests
	**Storage conditions**
	**Consult instructions for use** This information must be observed to avoid improper use of the kit and the kit components.
	**Use by**
	**Lot number** Lot number of the kit or component
	**IVD symbol** This kit is an *in vitro* diagnostic medical device
	Manufactured by
	Note / Attention Observe the notes marked in this way to ensure correct function of the device and to avoid operating errors for obtaining correct results.

The following abbreviations are used in the IFU:

Ct	Threshold cycle value
CV	Coefficient of variation
dNTP	2'-deoxynucleotide 5'-triphosphate
HBV	Hepatitis B Virus
IC	Internal Control
IFU	Instruction for use
IU	International Units
NA	Nucleic acid
NTC	Non-template control
PEI	Paul-Ehrlich-Institut, Langen, Germany
WHO	World Health Organization

2 Safety precautions

NOTE
Read through this chapter carefully prior to guarantee your own safety and a trouble-free operation.
Follow all the safety instructions explained in the IFU, as well as all messages and information, which are shown.

Human plasma and serum samples have to be considered as potentially infectious. Thus, always wear lab coat and gloves.

Always use clean and nuclease-free equipment.

Set up of template preparation, PCR reagent assembly, amplification and detection should be performed in different rooms.

Please be careful while pipetting your specimen material to avoid carry-over contaminations.

Discard sample and assay waste according to your in-house safety regulations.

Please carry out regular maintenance of your equipment to ensure that the required temperatures, centrifugation speeds and mixing intensities (rpm) are maintained correctly.

ATTENTION
Do not eat or drink components of the kit!
The kit shall only be handled by educated personnel in a laboratory environment!

3 Test description and principle

3.1 Principle of the TaqMan® assay

TaqMan® real-time PCR is a highly sensitive assay that combines amplification with fluorescence-based online detection of the nucleic acid of interest (target, template). The assay is based on a conventional set of target and internal control-specific primers in combination with fluorescence-labelled oligonucleotide probes, complementary to the desired target sequences. In the presence of targets the probes hybridize with their target-complementary sequences. The Taq DNA polymerase from the RT PCR Enzyme possesses a 5`→ 3` exonuclease activity that hydrolyses the probes and displaces the fluorescent dye from the quencher. This event results in an increase of the fluorescence signal, which is directly proportional to the target amplification during each PCR cycle.

3.2 Explanation of the HBV DNA quantification test

The RoboGene HBV DNA Quantification Kit 3.0 is an amplification test for the quantification of HBV DNA in human plasma and serum samples. The assay is able to detect all known 9 genotypes of HBV, A-I, by applying primers and probes specific for the S-Gene of the viral genome. Quantification of specimen is performed by amplification of the included quantification standard in parallel.

A synthetic internal control is included to control the whole procedure from NA extraction to the real-time PCR. Thus, the risk concerning false-negative results is drastically reduced, yielding in an increase of diagnostic correctness. Amplification of HBV DNA in samples and standards and of IC is measured independently at different wavelengths due to probes labelled with different fluorescent reporter dyes. HBV DNA detection is performed in the FAM channel. For monitoring the Internal Control the kit provides two options depending on the set up of the real-time PCR device and allows detection in Yakima Yellow/VIC/JOE or Cy5 channel.

Manual sample preparation should be conducted with the INSTANT Virus RNA/DNA Kit. NA extraction must be performed strictly according to manufacturer's instructions using 'Protocol 2: Isolation of viral RNA/DNA from 400 µl of serum/plasma using IC Spiking Tube'.

Automated sample preparation should be conducted with the INSTANT Virus RNA/DNA Kit – FX in combination with the automated pipetting device "CyBio FeliX Basic Unit with Enclosure" together with the extraction add-on module "CyBio FeliX Extraction Set".

3.3 Restrictions

This test is validated for the usage with either human EDTA-or citrate-plasma or serum. Heparinized plasma has to be excluded from analysis (see under point robustness of the test). Very high concentrations of lipids may act inhibitory on the quantification results. If other than the recommended sample types are used incorrect results may be obtained. The product is to be used only by personnel specially instructed and trained in *in vitro* diagnostics procedures. Strict compliance with the IFU is required for optimal PCR results. This kit may be used only with the mentioned real-time PCR devices and the recommended PCR consumables. Do not use expired components or mix with components from different batches.

4 Performance assessment

The RoboGene HBV DNA Quantification Kit 3.0 was validated according to the common technical specifications (CTS) for *in vitro* diagnostic medical devices (2002/364/EC) [3].

The validation of the RoboGene HBV DNA Quantification Kit 3.0 was executed with two purification procedures, manual purification using the INSTANT Virus RNA/DNA Kit and automated purification using the INSTANT Virus RNA/DNA Kit - FX. The RoboGene HBV DNA Quantification Kit 3.0 achieves comparable results with both purification procedures as shown in the Analytical Sensitivity.

4.1 Analytical Sensitivity

The analytical sensitivity of the RoboGene HBV DNA Quantification Kit 3.0 was determined by analyzing dilution series of the PEI Reference Material HBV DNA (#3620/05, genotype D). Analytical sensitivities for used qPCR devices were determined as summarized below in Table 1a for manual nucleic acid purification and Table 1b for automated extraction.

Table 1a: Determined device specific limits of detection and confidence intervals using manual nucleic acid purification.

qPCR device	**Limit of detection (LOD) (IU/ml)**	**95 % confidence interval (IU/ml)**	
qTOWER³ (qT)	10.0	6.9	13.1
CFX96 (CFX)	10.4	8.4	12.4
LightCycler® 480 (LC)	8.0	6.4	9.6
7500 Fast (FS)	7.3	5.8	8.7
Rotor-Gene® 3000 (RG)	10.4	8.4	12.4

Table 1b: Determined device specific limits of detection and confidence intervals using automated nucleic acid purification

qPCR device	Limit of detection (LOD) (IU/ml)	95 % confidence interval (IU/ml)	
qTOWER³ (qT)	6.1	4.9	7.2
CFX96 (CFX)	6.8	5.6	8.0
LightCycler® 480 (LC)	6.7	5.7	7.8
7500 Fast (FS)	8.3	7.2	9.3
Rotor-Gene® 3000 (RG)	6.6	5.4	7.9
RealLine Cycler 48	16.3	11.8	20.8
RealLine Cycler 96	11.4	9.3	13.5
QuantStudio 5	14.3	12.0	16.6

Detection limit was calculated by PROBIT analysis of at least 24 replicates of each dilution of reference material on each qPCR device with confidence of 95 % (see Figure 1).

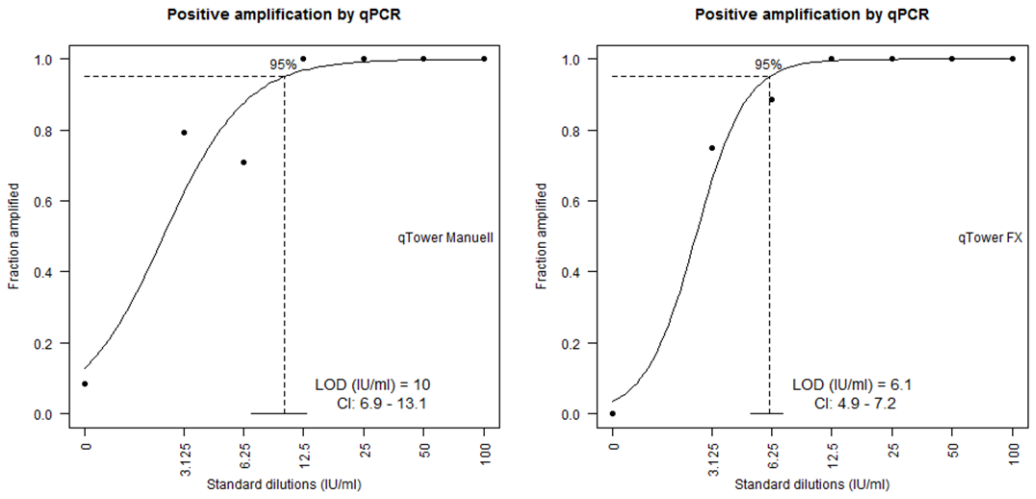


Figure 1: PROBIT analysis for the determination of PCR device specific limits of detection (LOD) with confidence of 95 % shown for an qTOWER³ device exemplarily for manual (left) and automated (FX) purification (right).

Individual values below the detection limit may be plausible but with a higher probability of error. To reduce this error probability 3 replicates of such samples are recommended.

4.2 Linear Range

The linear range for the quantification of HBV DNA was determined by analyzing dilution series of synthetic HBV DNA ranging from $2.5x10^9$ to 8 IU/ml and of native sample material from $1x10^8$ to 10 IU/ml. Manual nucleic acid purification was performed for each concentration level in triplicate, and all eluates were quantified with n=1 using CFX96, qTOWER^3, LightCycler® 480, 7500 Fast and Rotor-Gene® 3000 real-time PCR devices. The analysis was carried out according to the CLSI guideline EP06-A [4].

The RoboGene HBV DNA Quantification kit 3.0 was shown to be linear over 8 log_{10} steps across the range of HBV DNA concentrations from 9 IU/ml to $2.5x10^9$ IU/ml. The assay specific lower limit of quantification (LLOQ) is equivalent to the general LOD.

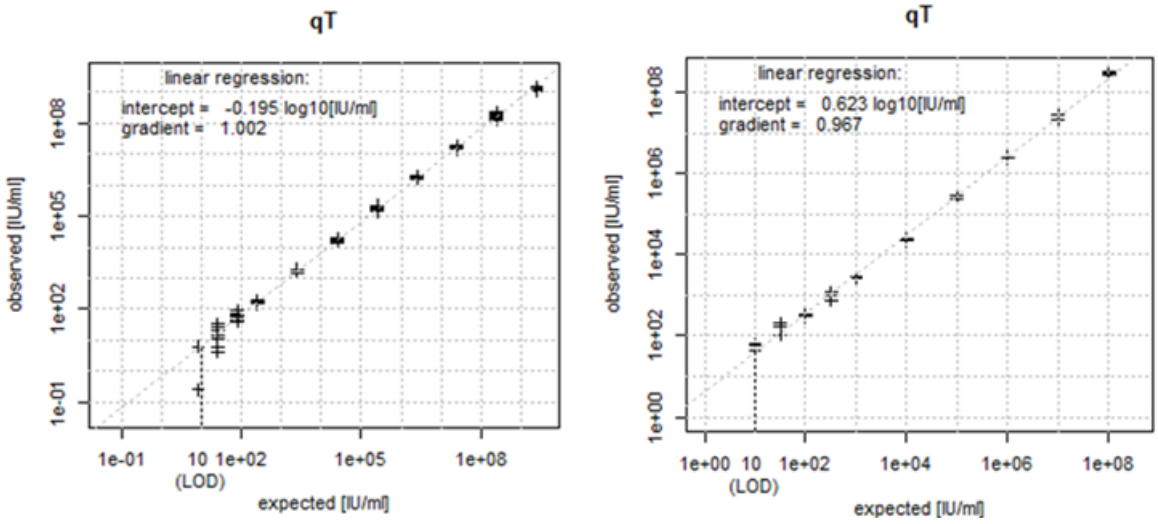


Figure 2: Linear range of quantification of the HBV DNA Quantification kit 3.0, exemplarily shown for qTOWER device. Data were obtained using a 8-log_{10} dilution series (8 IU/ml – $2.5x10^9$ IU/ml) of synthetic HBV DNA (left) and a 7-log_{10} dilution series (10 IU/ml – $1x10^8$ IU/ml) of a high-titer HBV-patient specimen (right). The assay was linear over the 8 log_{10} steps tested with an assay specific lower limit of quantification (LLOQ) equivalent to the general LOD.

4.3 Specificity

4.3.1 Genotype detection and quantification

The specificity of the RoboGene HBV DNA Quantification Kit 3.0 for the detection and quantification of known HBV genotypes/subtypes was tested using the 1st WHO International Reference Panel for Hepatitis B Virus Genotypes for Nucleic Acid Amplification Techniques – Based Assays PEI code 5086/08 (Version 2, 28th Nov 2011). The genotype panel includes 15 samples of the most prevalent HBV genotypes (A-G).

Half-log dilution series of all specimens were prepared and DNA was extracted using the manual extraction method of the INSTANT Virus RNA/DNA Kit by means of starting sample volume of 400 µl.

The quantification results of the RoboGene HBV DNA Quantification Kit 3.0 were within the ± 0.6 log_{10} acceptance interval of accuracy compared to the quantification results obtained in the WHO study

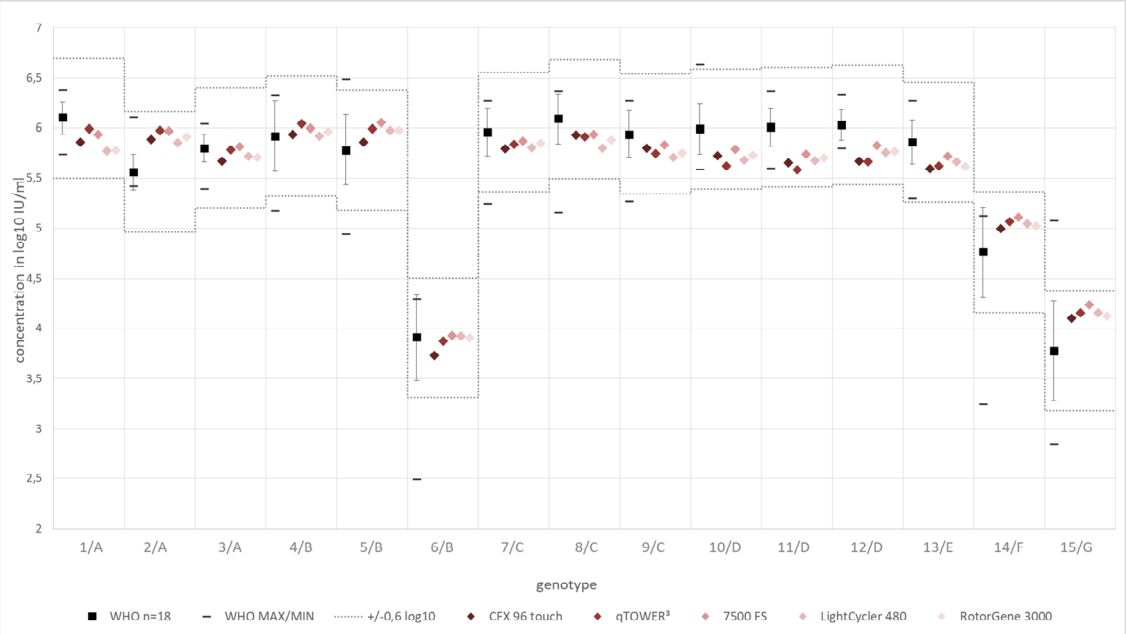


Figure 3: Comparison of the quantification results of the WHO study (mean of n = 18 participants, standard deviation, minimum and maximum values) with the quantification results of the RoboGene HBV DNA Quantification Kit 3.0 for all real-time PCR devices.

The theoretical specificity for the detection of all other confirmed HBV genotypes and subtypes was proven by aligning the sequences of the underlying oligonucleotides against sequence data of respective subtype reference strains (data not shown).

4.3.2 Analytical specificity

Analysis of 13 non-HBV positive human DNA-virus samples (HSV type 1 and 2; PVB19; EBV; HPV16; HPV18; HCMV) and two RNA virus samples (HIV; HCV) confirmed 100 % analytical specificity of the RoboGene HBV DNA Quantification Kit 3.0 (see Table 2).

Table 2: Results of analysis of 15 non-HBV positive human DNA/RNA-virus samples.

Sample	HBV detection	IC detection
HBV positive control (n = 1)	1/1	1/1
Virus negative control (n = 1)	0/1	1/1
HSV type 1(n = 1)	0/1	1/1
HSV type 2 (n = 1)	0/1	1/1
PVB19 (n = 2)	0/2	2/2
EBV (n = 1)	0/1	1/1
HPV16 (n = 1)	0/1	1/1
HPV18 (n = 1)	0/1	1/1
HCMV (n = 4)	0/4	4/4
HIV (n = 1)	0/1	1/1
HCV (n = 1)	0/1	1/1

4.3.3 Diagnostic specificity

Diagnostic specificity is expressed as a negative result in absence of the target. 100 patient samples tested negative for HBV by Biomex GmbH with the ABBOTT PRISM® HBsAg Assay Kit were determined with the RoboGene HBV DNA Quantification Kit 3.0. All samples showed negative results for HBV DNA while positive for Internal Control (see Table 3).

Table 3: Diagnostic Specificity

Sample	HBV detection	IC detection
HBV negative patient samples (n = 100)	0/100	100/100

The RoboGene HBV DNA Quantification Kit 3.0 showed analytical and diagnostic specificities of 100%.

4.4 Precision

The precision data represent the complete test procedure of HBV DNA quantification with the RoboGene HBV DNA Quantification Kit 3.0, using both the INSTANT Virus RNA/DNA Kit for manual NA extraction and the INSTANT Virus RNA/DNA Kit-FX for automated NA extraction with CyBio FeliX.

Dilution series of a HBV-positive patient specimen consisting of 5 different viral load levels and a range of 4-$\log_{10}$ steps were measured with 3 different batches of RoboGene HBV DNA Quantification Kit 3.0 on 3 different days and on 5 different real-time PCR devices (CFX96, qTOWER^3, LightCycler® 480, 7500 Fast and Rotor-Gene® 3000) for estimation of accuracy and precision (see Table 4).

Results showed overall reliant precision for RoboGene HBV DNA Quantification Kit 3.0 within the ± 0.6 $\log_{10}$ acceptance interval of accuracy independent of different parameters (manual and automated NA extraction; different processing days, batches, PCR devices).

Table 4: Mean precision and accuracy, as well as the accuracy range (difference between largest and smallest mean value) of the RoboGene HBV DNA Quantification Kit 3.0 over the individual influencing factors

Influencing factors	**Accuracy**	**Accuracy Range**	**Precision**
	[log_{10} IU/ml]	**[log_{10} IU/ml]**	**[CV%]**
Automated	0.05	Extraction method	13%
Manual	0.20	(0.15)	12%
batch 1	-0.01	Charge	14%
batch 2	0.16	(0.23)	12%
batch 3	0.22		11%
day 1	0.15	repeat	12%
day 2	0.12	(0.04)	13%
day 3	0.11		13%
CFX	0.16	PCR device	13%
qT	0.08	(0.15)	13%
FS	0.10		10%
LC	0.22		11%
RG	0.07		15%
Dil1 (588,235 IU/ml)	0.08	dilution	8%
Dil2 (58,824 IU/ml)	0.07	(0.12)	9%
Dil3 (5,882 IU/ml)	0.12		9%
Dil4 (588 IU/ml)	0.17		12%
Dil5 (58.8 IU/ml)	0.19		23%

For expected concentration levels above 100 IU/ml the mean coefficient of variation was about 10%. At the lowest concentration of about 60 IU/ml, the coefficient of variation increased to 23% (see Figure 4). Therefore, if a precise quantification result in the range between 100 IU/ml to LOD is required, it is recommended to repeat the sample with triplicate measurements.

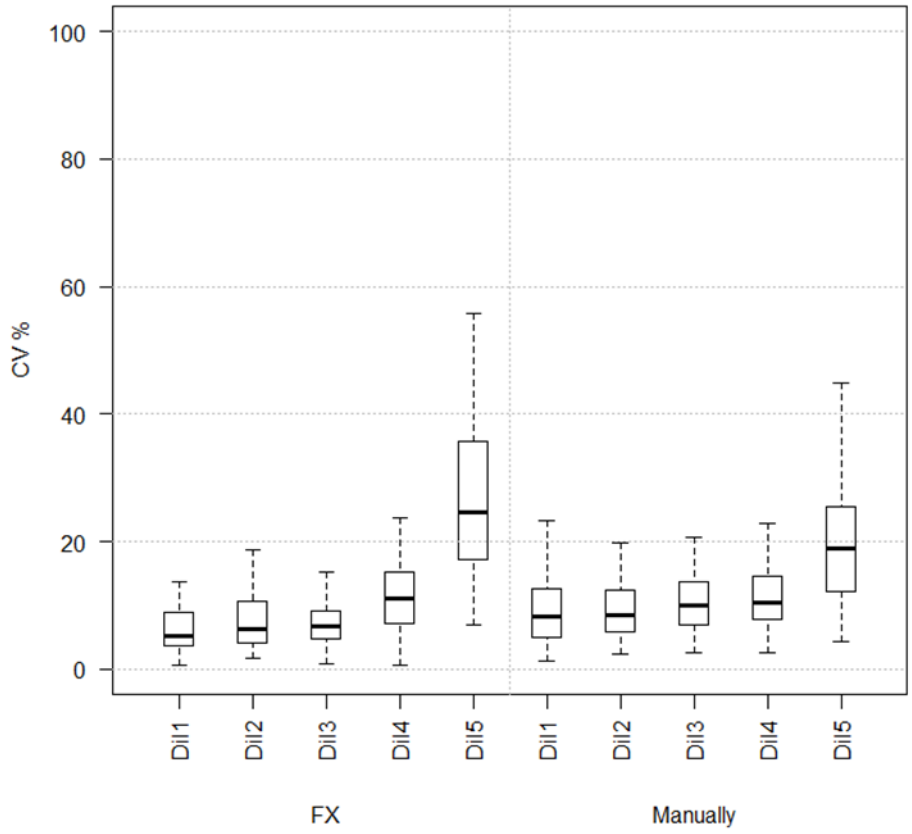


Figure 4: Precision data of the RoboGene HBV DNA Quantification Kit 3.0: Distribution of CV values as a function of the dilution stage and extraction method.

4.5 Robustness

The robustness expresses the total failure rate of the RoboGene HBV DNA Quantification Kit 3.0 and was tested for the complete test procedure using the INSTANT Virus RNA/DNA Kit-FX for automated NA extraction with CyBio FeliX.

A total number of 112 samples, containing reference plasma diluted to 25 IU/ml (representing the 3-fold virus concentration of the 95 % cut-off value of the test) were analyzed on CFX96, qTOWER^3, LightCycler® 480, and Rotor-Gene® 3000. Another 111 or 112 samples, containing reference plasma diluted to 30 IU/ml were analyzed with RealLine Cycler 48 / 96 as well as QuantStudio 5. Results of analysis are shown in Table 5.

Table 5: Results of failure rate study using RoboGene HBV DNA Quantification Kit 3.0

	(+) Results	Failure rate
CFX96		
HBV-DNA (FAM)	112/112	0 %
IC (VIC/ Cy5)	112/112	
qTOWER^3		
HBV-DNA (FAM)	112/112	0 %
IC (YY/ Cy5)	112/112	
LightCycler® 480		
HBV-DNA (FAM)	112/112	0.9 %
IC (YY/ Cy5)	111/112	
7500 FS		
HBV-DNA (FAM)	112/112	0 %
IC (VIC/ Cy5)	112/112	
Rotor-Gene® 3000		
HBV-DNA (FAM)	112/112	0 %
IC (YY/ Cy5)	112/112	

RealLine Cycler 48		
HBV-DNA (FAM)	111/111	0 %
IC (YY/ Cy5)	111/111	
RealLine Cycler 96		
HBV-DNA (FAM)	112/112	0 %
IC (YY/ Cy5)	112/112	
QuantStudio 5		
HBV-DNA (FAM)	112/112	0 %
IC (YY/ Cy5)	112/112	

Amplification of HBV DNA using RoboGene HBV DNA Quantification Kit 3.0 could not be reduced by the addition of EDTA, bilirubin and hemoglobin.

Quantification results from samples with high concentrations of lipids might be decreased considerably. Thus, results obtained from lipaemic plasma or serum samples should be interpreted carefully. Heparinized plasma has to be excluded from analysis, because of its inhibitory effect on the activity of Taq polymerases.

The performance of the RoboGene HBV DNA Quantification Kit 3.0 during seroconversion was analyzed using pre-seroconversion panels PHM 937, PHM 939 and PHM 941 obtained from SeraCare Life Sciences, Inc. (USA). HBV DNA quantification data were compared to the NAT assay used for initial viral load quantification of the respective seroconversion panel. Results are shown in Figure 5.

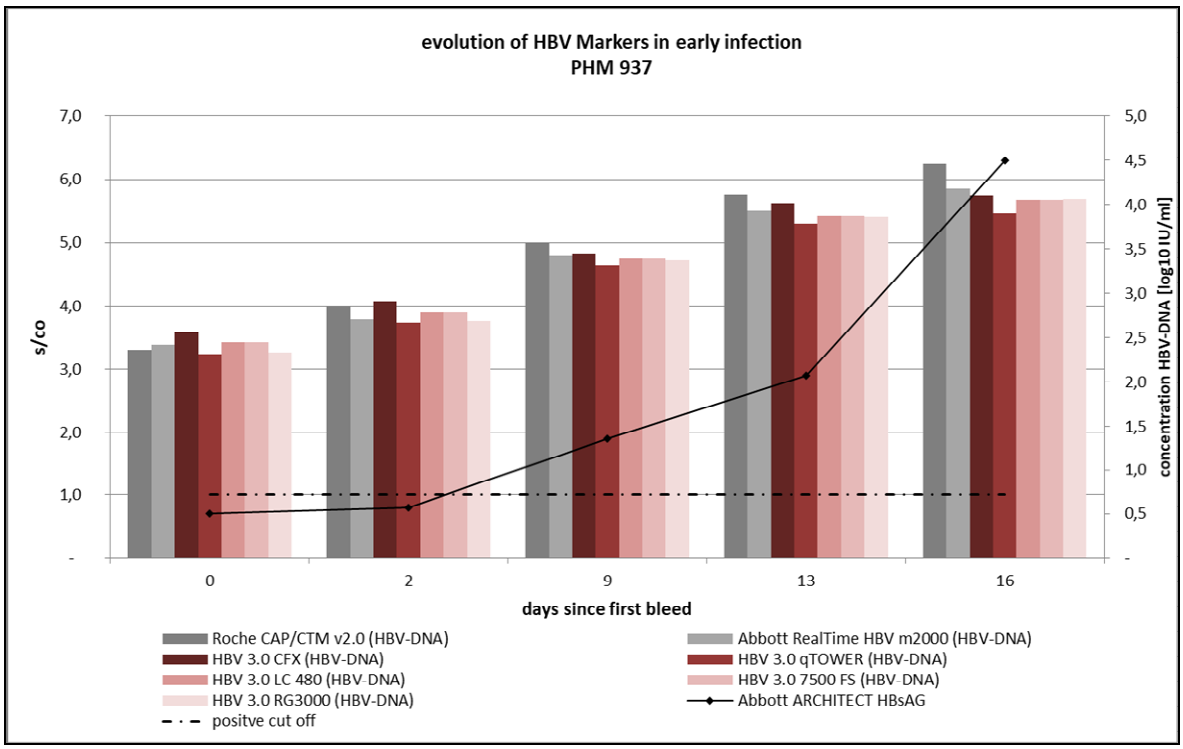


Figure 5: Performance of the RoboGene HBV DNA Quantification Kit 3.0 during seroconversion. For each PCR device the HBV DNA quantification results for each sample were blotted against the initially measured HBsAG-antibody levels (Abbott ARCHITEKT HBsAG)and compared with the results of the other real-time qPCR assays (CAP/CTM v2.0 assay (Roche) and Abbot RealTime HBV m2000). Results are exemplarily shown for the seroconversion panel PHM937.

In comparison to the initially applied NAT assays Abbott m2000, CAP/CTM v2.0 assay (Roche) and Cobas® AmpliPrep/TaqMan (Roche) the RoboGene HBV DNA Quantification Kit 3.0 revealed a comparable performance.

4.6 Diagnostic Evaluation

The diagnostic sensitivity and linearity of the RoboGene HBV DNA Quantification Kit 3.0 were analyzed with 106 HBV DNA positive patient samples on CFX96, qTOWER^3, LightCycler® 480, 7500 Fast and Rotor-Gene® 3000.

The diagnostic evaluation was performed with 59 samples for manual extraction and 51 samples for automated extraction with CyBio FeliX. Quantitative data were compared with results obtained

in advance with CE certified CAP/CTM v2.0 assay (Roche) performing Deming regression. Results are shown in Figure 6.

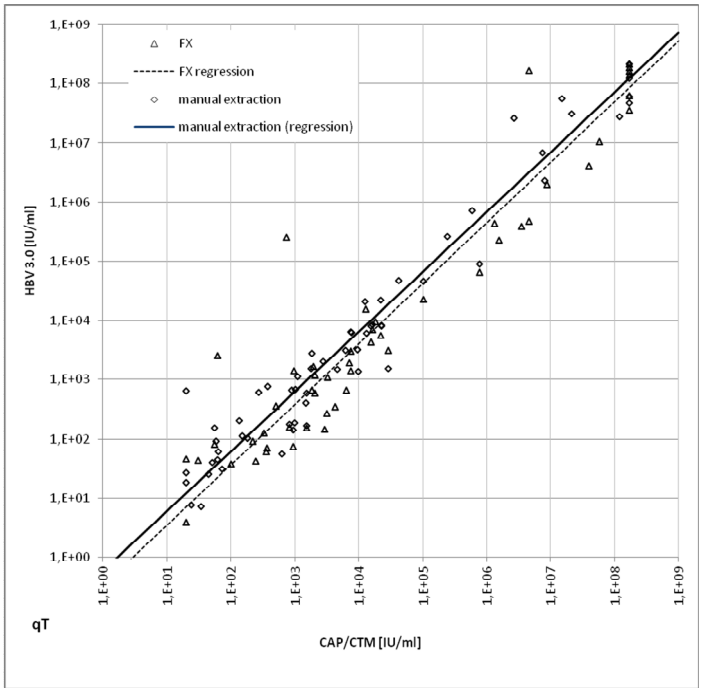


Figure 6: Diagnostic evaluation: Deming Regression scatter plot comparing the RoboGene HBV DNA Quantification Kit 3.0 with the CAP/CTM (Roche) assay. Data are shown exemplarily for the qTOWER quantification.

Deming regression showed a high degree of correlation between the results of the CAP/CTM (Roche) assay and the RoboGene HBV DNA Quantification Kit 3.0 for manual (see Table 6a) as well as automated nucleic acid extraction with CyBio FeliX (Table 6b).

Table 6a: Diagnostic evaluation: Comparison of the RoboGene HBV DNA Quantification Kit 3.0 with CAP/CTM v2.0 assay (Roche) for manual nucleic acid extraction.

Manual extraction	**CFX96**	**qTOWER^3**	**7500 Fast**	**LightCycler® 480**	**Rotor-Gene® 6000**
Correlation	0.959	0.973	0.970	0.972	0.972
Out of ± $\log_{10}$	4/59	3/59	3/59	3/59	3/56

Table 6b: Diagnostic evaluation: Comparison of the RoboGene HBV DNA Quantification Kit 3.0 with CAP/CTM v2.0 assay (Roche) for automated nucleic acid extraction.

FX	**CFX96**	**qTOWER^3**	**LightCycler® 480**	**Rotor-Gene® 6000**
Correlation	0.94	0.95	0.95	0.95
Out of ± $\log_{10}$	4/50	3/51	3/51	3/51

NOTE

The quantification standards of the RoboGene HBV DNA Quantification Kit 3.0 are calibrated against the PEI HBV-DNA reference material (#3620/05) that itself was calibrated against the 1st WHO International Standard for HBV DNA (NIBSC-code: 97/746).

Parallel the 4th WHO International Standard for HBV DNA (NIBSC-code: 10/266) was tested. Outcomes showed similar results as for the PEI reference material.

5 Kit components, storage and stability

Each kit contains two small inner boxes (1 and 2) and a small bag for storage of the following components:

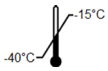
- box 1 for RT PCR Enzyme and IC
- box 2 for HBV/IC RM and PCR grade H_2O,
- bag for HBV/IC STD 1-4.

NOTE
RT PCR Enzyme has to be RE-PACKED to box 1 after arrival.

RoboGene HBV DNA Quantification Kit 3.0 is available in 3 sizes summarized in Table 7.

Table 7: Kit versions and components.

		Σ 32	Σ 96	Σ 192
REF		847-0207710032	847-0207710096	847-0207710192
IC [1]		IC Spiking Tube for 1 x 0.50 ml working solution	IC Spiking Tube for 3 x 0.50 ml working solution	IC Spiking Tube for 6 x 0.50 ml working solution
HBV/IC STD 1 - 4		4 Strips (4 x 4 wells)	4 Strips (4 x 4 wells)	4 Strips (4 x 4 wells)
HBV/IC RM[2]		Reagent Mix for 1 x 0.05 ml working solution	Reagent Mix for 2 x 0.05 ml working solution	Reagent Mix for 4 x 0.05 ml working solution
PCR grade H_2O[3]		1 x 1.5 ml	2 x 1.5 ml	4 x 1.5 ml
RT PCR Enzyme[4]		1 x 0.235 ml	1 x 0.660 ml	2 x 0.660 ml
IFU		1	1	1



STORAGE CONDITIONS

The RoboGene HBV DNA Quantification Kit 3.0 is shipped at room temperature, except the RT PCR Enzyme which is shipped on dry ice. After arrival store the RoboGene HBV DNA Quantification Kit 3.0 including the **RT PCR Enzyme** at -15 °C to -40 °C in the dark. The kit is stable until the expiry date when stored under these conditions.

IMPORTANT

[1] An appropriate amount of **IC** should be dissolved in **PCR grade H_2O** shortly before use. Remaining dissolved **IC** can be aliquoted properly and stored at -20 °C. Stored aliquots can be used up to 60 days. Repeated freezing and thawing up to 5 times is possible.

[2] An appropriate amount of Reagent Mix **HBV/IC RM** should be dissolved in **PCR grade H_2O** shortly before use. Remaining dissolved **HBV/IC RM** can be stored at -20 °C. Frozen **HBV/IC RM** can be used up to 60 days. Repeated freezing and thawing up to 5 times is possible. Always protect from light!

[3] Repeated freezing and thawing of **PCR grade H_2O** is possible.

[4] **RT PCR Enzyme** in general should be stored at -20 °C. Repeated freezing and thawing up to 5 times is possible. Nevertheless, **RT PCR Enzyme** should always be kept on ice-cold racks during usage.

6 Necessary laboratory equipment and additives

- HBV-positive control plasma (e.g. WHO International Standard for Hepatitis B Virus DNA for NAT testing or PEI Reference Preparation HBV DNA [PEI code #3620/05]). Provided quantification standards may be considered as positive control.
- HBV-negative control (e.g. human plasma or serum free of HBV DNA)
- For automated purification:
    - "CyBio® FeliX Basic Unit with Enclosure" (Analytik Jena GmbH)
    - „Laptop - english operation system" with software „Application Studio CyBio FeliX eXtract"
    - „CyBio FeliX Extraction Set"
- qTOWER 2 & 3 (Analytik Jena), CFX96 (Bio-Rad), LightCycler® 480 (Roche), 7500 Fast (Applied Biosystems) or Rotor-Gene® 3000/6000/Q (Corbett Research/ Qiagen)
- Real-time instrument specific software for data analysis and reporting
- Recommended real-time instrument specific PCR consumables (see table below)
- Suitable pipetting tools and sterile pipette aerosol-barrier tips
- Micro centrifuge
- Plate centrifuge
- Thermal mixer
- Vortex mixer
- 1.5 ml tubes
- 2.0 ml tubes
- Gloves, lab coat

Table 8: Recommended PCR consumables and ordering information.

Real-time PCR platform	PCR plastics	Sealing
qTOWER 2 & 3, CFX 96	96 Well PCR Plate 0.2 ml, fullskirt, white	Optical sealing foil
	Source: Analytik Jena Order number 844-70038-0	Source: Analytik Jena Order number 846-050-258
LightCycler® 480	LightCycler® 480 Multiwell Plate 96, white	LightCycler® 480 Sealing Foil
	Source: Roche Order number 04729692001	Source: Roche Order number 04729757001
7500 Fast	MicroAmp Fast Optical 96-Well Reaction Plate 0.1 ml (clear)	LightCycler® 480 Sealing Foil
	Source: ThermoFisher Order number 4346907	Source: Roche Order number 04729757001
Rotor-Gene® 3000/6000/Q	Strip Tubes and Caps, 0.1 ml	Source: Qiagen Order number 981103
RealLine Cycler 48 / 96 **QuantStudio 5**	96 x 0.2ml Transformer Netflex Plate White, High Profile	Axygen™ Microplate Sealing Film and Tapes
	Source: BIOplastics Order number B58709	Source: Axygen UC 500 Order number 14-222-873

7 Procedure

7.1 Collection and handling of clinical samples

- Collect 5-10 ml blood with standard specimen collection tubes.
- For plasma preferably EDTA or citrate anticoagulant has to be used; heparin is non-applicable, because of its inhibitory effect on PCR.
- Serum: after collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature for up to 30 minutes. Remove the clot by centrifuging at 1,000–2,000 x g for 10 minutes [5].
- Plasma: cells are removed from plasma by centrifugation for 10 minutes at 1,000–2,000 x g. Transfer to sterile tubes [5].
- Store serum and plasma at 2-8 °C. Test within 5 days [5].
- Plasma or serum samples may be stored deeply frozen for several months at -70 °C to -20 °C depending on the storage temperature. Avoid repeated freezing and thawing [6].

7.2 HBV DNA purification from clinical samples

The RoboGene HBV DNA Quantification Kit 3.0 has been validated with a manual and an automated purification method.

For manual purification use the INSTANT Virus RNA/DNA Kit (Roboscreen GmbH, Order number: 847-0259200602 for 50 reactions; 847-0259200603 for 250 reactions). Perform the HBV DNA purification steps according to the respective IFU using 'Protocol 2: Isolation of viral RNA/DNA from 400 µl of serum/plasma using IC Spiking Tube'.

For automated purification use the INSTANT Virus RNA/DNA Kit-FX (Roboscreen GmbH, Order number: 847-0259200902). Perform the HBV DNA purification steps according to the respective IFU.

7.3 Internal Control

The RoboGene HBV DNA Quantification Kit 3.0 contains the **IC** Spiking Tube stably coated with Internal Control nucleic acid and carrier nucleic acid.
Using **IC** together with the INSTANT Virus RNA/DNA Kit or INSTANT Virus RNA/DNA - FX always allows to control the whole procedure and to detect false-negatives due to failed extraction or excess of inhibitors within the sample. To judge the purification, the Ct value of the Internal Control purified together with HBV DNA negative or positive plasma should be in the instrument-specific ranges summarized in Table 16.

7.4 General procedure of quantitative analysis

The four quantification standards are provided as standard strips stably coated with defined amounts of synthetic HBV DNA. The standards are calibrated against the PEI reference material HBV DNA (#3620/05, calibrated against the 1st WHO International Standard for HBV DNA (NIBSC-code: 97/746)). The standard values are given in IU/ml, i.e. the HBV DNA concentration of the analyzed sample may be directly calculated from the reference curve without the need for subsequent conversion by an equation.

NOTE
Please note that the given standard values, and thus quantification, are dependent on the NA purification kit used together with the RoboGene HBV DNA Quantification Kit 3.0. Therefore quantification results are only valid when the INSTANT Virus RNA/DNA Kit or INSTANT Virus RNA/DNA Kit-FX in combination with indicated real-time PCR devices and device specific consumables were used.

8 Protocol

8.1 Preparation of Internal Control

NOTE
The RoboGene HBV DNA Quantification Kit 3.0 has been evaluated together with the INSTANT Virus RNA/DNA Kit as well as the INSTANT Virus RNA/DNA Kit - FX for nucleic acid extraction. The Internal Control is provided as **IC** Spiking Tube within the RoboGene HBV DNA Quantification Kit 3.0. Prepare the **IC** Tube according to the instructions below and extract NA following the instructions of the INSTANT Virus RNA/DNA Kit or INSTANT Virus RNA/DNA Kit - FX.

1. Centrifuge the IC Spiking Tube briefly at full speed to collect the lyophilized **IC** on the bottom of the tube.
2. Add 520 µl **PCR grade H_2O** to the vial; close the tube, mix by vortexing briefly followed by brief centrifugation.
3. Incubate at 37 °C for 5 min using a shaking platform (800-1000 rpm), mix by vortexing briefly followed by brief centrifugation.

8.2 Application of the dissolved IC for manual purification

1. Add 10 µl of resuspended IC per 400 µl sample volume to the Lysis Solution of the corresponding INSTANT Virus RNA/DNA Kit.
2. Follow instructions of the extraction kit 'Protocol 2: Isolation of viral RNA/DNA from 400 µl of serum/plasma using IC Spiking Tube' purification.

8.3 Application of the dissolved IC for automated purification

Follow instructions of the INSTANT Virus RNA/DNA Kit - FX.

8.4 Preparation of 25x Reagent Mix

1. Centrifuge the **HBV/IC RM** briefly at full speed to collect the lyophilized Reagent Mix on the bottom of the tube.
2. Add 53 µl **PCR grade H_2O** to **HBV/IC RM**; close the tube, mix by brief vortexing followed by brief centrifugation.
3. Incubate at 37 °C for 5 min using a shaking platform (800-1000 rpm), mix by brief vortexing followed by brief centrifugation.

8.5 Preparation of 1x Master Mix

1. Before setting up the Master Mix gently invert **RT PCR Enzyme** several times and centrifuge briefly.
2. Prepare the 1x Master Mix according to the following table. Mix by vortexing for at least 10 sec followed by brief centrifugation.

Table 9: Composition of 1x Master Mix per reaction.

Reagent	Volume for 1x rxn (µl)	Final concentration
PCR grade H_2O	7.75	-
HBV/IC RM Reagent Mix, 25x	1	1x
RT PCR Enzyme	6.25	1x
Total	15	

8.6 Preparation of Quantification Standards

1. Uncover standard strip **HBV/IC STD 1 -4** and place the strip onto a suitable ice-cold rack.
2. Add 25 µl **PCR grade H_2O** to each well of the quantification standard **HBV/IC STD 1 - 4**; mix by pipetting up and down several times.

NOTE
Be careful to use a new pipette tip for each standard to avoid carry over contamination.
It is important to mix quantification standards **HBV/IC STD 1 - 4** by pipetting up and down several times. Do not vortex the quantification standards!
Store quantification standard on ice or an ice-cold rack until introduction into PCR!

8.7 Preparation of reaction set

1. Place real-time PCR consumables (not provided) onto a suitable ice cold rack.
2. Add 15 µl 1x Master Mix to wells intended for sample quantification, NTCs and additional four wells for each of the quantification standards **HBV/IC STD 1 – 4**.
3. Add 10 µl **PCR grade H_2O** to wells that serve as NTC. Add 10 µl of resuspended **HBV/IC STD 1 - 4** to all wells that serve as quantification standards containing the 1x Master Mix. Do not exceed a final reaction volume of 25 µl. Make sure PCR grade H_2O and quantification standard solutions are mixed properly with the Master Mix by pipetting up and down several times.

NOTE
After usage discard the remaining solution of **HBV/IC STD 1-4**. To avoid contaminations, we recommend to seal the quantification standard with a suitable cover (e.g. parafilm, not included in the kit).

4. Add 10 µl eluate from NA isolation (INSTANT Virus RNA/DNA Kit or INSTANT Virus RNA/DNA Kit - FX) to the respective sample wells containing the 1x Master Mix. Do not exceed a final reaction volume of 25 µl. Make sure master mix and eluate is mixed properly.
5. Cover the real-time PCR consumables. Centrifuge PCR plates for 1 min at 1,000 rpm to collect the PCR mix on the bottom of each well (not necessary for Rotor-Gene® tubes).
6. Program the applied real-time PCR platforms as indicated in Table 10 to 13 and start the program.

NOTE
The essential in-run standard curve provides run validation criteria slope and R2 value (see Table 16).
Never use external standard curves for quantification.

Table 10: PCR program for qTOWER 2 & 3, CFX96

Step	Cycle	Profile	Temperature	Time	Ramping
1	1	Reverse transcription	47 °C	15 min	5 °C/sec
2	1	Taq activation	95 °C	2 min	5 °C/sec
3	45	Denaturation	95 °C	15 sec	2.5 °C/sec
		Annealing/ Elongation*	57 °C	1 min	5 °C/sec

* Data acquisition: Fluorescence Detection (FAM; Cy5) for qTOWER 2 & 3
# for qTOWER 2 & 3 the following device specific presetting are recommended: Open new project > Scan > Gain: for FAM = 4; for VIC/JOE = 3; for Cy5 = 5.

Table 11: PCR program for 7500 Fast and Rotor-Gene® 3000/6000/Q, RealLine Cycler 48 / 96

Step	Cycle	Profile	Temperature	Time	Ramping
1	1	Reverse transcription	47 °C	15 min	Not adjustable
2	1	Taq activation	95 °C	2 min	
3	45	Denaturation	95 °C	15 sec	
		Annealing/Elongation*	57 °C	1 min	

* Data acquisition: Fluorescence Detection (FAM; Cy5) for RealLine Cycler 48 / 96 and for 7500 Fast and Rotor-Gene® 3000/6000/Q (FAM; VIC/JOE and/ or Cy5)

Table 12: PCR program for QuantStudio 5.

Step	Cycle	Profile	Temperature	Time	Ramping
1	1	Reverse transcription	47 °C	15 min	3.16°C/sec
2	1	Taq activation	95 °C	2 min	3.16°C/sec
3	45	Denaturation	95 °C	15 sec	3.16°C/sec
		Annealing/Elongation*	57 °C	1 min	2.45°C/sec

* Data acquisition: Fluorescence Detection (FAM; VIC and/ or Cy5)

Table 13: PCR program for LightCycler® 480

Step	Cycle	Profile	Temperature	Time	Ramping
1	1	Reverse transcription	47 °C	15 min	4.4°C/sec
2	1	Taq activation	95 °C	2 min	4.4°C/sec
3	45	Denaturation	95 °C	15 sec	2.5°C/sec
		Annealing/Elongation*	57 °C	1 min	2.2°C/sec
4	1	Cooling	40 °C	30 sec	Max

* Data acquisition: Fluorescence Detection (FAM; VIC and/ or Cy5)

9 Data analysis

Each DNA amplification is associated with generation of a fluorescence signal measurable in FAM channel (for HBV DNA) and in YY/VIC/JOE and/ or Cy5 channel (for IC) resulting in a sigmoid growth curve (log scale). The data analysis is performed according to manufacturer's instructions of the real-time PCR instrument using the respective software. Check the obtained data to ensure that the run is valid and to interpret results (Table 14).

Table 14: Interpretation of the results.

FAM channel	YY/VIC/JOE or Cy5 channel	Interpretation
Interpretation of detection results		
x	**x**	Sample valid - detection of sample HBV DNA
x	**-**	Sample invalid - repeat sample
-	**x**	Sample valid - only detection of IC, HBV DNA not detectable/ HBV negative sample
-	**-**	Sample invalid - no amplification/ detection at all, repeat sample
Interpretation of quantification results		
< LOD/LLoQ	**x**	Below lower limit of detection of the assay. Three replicates of analysis are recommended to confirm positive result.
< 100 IU/ml	**x**	Fluctuation prone quantification area. Three replicates of analysis are recommended for precise quantification.
≥ 9 IU/ml and ≤ $2.5x10^9$ IU/ml	**x**	Calculated results are within the linear range of the assay.
> $2.5x10^9$ IU/ml	**x**	Above upper limit of covered linear range of the assay. Dilution of original sample is recommended.

HBV DNA concentration of clinical specimens is determined based upon a standard curve resulting from analysis of the quantification standard strip and the Ct values of the respective samples. The HBV DNA concentration is expressed in IU/ml. Table 16 lists the concentrations of HBV DNA quantification standards in case of using the INSTANT Virus RNA/DNA Kit or INSTANT Virus RNA/DNA Kit-FX.

Table 15: HBV DNA quantification standard concentrations.

HBV/IC STD 1 - 4	HBV DNA [IU/ml]
1	25,000,000
2	250,000
3	2,500
4	250

NOTE

Setting of threshold may markedly influence Ct values. Recommendation for setting thresholds is shown below:

- qTOWER 2 & 3: FAM: 6-7; YY: 5; Cy5: 5
- CFX96: FAM: 400-700; YY:150-250; Cy5: 100-150
- LightCycler® 480:

Channel	Noiseband	Threshold	Fit Points
FAM	0.7-1.6	1.5-5.9	4
YY	0.8-1.5	1.4-2.6	4
Cy5	0.5-0.9	1.0-2.4	4

- 7500 Fast: FAM: 0.1-0.2; YY: 0.04-0.05; Cy5: 0.04-0.1
- Rotor-Gene® 3000/6000/Q: FAM: 0.03-0.05; YY: 0.015-0.03; Cy5: 0.03-0.05
- RealLine Cycler 48 / 96: FAM: ~500; Cy5: ~700
- QuantStudio 5: FAM: ~200.000; YY: ~60.000; Cy5: ~90.000

Criteria for run validation are the slope and R^2 value of the standard curve (see Table 16). The areas of expected Ct values of the standards refer to own validation data and should be used as guidelines for setting threshold values (see Tables 17 to 19).

If slope and/or R^2 are out of range (Table 16), one of the four quantification standards may be excluded (most outlying of regression line), as three quantification standards are sufficient for valid results.
In such case no right for warranty of the whole product may be deduced.

Table 16: Criteria for run validation.

Parameter	**qTOWER 2 & 3, CFX96, LightCycler® 480, 7500 Fast, Rotor-Gene® 3000/6000/Q, RealLine Cycler 48 / 96, QuantStudio 5**
Range of slope	-3.10 to -3.60
The linear regression coefficient (R^2) of the reference curve should be between 0.98 and 1.00 (not applicable to LightCycler®480 analysis).	
Expected Ct values for IC of the quantification standards (dependent on the set threshold value, see above)	
YY/VIC/JOE	≤ 40
Cy5	≤ 38
Expected Ct values for IC in HBV negative patient samples and HBV positive samples (dependent on the set threshold value, see above)	
YY/VIC/JOE	≤ 40
Cy5	≤ 38

Table 17: Guidance Ct values of the quantification standards on qTOWER 2 & 3, LightCycler® 480 and CFX 96.

HBV/IC STD 1 - 4	Expected increment between Ct values	qTOWER 2 & 3		LightCycler® 480		CFX96	
		mean	from – to	mean	from – to	mean	from – to
1		14.8	14.0 – 15.6	15.4	13.7 – 17.2	15.2	14.2 – 16.1
2	1 to 2 + ~ 6.64	21.4	20.7 – 22.2	22.2	20.5 – 23.8	21.8	21.0-22.7
3	2 to 3 + ~ 6.64	28.3	27.5 – 29.1	28.9	27.2 – 30.7	28.5	27.6-29.5
4	3 to 4 + ~ 3.32	31.6	30.7 – 32.4	32.3	30.6 – 34.0	32.0	31.0-33.0

Table 18: Guidance Ct values of the quantification standards on 7500 Fast and Rotor-Gene® 3000/6000/Q.

HBV/IC STD 1 - 4	Expected increment between Ct values	7500 Fast		Rotor-Gene® 3000/6000/Q	
		mean	from – to	mean	from – to
1		14.7	13.7-15.6	12.5	11.4-13.6
2	1 to 2 + ~ 6.64	21.4	20.6-22.2	19.1	17.9-20.4
3	2 to 3 + ~ 6.64	28.1	27.3-29.0	25.6	24.6-26.7
4	3 to 4 + ~ 3.32	31.5	30.6 – 32.3	28.9	27.7-30.1

Table 19: Guidance Ct values of the quantification standards on RealLine Cycler 48 / 96 and Quant Studio 5.

HBV/IC STD 1 - 4	Expected increment between Ct values	RealLine Cycler 48		RealLine Cycler 96		Quantstudio 5	
		mean	from – to	mean	from – to	mean	from – to
1		13.8	12.3 – 14.8	13.9	13.4 – 14.8	14.4	13.5 – 15.4
2	1 to 2 + ~ 6.64	20.6	19.2 – 21.3	20.9	20.3 – 21.2	21.4	20.4 – 22.3
3	2 to 3 + ~ 6.64	27.3	25.8 – 28.1	27.7	27.1 – 28.0	28.1	27.2 – 29.1
4	3 to 4 + ~ 3.32	30.7	29.1 – 31.3	20.9	30.7 – 31.5	31.6	30.9 – 32.4

10 Troubleshooting

Problem / probable cause	Comments and suggestions
No signal at all	
▪ Fluorescence measurement not activated	Read the user guide of the real-time PCR device.
▪ False channels selected	Select FAM channel for HBV DNA and YY/VIC/JOE or Cy5 channel for IC .
▪ Incorrect cycling program	Check instrument settings, repeat run.
▪ Incorrect application of the kit	Read instruction for use.
▪ Storage conditions did not comply with instructions, expiry date of detection kit is exceeded	Check storage conditions and expiry date.
Low fluorescence signal recorded for both target and IC, target copy number underestimated	
▪ Target DNA degraded	Use DNase and RNase free consumables and reagents, store DNA on ice. Read instruction for use of the extraction kit.
▪ Optical lenses contaminated (Rotor-Gene®)	See chapter “Maintenance” of respective instrument brochure, alternatively clean lense once per month using absolute isopropanol and cotton swabs.
▪ Thermal block and/or optics polluted (96-well block format)	See chapter “Maintenance” of respective instrument brochure, alternatively fill each well with isopropanol, incubate 10 min at 50 °C, remove isopropanol and rinse with H_2O.
No or weak signal for IC in HBV-negative sample	
▪ Incorrect cycling program	Check instrument settings, repeat run.
▪ Excess of inhibitors in the sample/ loss of DNA during extraction	Use the recommended extraction kit and follow exactly manufacturer’s instructions.

▪ Incorrect sample material (e.g. heparinized plasma)	Request for fresh EDTA- plasma or serum.
▪ Storage conditions did not comply with instructions, expiry date of detection kit is exceeded	Check storage conditions and expiry date.
Unexpectedly low Ct values for IC particularly with high standards or high viral load samples	
▪ Cross talk between target and IC recording channels (especially YY/VIC/JOE)	Calibrate instrument using pure fluorescence dyes or repeat run using Cy5 channel for IC detection.
Non-sigmoidal growth curves of quantification standards, unacceptable high deviation of Ct from expected values	
▪ Frequent freezing/thawing or incorrect storage of dissolved reagent mix	Read IFU, check storage conditions, prepare new reagent mix.
▪ Storage conditions did not comply with instructions, expiry date of detection kit is exceeded	Check storage conditions and expiry date.
Different amplification behavior of sample HBV DNA and standards, non-parallel growth curves in exponential phase of reaction	
▪ Excess of inhibitors in the sample	Use the recommended extraction kit, follow exactly the manufacturer's instructions; consult attending doctor for patient medication.
▪ Incorrect sample material	Use recommended sample type.
FAM signal for HBV-negative samples / NTC recorded	
▪ Contamination with HBV DNA or DNA amplicons	Repeat extraction and/or PCR with new reagents; decontaminate instruments and work space.

If you have any further questions which are not answered, please contact our technical service.

11 References

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