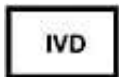


September 2010

RoboGene[®] HBV DNA Quantification Kit

TripleHyb[®] version

INSTRUCTION MANUAL



For quantitative in vitro diagnostic use



For 100/50 HBV DNA quantification reactions



For use with ABI PRISM[®] 7000/7300 SDS

Cat.no. 0207300102 100 tests

Cat.no. 0207300104 50 tests

For use with 7500 Fast/LP (0.1 ml)

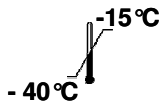
Cat.no. 0207300162 100 tests

Cat.no. 0207300164 50 tests

For use with Rotor-Gene[™] 3000/6000

Cat.no. 0207300142 100 tests

Cat.no. 0207300144 50 tests



Recommended storage temperature



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INSTRUCTION MANUAL

RoboGene[®] Hepatitis B Virus (HBV) DNA Quantification Kit

Kit for quantification of HBV DNA in human plasma or serum samples via Hepatitis B surface antigen (HBsAg) gene by real-time PCR

Trademarks

Roboscreen[®], RoboGene[®], and TripleHyb[®] are registered marks of the AJ Roboscreen GmbH. The Roboscreen[®] "Intelligent tube" technology is covered by patent and patent application DE 198 40 531 and WO 0012756. The TRIPLEHYB[®] technology is covered by German and US patent pending applications. ABI PRISM[®], FAM[™], and VIC[™] are registered trademarks of the Applied Biosystems Corporation. Rotor-Gene is a trademark of Qiagen B.V.. Some of the applications which may be performed with this product may be covered by applicable patents in certain countries. The purchase of this product does not include or provide a license to perform patented applications. Users may be required to obtain a license depending on the country and/or application. AJ Roboscreen does not encourage the unlicensed use of patented applications.

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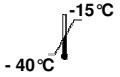
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Intended use

The RoboGene® HBV DNA Quantification Kit is intended for real-time PCR quantification of Hepatitis B Virus (HBV) DNA in human EDTA plasma or serum samples. Three kit versions are available for application to Rotor-Gene™ 3000/6000, ABI PRISM® 7000/7300 Sequence Detection System and 7500 Fast/LP (0.1ml), respectively. The level of HBV DNA in plasma or serum can be used in conjunction with other clinical markers and clinical findings to distinguish between acute and chronic HBV infection and to assess the viral response to antiviral treatment.

The quantification kit is not intended for screening of blood or blood products for HBV DNA or for confirmation of a HBV infection.

Symbols and Abbreviations

	for X detections
	use by
	consult instructions
IVD	for <i>in vitro</i> diagnostic use
REF	catalogue number
LOT	batch code
	manufacturer
	upper and lower limit of storage temperature
COMP	component
VOL	volume

C _T	Threshold cycle value
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuracyl 5'-triphosphate
dNTP	2'-deoxynucleotide 5'-triphosphate
HBV	Hepatitis B Virus
IC	Internal Control
IU	International Units
LP	"Low profile" PCR tubes (0.1 ml) supporting instruments
NTC	Non-template control
PEI	Paul-Ehrlich-Institut, Langen, Germany
SD	Standard deviation
HBV-S gene	Hepatitis B Virus surface antigen gene
HBsAg	HBV-S antigen coded by the HBV-S gene

Kit contents

Table 1.1: Kit components for application to Rotor-Gene™ 3000/6000


Label	Package size 100 tests	Package size 50 tests	Package format	Description	Box No.
HBV_D1	100 tubes	50 tubes	plastic bag	Extraction tubes coated with IC DNA and carrier nucleic acid	2
HBV_D2_RG	100 tubes	50 tubes	plastic bag	Sample tubes coated with amplification enhancer	1
HBV_D3_RG	4 strips (4x 8 tubes)	2 strips (2x 8 tubes)	plastic box	Quantitation standard tubes coated with different amounts of HBV DNA, IC DNA and amplification enhancer	1
HBV_D4	4 vials	2 vials	amber vial/amber cap	Reagent mix lyophilized with HBV/IC specific primers, probes and dNTPs	1
Taq Polymerase FS1	2x 0.03 ml	1x 0.03 ml	clear vial/clear cap	Taq polymerase, 5 U/μl	separate
10x PCR buffer FS1	1x 0.50 ml	1x 0.50 ml	clear vial/orange cap	10x PCR buffer with MgCl ₂	separate
PCR grade water	4x 1.50 ml	2x 1.50 ml	clear vial/clear cap	PCR grade water	1
	1	1	handbook		1

Table 1.2: Kit components for application to ABI PRISM® 7000/7300 SDS



Label	Package size 100 tests	Package size 50 tests	Package format	Description	Box No.
HBV_D1	100 tubes	50 tubes	plastic bag	Extraction tubes coated with IC DNA and carrier nucleic acid	2
HBV_D2_AB	13 strips (13x 8 tubes)	7 strips (7x 8 tubes)	plastic bag	Sample tubes coated with amplification enhancer	1
HBV_D3_AB	4 strips (4x 8 tubes)	2 strips (2x 8 tubes)	plastic box	Quantitation standard tubes coated with different amounts of HBV DNA, IC DNA and amplification enhancer	1
HBV_D4	4 vials	2 vials	amber vial/amber cap	Reagent mix lyophilized with HBV/IC specific primers, probes and dNTPs	1
Taq Polymerase FS1	2x 0.03 ml	1x 0.03 ml	clear vial/clear cap	Taq polymerase, 5 U/μl	separate
10x PCR buffer FS1	1x 0.50 ml	1x 0.50 ml	clear vial / orange cap	10x PCR buffer with MgCl ₂	separate
PCR grade water	4x 1.50 ml	2x 1.50 ml	clear vial/clear cap	PCR grade water	1
10x ROX	1x 0.50 ml	1x 0.50 ml	amber vial/green cap	10x ROX passive reference dye	1
OT_AB	2	1	plastic bag	Optical tape	1
	1	1	handbook		1

Table 1.3: Kit components for application to 7500 Fast/LP (0.1 ml)

Label	Package size 100 tests	Package size 50 tests	Package format	Description	Box No.
HBV_D1	100 tubes	50 tubes	plastic bag	Extraction tubes coated with IC DNA and carrier nucleic acid	2
HBV_D2_LP	13 strips (13x 8 tubes)	7 strips (7x 8 tubes)	plastic bag	Sample tubes coated with amplification enhancer	1
HBV_D3_LP	4 strips (4x 8 tubes)	2 strips (2x 8 tubes)	plastic box	Quantitation standard tubes coated with different amounts of HBV DNA, IC DNA and amplification enhancer	1
HBV_D4	4 vials	2 vials	amber vial/amber cap	Reagent mix lyophilized with HBV/IC specific primers, probes and dNTPs	1
Taq Polymerase FS1	2x 0.03 ml	1x 0.03 ml	clear vial/clear cap	Taq polymerase, 5 U/μl	separate
10x PCR buffer FS1	1x 0.50 ml	1x 0.50 ml	clear via / orange cap	10x PCR buffer with MgCl ₂	separate
PCR grade water	4x 1.50 ml	2x 1.50 ml	clear vial/clear cap	PCR grade water	1
10x ROX	1x 0.50 ml	1x 0.50 ml	amber vial/green cap	10x ROX passive reference dye	1
OT_AB	2	1	plastic bag	Optical tape	1
	1	1	handbook		1

Storage and stability

The RoboGene[®] HBV DNA Quantification Kit is delivered at room temperature except the Taq polymerase and PCR buffer which are shipped on dry ice. Store the HBV DNA Quantification Kit and taq polymerase at -15°C to -40°C in the dark immediately upon arrival. The kit is stable until the expiration date when stored under these conditions.

Reagents not contained in the test kit should be stored according to the respective manufacturer's recommendations.

- **NOTE** An appropriate amount of Reagent mix (HBV_D4) should be dissolved in PCR grade water shortly before use. Remaining dissolved reagent mix can be stored at 2 - 8°C up to 30 days (do not freeze!). Always protect from light!

Product use limitations

All reagents may exclusively be used for in vitro diagnostic applications. This test is validated for use together with human EDTA plasma or serum. Heparinized and lipaemic plasma or serum has to be excluded from analysis. If other than the recommended sample types are used wrong results may be obtained. The product is to be used by personnel specially instructed and trained in the in vitro diagnostics procedures (EN375) only. Strict compliance with the user manual is required for optimal PCR results. The product may be used only with the mentioned real-time PCR instruments. Do not use expired components or mix with components from different batches.

Technical assistance

In case of any problem with the RoboGene® HBV Quantification Kit please contact our technical support team which consists of experienced scientists with long-time experience in the field of molecular diagnosis particularly of real-time PCR detection of pathogens. For technical assistance please contact us as shown on the back cover of the manual.

Sample collection and storage, safety information

Collection and handling of clinical samples

- Collect 5-10 ml blood with standard specimen collection tubes
- Preferably EDTA (red cap, Sarstedt or equivalent manufacturer) anticoagulant has to be used, heparin is non-applicable, because of its inhibitory effect on PCR
- Store whole blood at 2-25 °C not longer than 6 hours, centrifuge for 20 min at 800-1600 g to separate plasma or serum from blood cells and transfer to sterile tubes (e.g. Eppendorf)
- Plasma or serum samples may be transported at room temperature, do not exceed the time 6 hours after blood collection
- Plasma or serum HBV DNA may be stored deeply frozen for several months at -20°C to -70°C depending on the storage temperature.

Safety information

- Human plasma or serum samples have to be considered as potentially infectious! (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.
- Discard sample and assay waste according to your in-house safety regulations
- For more information please read the appropriate material safety data sheet.

Reagents and equipment to be supplied by the user

- HBV-positive control (e.g. current WHO international standard for Hepatitis B Virus DNA for NAT testing [97/750] or other HBV reference plasma or serum; provided quantitation standards [HBV_D3_RG / HBV_D3_AB] may be considered as positive control)
- HBV-negative control (e.g. human plasma or serum free of HBV DNA)
- Rotor-Gene™ 3000/6000, ABI PRISM® 7000/7300 SDS or 7500 Fast/LP (0.1 ml)
- Real-time instrument specific software for data analysis and reporting
- Suitable pipetting tools
- Micro centrifuge applicable for 0.1-ml, 0.2-ml- and 1.5-ml-tubes (also for 96-well-plates in case of application to ABI PRISM® 7000/7300 SDS or 7500 Fast/LP [0.1 ml])
- Vortex mixer
- 1.5-ml-tubes
- Sterile pipette aerosol-barrier tips
- Applicator for optical tape (for application to ABI PRISM® 7000/7300 SDS and 7500 Fast/LP [0.1 ml])
- Compression pad (for application to ABI PRISM® 7000/7300 SDS)
- Precision plate holder for tube strips (for application to 7500 Fast Real Time PCR System)
- Gloves, lab coat

HBV DNA purification from sample

The RoboGene® HBV DNA Quantification Kit is validated together with the INSTANT Virus DNA Kit (AJ Innuscreen, cat.no. 845-KS-4150050) and the QIAamp DSP Virus Kit (Qiagen, cat.no. 60704). Perform the HBV DNA purification steps according to the instructions of the INSTANT Virus DNA Kit manual or the QIAamp DSP Virus Kit handbook, respectively.

Internal DNA Control

The RoboGene® HBV DNA Quantification Kit is provided together with stabilized internal control DNA (IC DNA). The IC DNA is contained in the extraction tubes which are stably coated with IC DNA and carrier nucleic acid, respectively. The tubes are labelled with HBV_D1 and are contained in box 2 of the kit.

Applying the IC DNA containing extraction tubes together with the DNA extraction kit of choice always allows controlling for extraction yield, inhibitor load and judging the efficiencies of DNA extraction and subsequent PCR amplification, respectively. False-negatives due to failed extraction or excess of inhibitors in the sample may be excluded when getting positive amplification results for the internal control.

In case of using the INSTANT Virus DNA Kit replace the original lysis tubes labelled with "Extraction tubes" and in case of using the QIAamp DSP Virus Kit replace the original lysis tubes labelled with "LT" by the extraction tubes (HBV_D1) contained in box 2. To consider the purification successful, the C_T value of the IC DNA purified together with HBV negative plasma or serum should be in the instrument-specific ranges summarized in Table 10 (see page 25).

- **NOTE** Please add at first the lysis solution contained in the respective DNA purification kit to the extraction tubes containing the IC DNA and subsequently the patient sample. Do not add the sample directly to the extraction tube. Since the IC DNA is already contained in the extraction tube no additional pipetting steps are required.

Quantitative analysis

The quantification standards are provided as ready-to use standard strips which are stably coated with defined amounts of HBV standard DNA. The standards are calibrated using a WHO calibrated reference HBV DNA preparation obtained from the German Federal Agency for Sera and Vaccines (PEI) and are defined as IU/ml. The standard values are given as IU per ml, i.e. the HBV DNA concentration of the analyzed sample may be directly calculated from the reference curve without need of subsequent conversion by an equation.

- **NOTE** Please notice that the standard values are dependent on the DNA purification kit used together with the RoboGene® HBV DNA Quantification Kit. Using the INSTANT Virus DNA Kit please obtain the given standard concentration from Table 2.1. In case of using the QIAamp DSP Virus Kit we refer to Table 2.2. We guarantee correct results only when using one of the recommended DNA purification kits.
- **NOTE** Please notice also that individual values may be below the stated detection limit of the kit. Detection limit of e.g. 50 IU/ml means that from the statistical point of view at least 95% of samples containing 50 IU per ml are correctly detected with the kit at a probability of error of 5%. This means on the other hand that values below the stated detection limit may be plausible but with an unacceptable high probability of error!

Introduction

Pathogen information

The Hepatitis B virus (HBV) is an organism which infects the liver of humans, and causes an inflammation called hepatitis. This widely spread virus which proliferates in liver cells and destroys them has recently infected approximately 350 billion people worldwide. Chronic carriers are at high risk of long term health complications including liver cirrhosis and cancer. Quantitative PCR tests particularly based on real time PCR have been developed to detect and measure the amount of HBV DNA, called the viral load, in clinical specimens. Quantitative data are used to assess a person's infection status, to support therapeutic decisions and to monitor treatment.

Principle of the TripleHyb® assay

Real-time PCR is a highly sensitive homogenous assay that combines amplification with fluorescence-based online detection of the nucleic acid of interest (target, template) and it has become one of the leading techniques of molecular diagnostics.

The provided assay exploits the AJ Roboscreen owned, patent pending TripleHyb® detection format for real-time PCR (Figure 1). This format besides a conventional set of primers comprises a pair of labelled oligonucleotide probes, whereas the upstream or detector probe is labelled with a reporter dye at its 5'-end and the downstream or quencher probe is labelled with a quencher dye. Both probes consist of a target-complementary subsequence and a target-unrelated subsequence which has the capability to form a stem structure between both probes. In the presence of target the detector probe hybridizes by its target-complementary 5'-end and the quencher probe adjacently with its 3'-target-complementary end to the desired sequence. Simultaneously, an intermolecular stem structure is formed between the 3'-end of the upstream and the 5'-end of the downstream probe thus forming a stable tertiary detection complex with the target. A Taq DNA polymerase which possesses 5' → 3' exonuclease activity cleaves the detector probe and displaces the quencher probe from the target. The reporter dye is separated from the detector probe upon cleavage, resulting in an increase in fluorescence which may no longer be suppressed by the quencher probe dye and which is directly proportional to the target amplification during PCR. Consequently, the assay requires a 3-step PCR protocol whereas the low temperature step

of the protocol enables both the inter-probe stem formation and the subtractive detection of the cleavage product.

Explanation of the HBV DNA quantification test

The RoboGene[®] HBV DNA Quantification Kit is an amplification test for quantification of HBV DNA in human plasma or serum samples. All eight HBV genotypes (A-H) are amplified with equal efficiency applying probes and primers specific for a subsequence of the HBV-S gene encoding HBsAg. Quantitation standard consists of 8 tubes coated with given amounts of synthetic HBV-S DNA, which must be amplified in parallel.

A synthetic internal control is included via Extraction tubes HBV_D1 to control DNA extraction and to indicate for inhibitory effect on detection. Thus, the risk concerning false-negative results is clearly reduced yielding in increase of diagnostic correctness. Amplification of HBV DNA in samples and standards and of IC DNA is measured independently at different wavelengths due to probes labelling with different fluorescence reporter dyes (HBV DNA: FAM/Green channel, IC DNA: JOE/VIC/Yellow channel, depending on the real-time PCR instrument in use). In the protocols the channels are just denoted as FAM and JOE/VIC, respectively.

For sample preparation the “INSTANT Virus DNA Kit” (AJ Innuscreen) or “QIAamp DSP Virus Kit” (QIAGEN) are recommended. DNA extraction must be performed with the respective starting sample volume strictly according to manufacturer’s instructions. For final elution of filter bound DNA the use of 60 µl of elution buffer is suggested respectively, to make use of quantification standard concentrations are listed in tables 2.1 and 2.2, respectively.

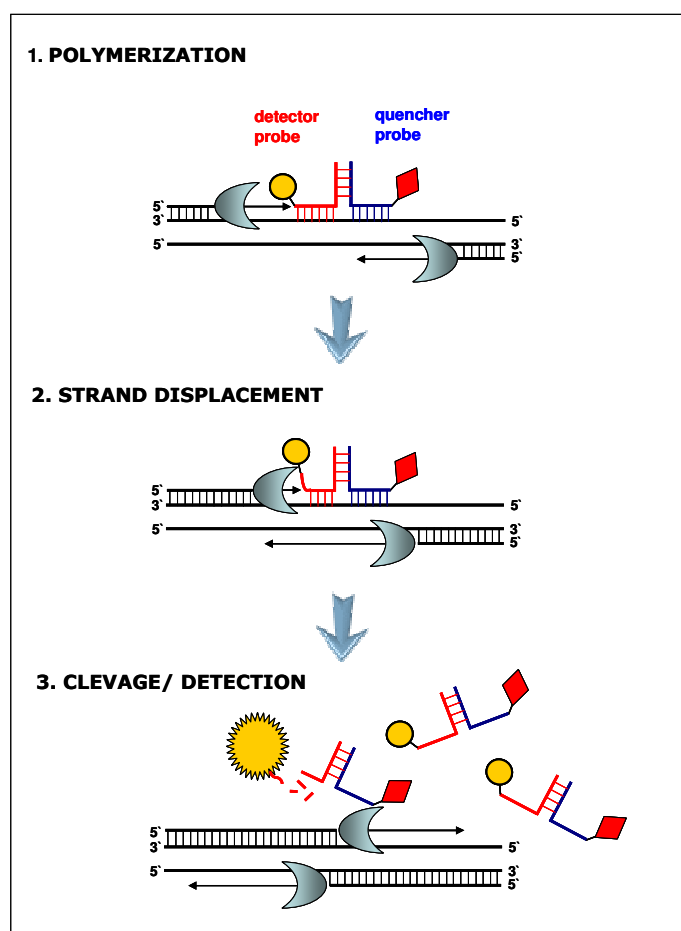


Figure 1: Schematic diagram illustrating the TripleHyb[®] fluorescence PCR process. The measured reporter fluorescence signal obtained from hydrolyzed detector probe which may no longer be suppressed by the hybridized quencher probe dye is proportional to the initial target concentration.

Protocol

- **NOTE** Include at least one replicate each of positive control plasma, negative control plasma, and NTC per run. Control plasma is not contained in the kit.

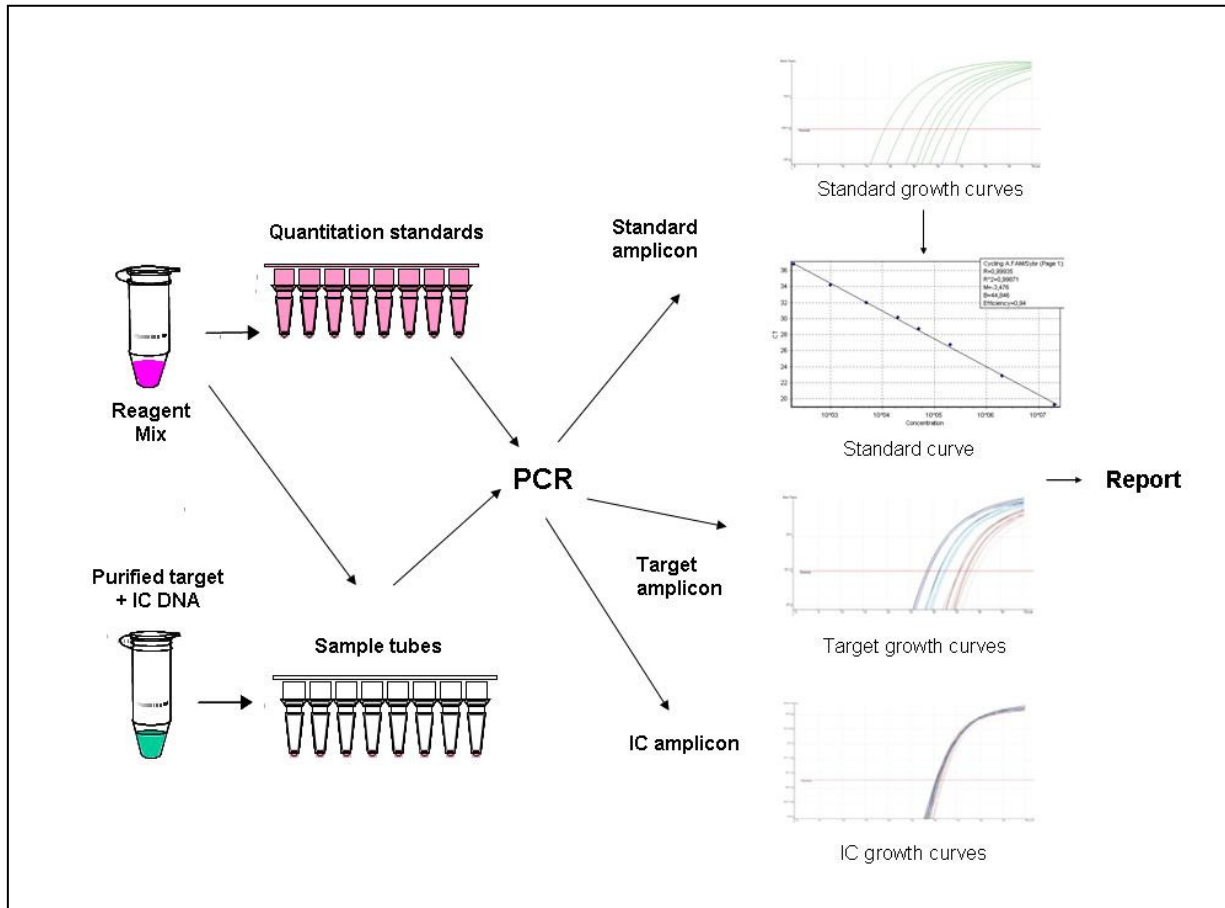


Figure 2: HBV DNA quantification by real-time PCR

Table 2.1: HBV DNA (IU/ml) given in quantitation standard in case using the INSTANT Virus DNA Kit.

Standard-Tube No.	HBV standard DNA [IU/ml] ¹⁾	HBV standard DNA [copies/ml] ¹⁾
1 (A)	50,000,000	250,000,000
2	5,000,000	25,000,000
3	500,000	2,500,000
4	125,000	625,000
5	50,000	250,000
6	12,500	62,500
7	2,500	12,500
8	500	2,500

¹⁾ The given values for the standards are valid as long as the sample, the elution volume and the purified DNA sample volumes per reaction are 200 µl, 60 µl and 5 µl, respectively.

Table 2.2: HBV DNA (IU/ml) given in quantitation standard in case using the QIAamp DSP Virus Kit

Standard-Tube No.	HBV standard DNA [IU/ml] ²⁾	HBV standard DNA [copies/ml] ²⁾
1 (A)	10,000,000	50,000,000
2	1,000,000	5,000,000
3	100,000	500,000
4	25,000	125,000
5	10,000	50,000
6	2,500	12,500
7	500	2,500
8	100	500

²⁾ The given values for the standards are valid as long as the sample, the elution volume and the purified DNA sample volumes per reaction are 500 µl, 60 µl and 5 µl, respectively.

The conversion of IU to copies is based on the conversion factor of “5” given by the manufacturer of reference plasma used to calibrate the kit.

► **NOTE** The values for the quantitation standards of tables 2.1 and 2.2 are only valid together with the mentioned DNA purification kits and considering the recommended elution volumes (see footnotes under the tables).

Table 2.3: Expected C_T values of the standards listed in tables 2.1 and 2.2 depending on the used real-time PCR instrument

Standard-Tube No.	Rotor-Gene		ABI PRISM 7000/7300		7500 Fast/LP (0.1 ml)	
	mean ³⁾	from - to ³⁾	mean ³⁾	from - to ³⁾	mean ³⁾	from - to ³⁾
1 (A)	18.4	17.4 – 20.9	17.0	15.9 – 18.1	18.5	17.2 – 19.4
2	21.9	20.9 – 23.8	20.5	19.8 – 21.9	21.9	21.6 – 22.6
3	25.5	24.4 – 28.2	23.8	22.6 – 25.0	25.3	24.0 – 26.7
4	27.5	26.5 – 28.8	25.9	25.1 – 27.1	27.5	26.8 – 28.7
5	28.9	27.5 – 30.3	27.2	26.1 – 28.8	28.6	27.9 – 31.0
6	30.9	29.7 – 32.9	29.3	28.3 – 30.5	30.7	29.9 – 31.6
7	33.1	31.5 – 35.2	31.4	29.7 – 32.9	32.8	32.3 – 34.1
8	35.2	33.1 – 37.2	33.7	32.6 – 35.3	34.6	33.8 – 36.0

³⁾ Data valid only when the recommended data analysis settings (see notes on page 24) are selected.

Rotor-Gene™ 3000/6000

1. Preparing 5x reagent mix (HBV_D4, 5x)

Add 200 µl PCR grade water to the vial with lyophilized reagent mix (HBV_D4). Close the tube and incubate at 37°C for 20 min, mix by vortexing for 3 sec and centrifuge for 5 sec at full speed.

➤ **NOTE** Dissolved reagent mix can be stored at 2-8 °C and always protected from light up to 30 days (do not freeze!).

2. Preparing 1x master mix

Table 3: Reagents and volumes for 1x master mix

Reagent	Volume (µl)/Reaction	Final concentration
PCR grade water	12.1	-
10x PCR buffer FS1	2.5	1x
HBV_D4, 5x	5.0	1x
Taq polymerase FS1 (5 U/µl)	0.4	2.0 U per reaction

Mix by vortexing for 3 sec and centrifuge for 5 sec at full speed.

3. Identify sample tubes (HBV_D2_RG) and quantitation standard (HBV_D3_RG) carefully and place them onto a suitable rack.

➤ **NOTE** Attention should be paid to correct orientation of quantitation standard. Highest HBV DNA amount is given in the tube labelled with 1 on top of the strip, lowest amount is given in tube 8.

4. Add 20 µl 1x master mix to all sample tubes and quantitation standard tubes required for the respective run.

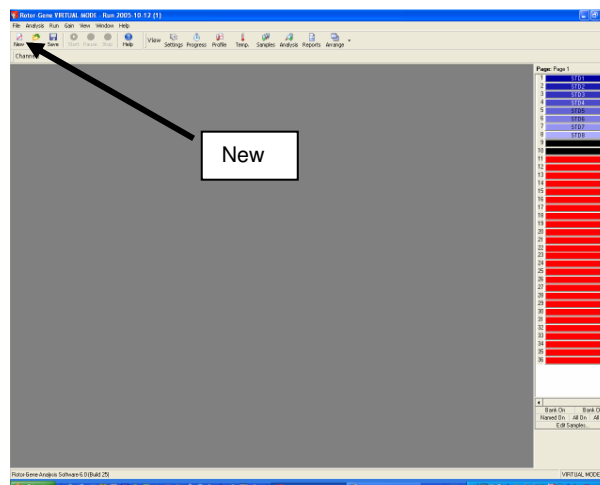
5. Add 5 µl PCR grade water to sample tubes which serves as NTC and to all quantitation standard tubes containing the 1x mastermix. Do not exceed a final reaction volume of 25 µl.

6. Add 5 µl of eluate from DNA isolation (e.g. using the INSTANT Virus DNA Kit) to the respective sample tubes containing the 1x mastermix. Do not exceed a final reaction volume of 25 µl.

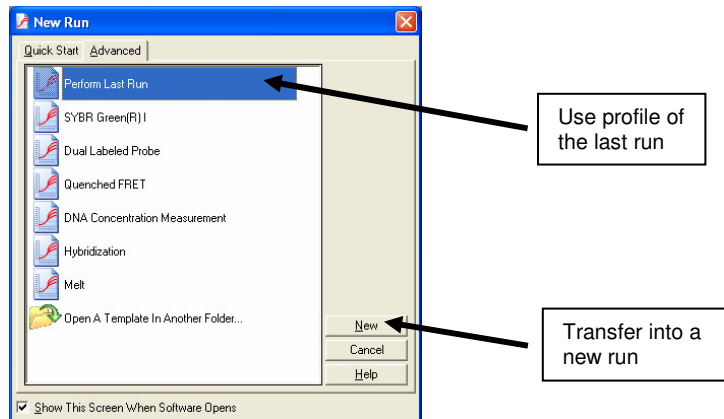
7. Cover tubes with attached caps.

8. Set up the run:

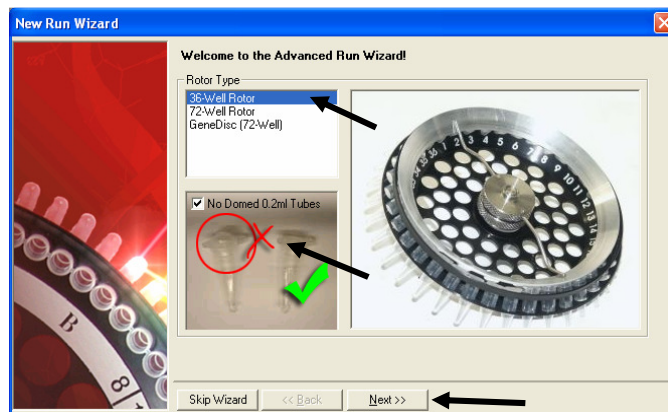
- 1) Switch on instrument by pressing the main switch, start Rotor-Gene software
- 2) Start a new run



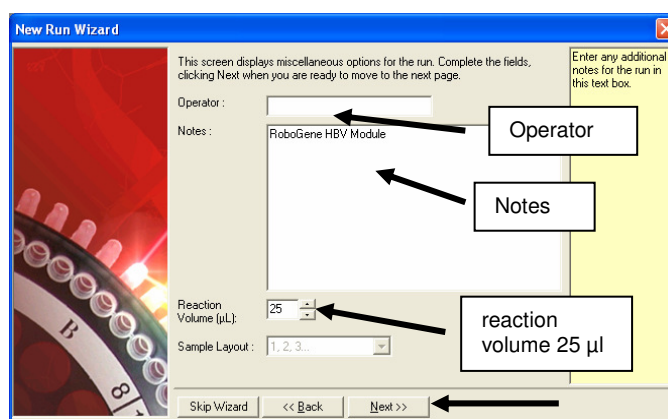
3) Select function „Perform Last Run“ and transfer into a new run



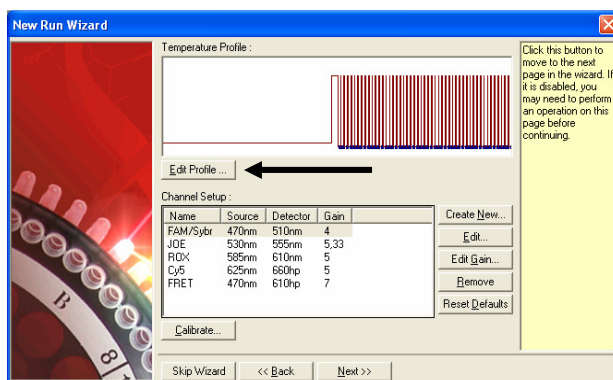
4) Select appropriate rotor



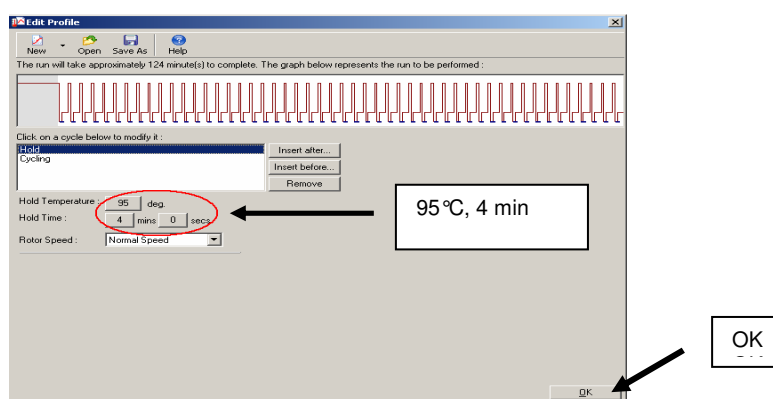
5) Input operator's name, notes, and reaction volume. Subsequently press "Next".



- 6) Enter amplification conditions (see Table 4)
Edit profile



Define step “Initial Denaturation/enzyme activation”



Define cycling conditions, select fluorescence reporter dyes (“FAM” and “JOE/VIC” - data acquired on the FAM and JOE/VIC channels), and select point of fluorescence signal measurement (different settings between Rotor-Gene 3000 and 6000 see Table 4)

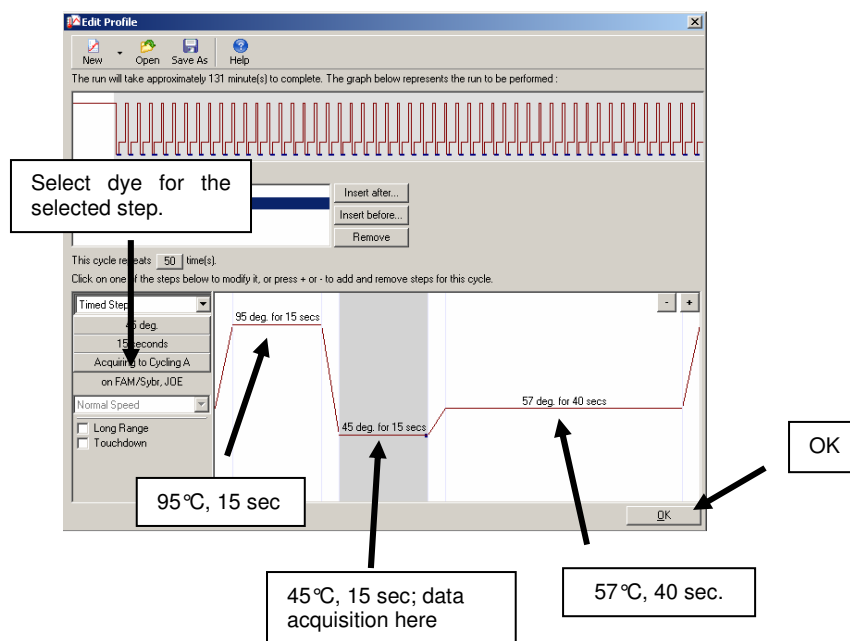


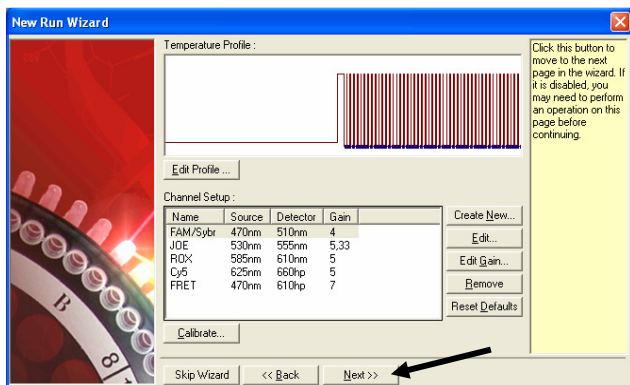
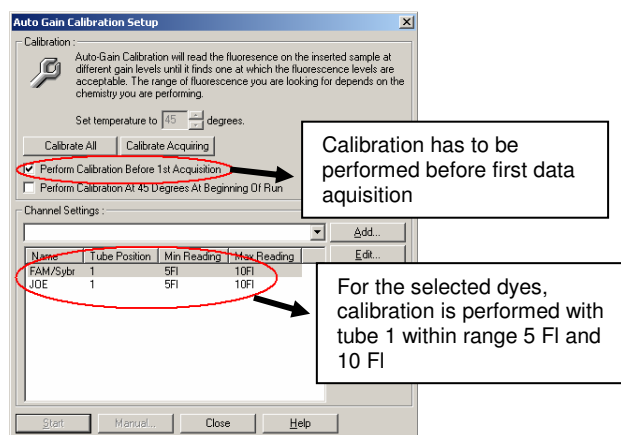
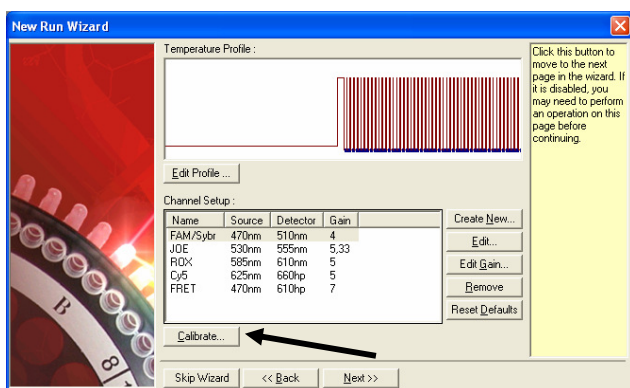
Table 4: Cycling conditions for quantification of HBV DNA using Rotor-Gene

Rotor-Gene™ 3000			
Step	Temperature	Time	Repeat
Taq activation	95 °C	4:00 min	1
Melting	95 °C	0:15 min	50
Stem formation, fluorescence detection (FAM; JOE/VIC)	45 °C	0:15 min	
Annealing / synthesis	57 °C	0:40 min	
Rotor-Gene™ 6000			
Taq activation	95 °C	4:00 min	1
Melting	95 °C	0:20 min	50
Stem formation, fluorescence detection (FAM; JOE/VIC)	45 °C	0:30 min	
Annealing / synthesis	57 °C	0:40 min	

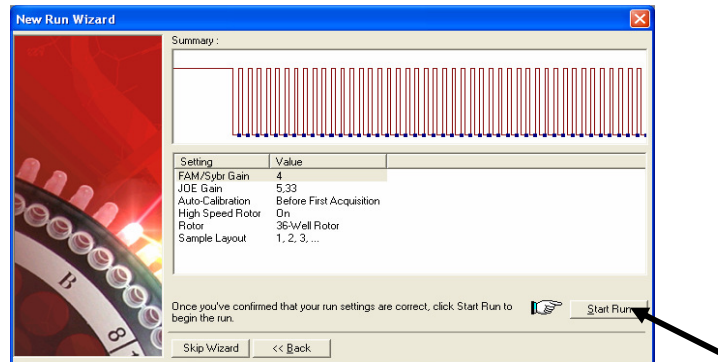
Run time about 2.5 hours

➤ **NOTE** Because the reaction is performed as duplex PCR always select both FAM and JOE/VIC dyes, respectively for each target.

7) Perform Auto-GAIN Calibration of the Rotor-Gene

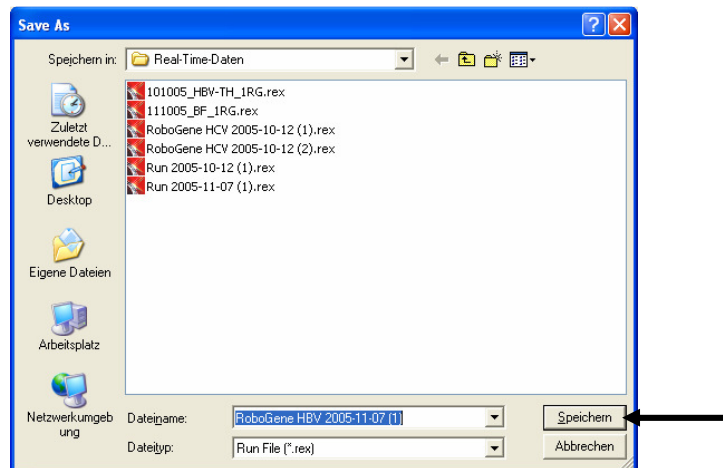


8) Place tubes carefully onto Rotor-Gene™ 3000/6000 rotor, close lid

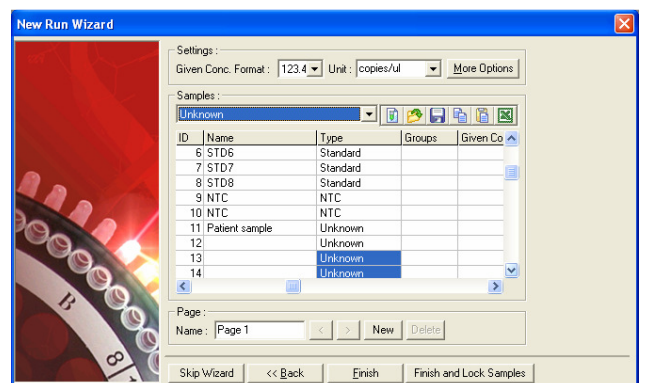
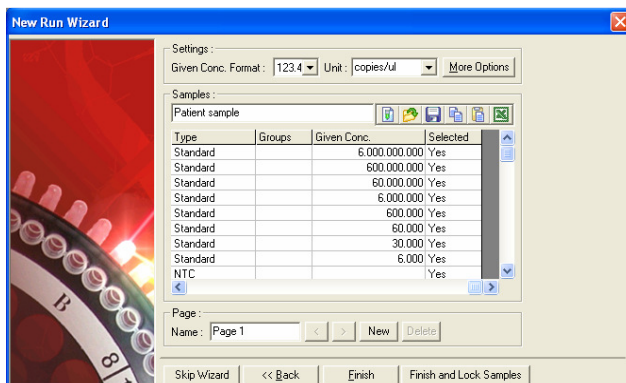


9) Start run

10) Save; run starts automatically



11) Enter sample positions and names (standards with respective amounts, NTC and samples = "unknown")



ABI PRISM® 7000/7300 SDS

1. Preparing 5x reagent mix

Add 200 µl PCR grade water to the vial with lyophilized reagent mix (HBV_D4) and close the lid. Incubate the vial at 37°C for 20 min, mix by vortexing for 3 sec and centrifuge for 5 sec at full speed.

➤ **NOTE** Dissolved reagent mix can be stored at 2-8 °C always protected from light up to 30 days (do not freeze!).

2. Preparing 1x master mix

Table 5: Reagents and volumes for 1x master mix

Reagent	Volume (µl)/Reaction	Final concentration
PCR grade water	9.6	-
10x PCR buffer FS1	2.5	1x
HBV_D4, 5x	5.0	1x
10x ROX	2.5	1X
Taq Polymerase FS1 (5 U/µl)	0.4	2.0 U

Mix by vortexing for 3 sec and centrifuge for 5 sec at full speed.

3. Identify sample tubes (HBV_D2_AB) and quantitation standards (HBV_D3_AB) carefully and place them onto a suitable rack (96-well reaction plate).

➤ **NOTE** Attention should be paid to correct orientation of quantitation standards. Highest HBV DNA amount of a standard is given in the tube labelled with A on top of the strip.

4. Add 20 µl 1x master mix to sample tubes and each tube of quantitation standard.

5. Add 5 µl PCR grade water to the desired amount of sample tubes which serve as NTC and all quantitation standard tubes. Do not exceed a final reaction volume of 25 µl.

6. Add 5 µl of eluate from DNA isolation (e.g. using the INSTANT Virus DNA Kit) to the respective sample tubes. Do not exceed a final reaction volume of 25 µl.

7. Cut optical tape (OT_AB) by using the white cutter blade contained in the box 1 according to the required size and cover sample and quantitation standard strips carefully. **Prevent cut-injuries!** Use of an appropriate applicator for fixing the tape at the tube surface of the strips is recommended.

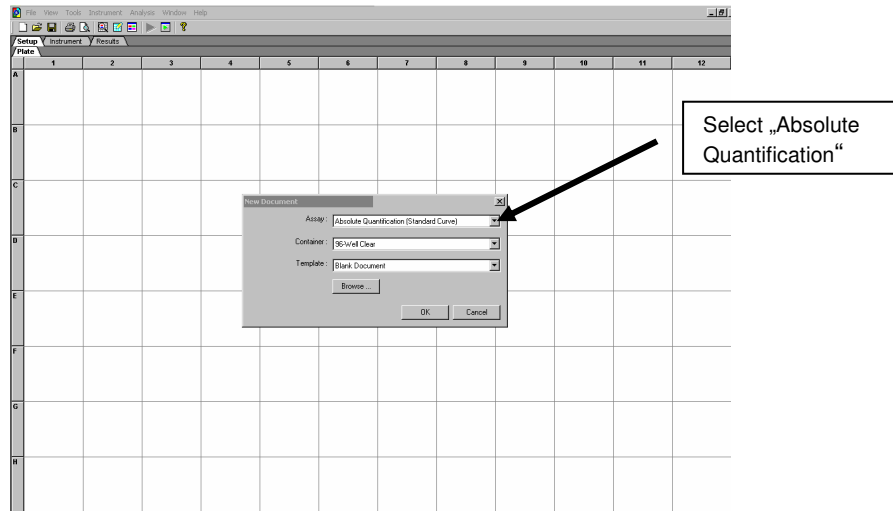
8. Centrifuge rack with loaded strips at 200x g for 1 min.

9. Set up the run:

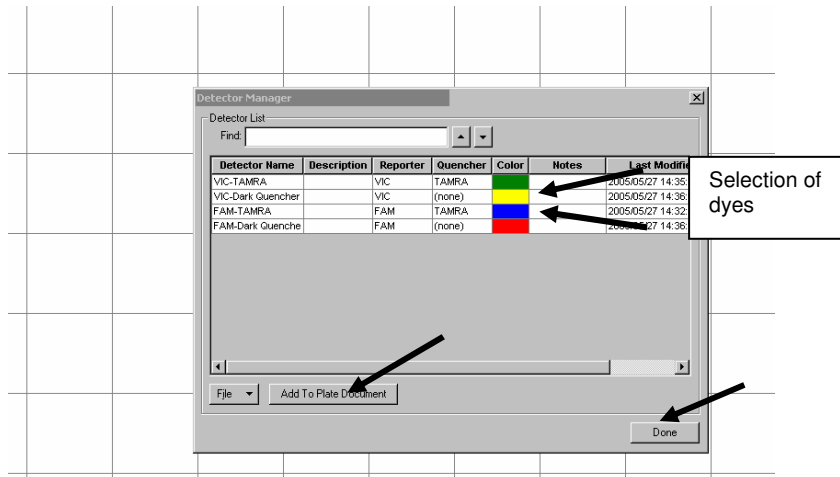
1) Switch on instrument by pressing the main switch, subsequently start ABI PRISM SDS software

2) Start a new run

Go to main menu, select "New", "New Document" from the "File" menu

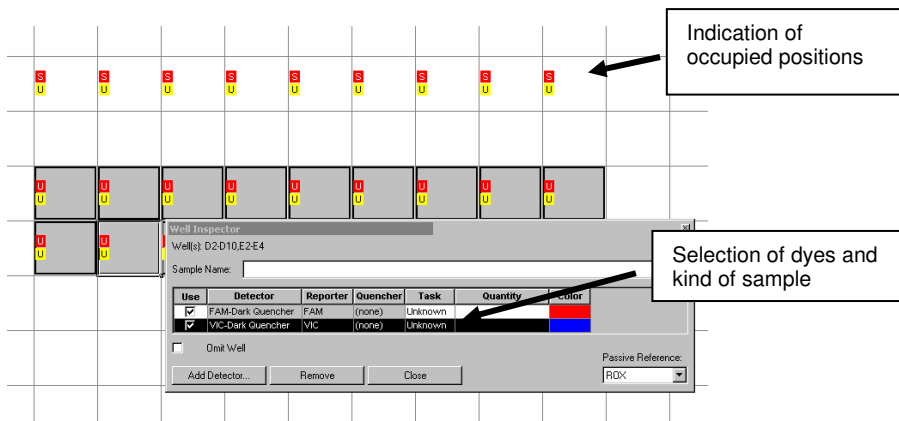


3) Selection of active dyes (ROX as passive reference dye is automatically lodged!)



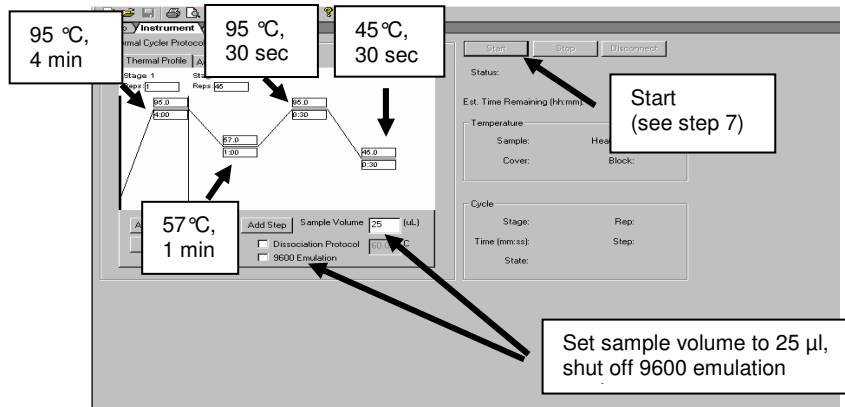
Setup: Main menu -> „Tools“ -> „Detector Manager“ -> Select “VIC-Dark Quencher” and “FAM-Dark Quencher” from detector list
 Transfer run by pressing the button „Add To Plate Document“ and close “Detector Managers” by choosing „Done“.

4) Enter sample positions and names (i.e. standards with respective amounts, NTC and samples = “unknown”), select fluorescence reporter dyes (“FAM” and “VIC” - data acquired on the FAM and VIC channels, respectively) and passive fluorescence dye (ROX)



➤ **NOTE** All sample positions to be measured and dyes require definition before starting the run. Otherwise non-defined positions are not measured. The kind of measured sample (i.e. standard/sample/NTC) may be defined after completing the run.

5) Enter cycling conditions (Stage 2, 3-step-PCR, see Table 6)



➤ **NOTE** Strictly keep the sequence of cycling steps (57 °C -> 95 °C ->45 °C) as shown in the figure above.

6) Save file

Define file name which should contain date, number of run and pathway, go to main menu, select "File" menu, "Save" from the "File" menu.

7) Start run

Place the 96-well reaction plate carefully into the ABI PRISM® 7000/7300 SDS sample block, Cover the plate with compression pad, slide the cover over the block and tighten the lid, start run.

Table 6: Cycling conditions for quantification of HBV DNA using ABI PRISM® 7000/7300 SDS

Step	Temperature	Time	Repeat
shut off « 9600 emulation » (window « instruments »)			
Taq activation	95 °C	4:00 min	1
Synthesis	57 °C	1:00 min	45
Melting	95 °C	0:30 min	
Stem formation, fluorescence detection(FAM; JOE/VIC)	45 °C	0:30 min	

Run time about 2.5 hours

7500 Fast/LP (0.1 ml)

1. Preparing 5x reagent mix

Add 200 µl PCR grade water to the vial with lyophilized reagent mix (HBV_D4) and close the lid. Incubate the vial at 37°C for 20 min, mix by vortexing for 3 sec and centrifuge for 5 sec at full speed.

➤ **NOTE** Dissolved reagent mix can be stored at 2-8 °C always protected from light up to 30 days (do not freeze!).

2. Preparing 1x master mix

Table 7: Reagents and volumes for 1x master mix

Reagent	Volume (µl)/Reaction	Final concentration
PCR grade water	11.85	-
10x PCR buffer FS1	2.5	1x
HBV_D4, 5x	5.0	1x
10x ROX	0.25	0.1x
Taq Polymerase FS1 (5 U/µl)	0.4	2.0 U

Mix by vortexing for 3 sec and centrifuge for 5 sec at full speed.

3. Identify sample tubes (HBV_D2_LP) and quantitation standards (HBV_D3_LP) carefully and place them onto a suitable rack (96-well reaction plate).

➤ **NOTE** Attention should be paid to correct orientation of quantitation standards. Highest HBV DNA amount of a standard is given in the tube labelled with A on top of the strip.

4. Add 20 µl 1x master mix to sample tubes and each tube of quantitation standard.

5. Add 5 µl PCR grade water to the desired amount of sample tubes which serve as NTC and all quantitation standard tubes. Do not exceed a final reaction volume of 25 µl.

6. Add 5 µl of eluate from DNA isolation (e.g. using the INSTANT Virus DNA Kit) to the respective sample tube strips. Do not exceed a final reaction volume of 25 µl.

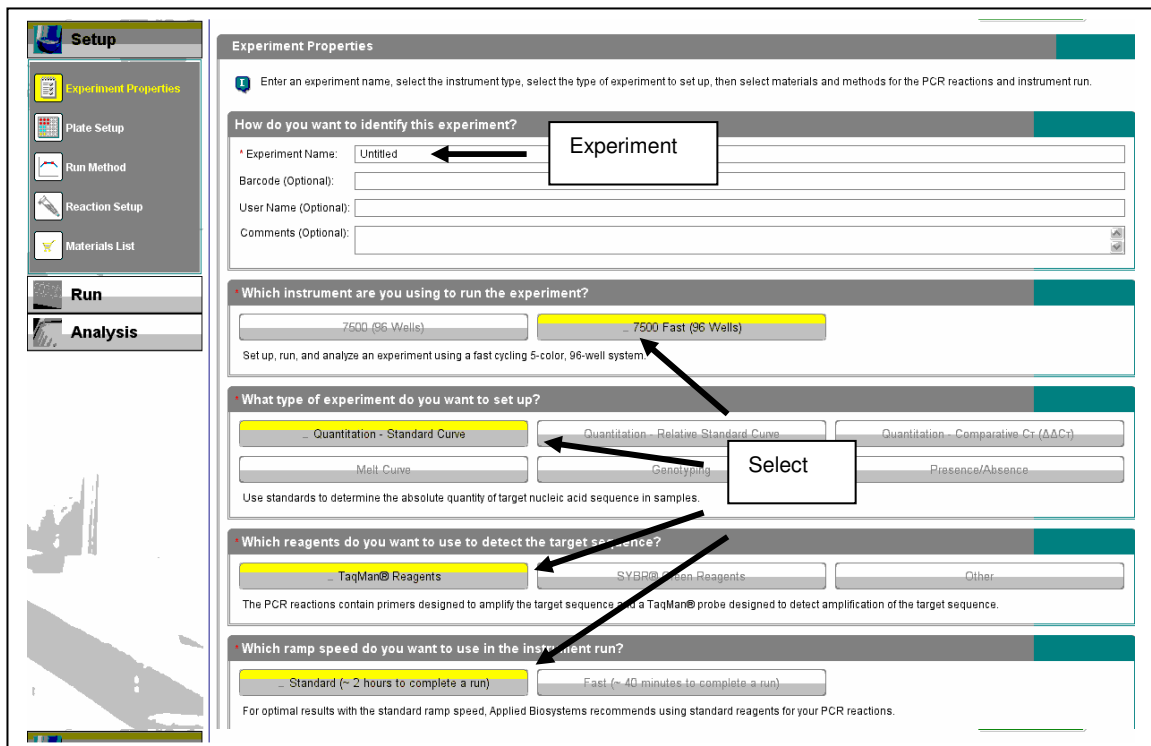
7. Cut optical tape (OT_AB) by using the white cutter blade contained in the box 1 according to the required size and cover sample and quantitation standard strips carefully. **Prevent cut-injuries!** Use of an appropriate applicator for fixing the tape at the tube surface of the strips is recommended.

8. Centrifuge rack with loaded strips at 200x g for 1 min.

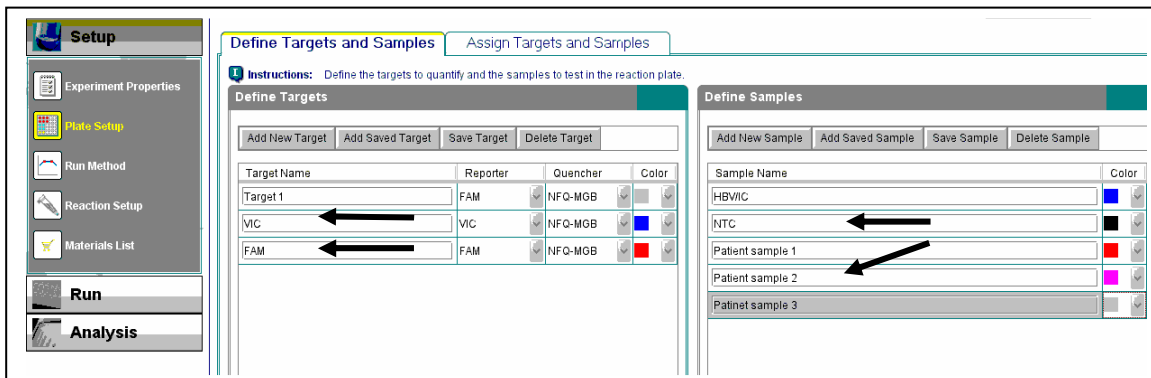
9. Set up the run:

- 1) Switch on instrument by pressing the main switch, subsequently start 7500 Fast Real Time PCR System software
- 2) Start a new run
Go to main menu, select "New Experiment"

- Open “Experiment properties” folder
select buttons “7500 Fast (96 wells)”, “Quantitation Standard Curve”, “TaqMan Reagents”,
“Standard (~ 2 hours to completion)” from the menu; define Experiment Name

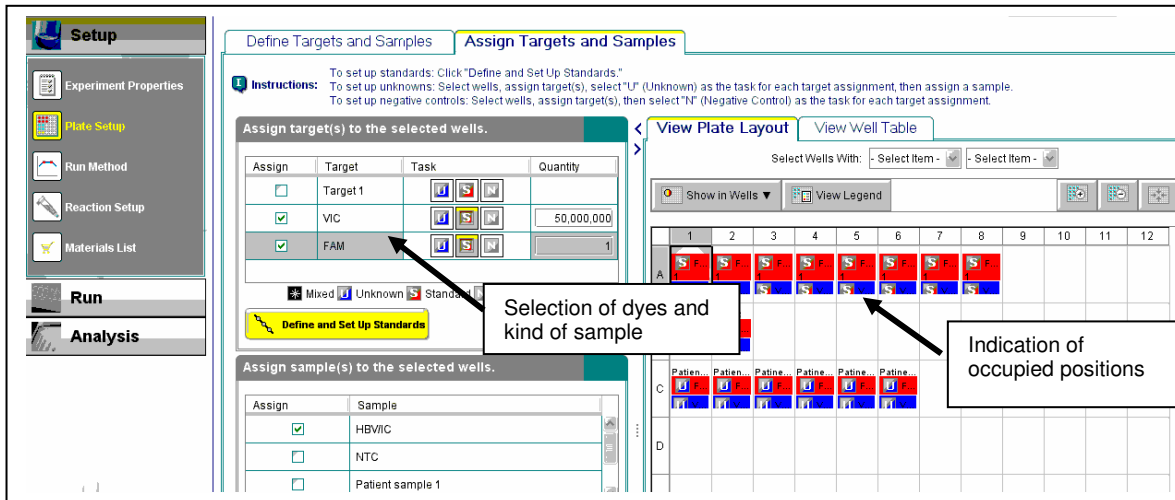


- Open “Plate Setup” folder, define targets and samples

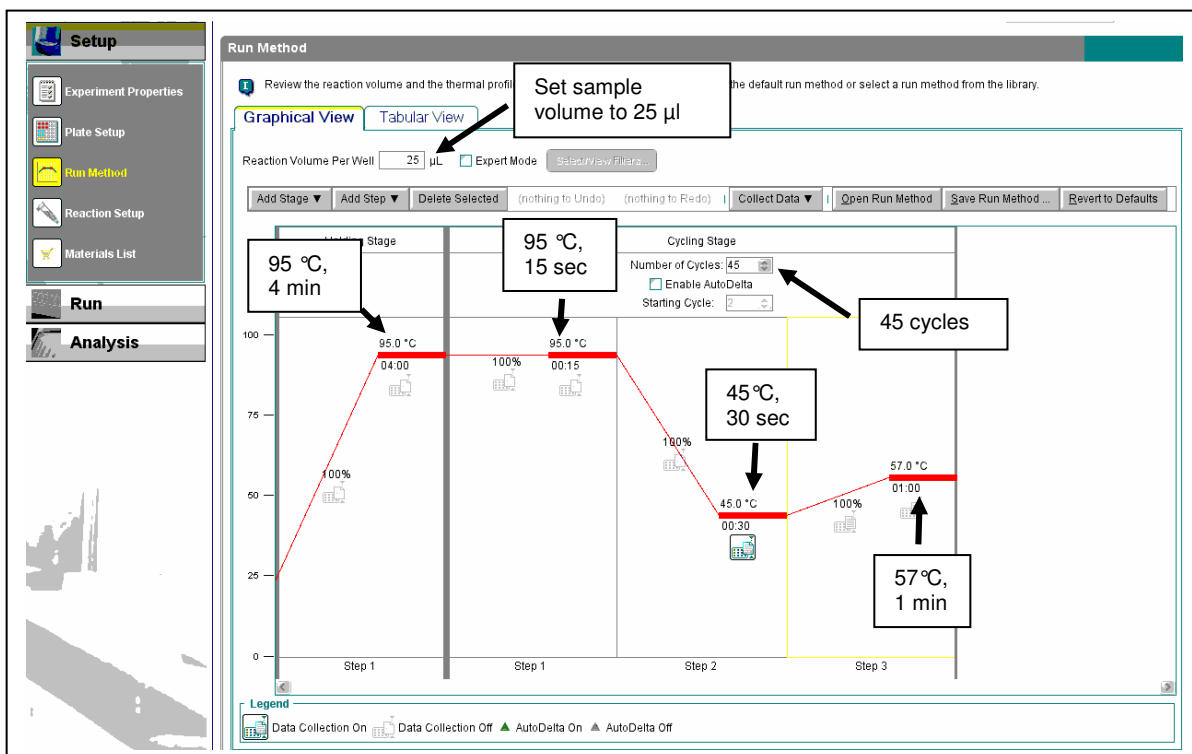


Select “VIC-Dark Quencher” and “FAM-Dark Quencher” from reporter/quencher list, define patient samples, NTC etc.

- 5) Assign targets and samples to the selected wells, define and set up standards and unknowns



- 5) Run method, enter cycling conditions (3-step-PCR, see Table 8), collect data on “hold 45 °C”



- 6) Prepare for the run
 Transfer the reaction strips from the rack to the precision plate holder for tube strips which is provided with the instrument. Open the tray door and place the loaded precision plate holder carefully into the 7500 Fast Real Time PCR System. Close the tray door and start run by pressing the green “Start Run” button.

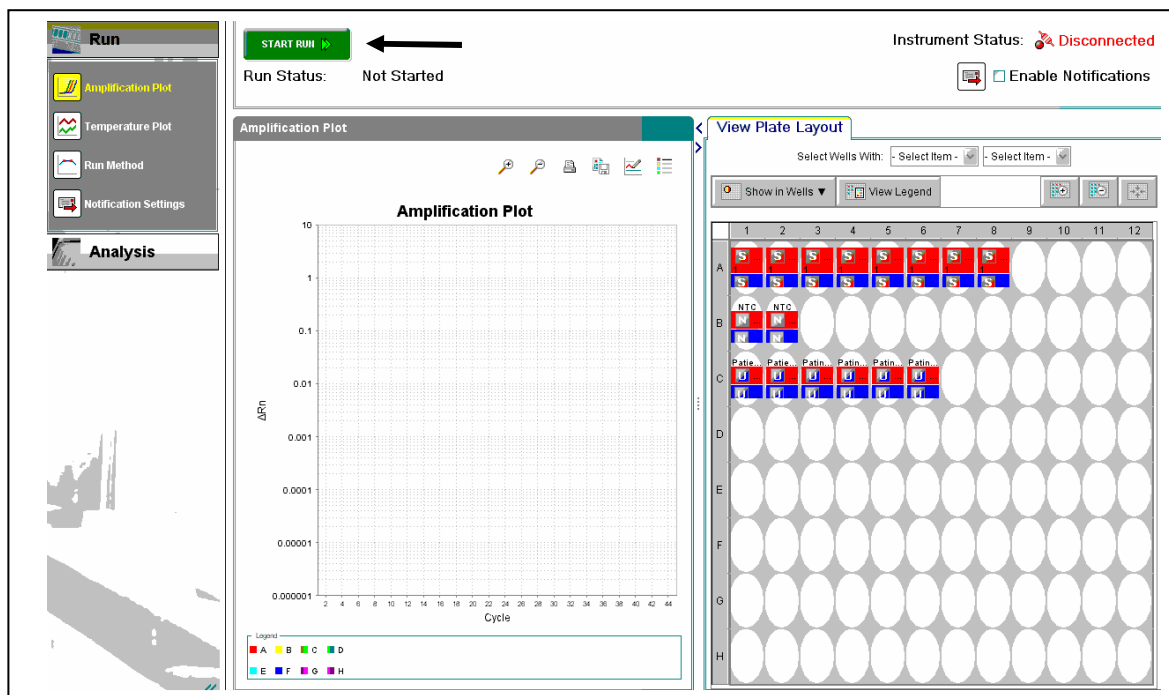


Table 8: Cycling conditions for quantification of HBV DNA using 7500 Fast/LP (0.1 ml)

Step	Temperature	Time	Repeat
Taq activation	95 °C	4:00 min	1
Melting	95 °C	0:15 min	45
Stem formation, fluorescence detection(FAM; JOE/VIC)	45 °C	0:30 min	
Synthesis	57 °C	1:00 min	

Run time about 2 hours, 25 min

Data analysis

Each DNA amplification is associated with generation of a fluorescence signal measurable in FAM channel (for HBV DNA) or in JOE/VIC channel (for IC DNA) resulting in a sigmoid growth curve (log scale). The data analysis is performed according to manufacturer's instructions (Operation manual Rotor-Gene™ 3000/6000, Corbett Research; User guide ABI PRISM® 7000/7300 SDS or Getting Started Guide of 7500/7500 Fast, Applied Biosystems, etc.) using the respective software and considering the recommendations of the following note. Check the obtained data to ensure that the run is valid and to interpret results (see Table 9). HBV DNA is determined based upon the C_T values for the sample HBV DNA and a standard curve resulting from analysis of quantitation standards and the assay specific calibration coefficient. HBV DNA concentration is expressed in IU/ml.

➤ **NOTE**

- Setting of threshold may markedly influence C_T values, set thresholds as follows:

ABI PRISM 7000: select *auto baseline* and set threshold manually to > 0.05

7500 Fast/LP (0.1 ml): select *auto baseline* and set threshold manually to ≥ 0.02

Rotor-Gene: select *Dynamic tube: Yes; Slope correction: Yes; Ignore first: 4; No template control threshold: 20%; Threshold modus: manual, HBV: 0.01-0.03, IC DNA: 0.01-0.04*

- Important criteria for run validation are the slope of the standard curve (see table 10), the range of expected C_T values of the standards (see Table 2.3), and the linear regression coefficient (R^2) of the reference curve which should be between 0.98 and 1.00.
- Due to manufacturing reasons the amplification of 1 standard of the standard strip may fail and should be omitted from calculation. In such case no right for warranty of the whole product may be deduced.

Table 9: Interpretation of the results

Signal in FAM channel	Signal in JOE/VIC channel ²⁾	Interpretation
x	x	valid, quantification of sample HBV DNA
x	-	valid, quantification of sample HBV DNA
-	x	valid, HBV-negative sample
-	-	Invalid, no quantification, repeat run
x ($< 17-100$ IU/ml) ¹⁾	x	below lower limit of quantification range of test
x ($> 1.0 \times 10^{11}$ IU/ml)	x ³⁾	above quantification range of test - dilute original sample on demand with HBV-negative human plasma or serum and test once again

¹⁾ depending on the used virus DNA purification kit and real-time PCR instrument

²⁾ accepted C_T value for IC DNA defined in Table 10

Table 10: Criteria for run validation

Parameter	Rotor-Gene 3000/6000	ABI 7000/7300	7500 Fast/LP (0.1 ml)
Slope standard curve from - to	-2.95 to -3.65	-3.05 to -3.58	-2.87 to -3.51
mean	-3.31	-3.33	-3.29
S.D.	0.13	0.12	0.15
N =	203	63	21
C_T value for IC DNA from - to ³⁾	27 – 34	30 – 37	28 – 33
N =	187	63	21

³⁾ IC DNA specific signal may be declined or missing in presence of HBV DNA concentrations of $> 1 \times 10^5$ IU per ml due to competition as tested with HBV DNA plasma on Rotor-Gene and ABI 7000, respectively.

Troubleshooting

Table 11: Troubleshooting

Problem	Potential cause	Solution
No signal at all	<ul style="list-style-type: none"> • Fluorescence measurement not activated • False channels selected • Incorrect cycling program • Incorrect application of the kit • Storage conditions did not comply with instructions, expiry date of detection kit is exceeded 	<ul style="list-style-type: none"> • Read the user guide • Select FAM channel for HBV DNA and JOE/VIC channel for IC DNA • Check instrument settings • Read instruction manual, • Check storage conditions and expiry date
Low fluorescence signal recorded for both target and IC, target copy number underestimated	<ul style="list-style-type: none"> • Target DNA degraded • Optical lenses contaminated (RG) • Thermal block and/or optics polluted (96-well block format) 	<ul style="list-style-type: none"> • Use DNase free consumables reagents, store DNA after purification on ice and for long time at -20 °C • See chapter “maintenance” of respective instrument brochure, alternatively clean lense once per month using absolute ethanol and cotton swabs • See chapter “maintenance” of respective instrument brochure, alternatively fill each well with isopropanol, incubate 10 min at 50 °C, remove isopropanol and rinse with dd H₂O
No or weak signal for IC DNA in HBV-negative sample	<ul style="list-style-type: none"> • Incorrect cycling program • Excess of inhibitors in the sample/ loss of DNA during extraction • Incorrect sample material (e.g. heparinized or lipaemic plasma or serum) • Wrong sequence of reagent addition to extraction tube • Storage conditions did not comply with instructions, expiry date of detection kit is exceeded 	<ul style="list-style-type: none"> • Compare with protocol (see Tables 4, 6, and 8 respectively) • Use one of the recommended extraction kits and follow exactly manufacturer’s instructions • Request for fresh EDTA blood or serum • Add lysis solution to extraction tubes prior to addition of the sample • Check storage conditions and expiry date
Unexpectedly low C _T values for IC DNA particularly with high standards or high viral load samples	<ul style="list-style-type: none"> • Cross talk between target and IC recording channels 	<ul style="list-style-type: none"> • Calibrate instrument using pure fluorescence dyes
Non-sigmoidal growth curves of quantitation standards, unacceptable high deviation of C _T from expected values	<ul style="list-style-type: none"> • Frequent freezing/thawing or incorrect storage of dissolved reagent mix • Storage conditions did not comply with instructions, expiry date of detection kit is exceeded 	<ul style="list-style-type: none"> • Read instruction manual, check storage conditions, prepare new reagent mix • Check storage conditions and expiry date

Different amplification behaviour of sample HBV DNA and standards, non-parallel growth curves in exponential phase of reaction	<ul style="list-style-type: none"> • Excess of inhibitors in the sample • Incorrect sample material 	<ul style="list-style-type: none"> • Use the recommended extraction kit, follow exactly the manufacturer's instructions; consult attending doctor for patient medication • use correctly taken sample
FAM signal for HBV-negative samples / NTC recorded	<ul style="list-style-type: none"> • Contamination with HBV DNA or DNA amplicons 	<ul style="list-style-type: none"> • Repeat extraction and/or PCR with new reagents; decontaminate instruments and work space

If you have any further questions which are not answered by Table 11, please contact our technical service.

Elimination of PCR carryover contamination (optional)

The reagent mix contains dUTP as substitute for dTTP resulting in amplicons including many uracil bases per molecule. This distinctive feature provides the basis for application of a heat-labile uracil-DNA glycosylase (UDG) protocol to eliminate potential PCR contaminants. For this purpose, UDG has to be added as a normal part of the PCR reaction mixture. We recommend an incubation step with 0.2 U UDG per assay at 37°C for 15 min prior to cycling (1). The initial denaturation step of the first PCR cycle should be extended to 10 min at 94°C to inactivate the UDG and to break the contaminants into small fragments thus preventing amplification.

➤ **NOTE** Do not apply the UDG protocol to quantitation standards which would result in degradation of the HBV quantitation standard DNA stabilized within the tube.

Performance assessment

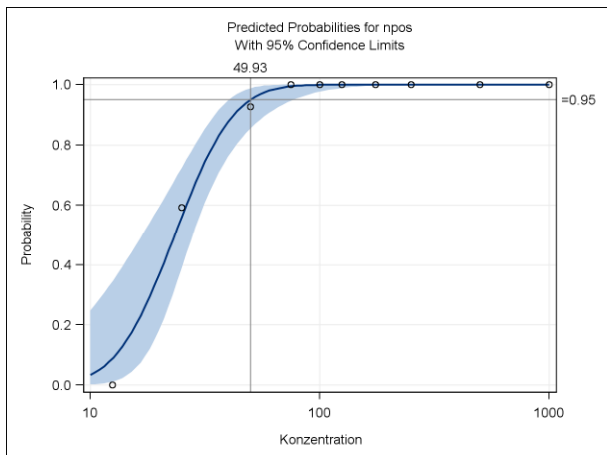
The RoboGene® HBV DNA Quantification Kit was evaluated according to the common technical specifications (CTS) for *in vitro* diagnostic medical devices (2009/108/EC).

Analytical sensitivity

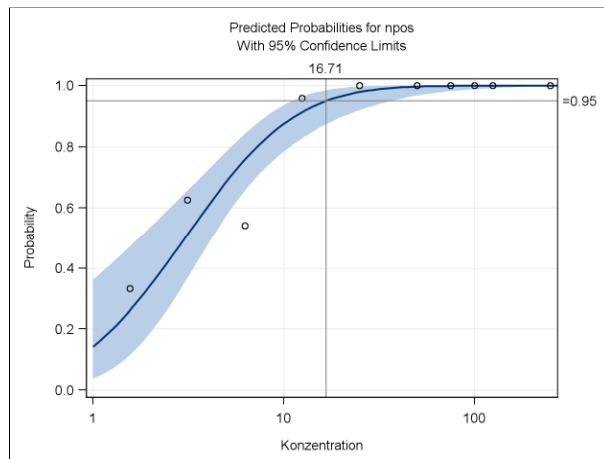
The analytical sensitivity is defined as the smallest amount of target that can be precisely detected in a sample. The analytical detection limit in consideration of DNA purification from the sample is determined using HBV-positive clinical specimens in combination with a particular extraction method. HBV DNA was extracted with either the "INSTANT Virus DNA Kit" (AJ Innuscreen) or the "QIAamp DSP Virus Kit" (QIAGEN) according to the manufacturer's instructions and the recommended elution/sample volumes specified by footnotes of Tables 2.1 and 2.2.

To determine the analytical sensitivity of the RoboGene HBV DNA Quantification Kit, a dilution series of PEI HBV reference plasma (#3620/05; Genotype D; 40.000 IU/vessel) was set up from 0 to 1000 IU/ml of HBV and analyzed with Rotor-Gene, ABI PRISM 7000 and 7500 Fast instruments, respectively. The results were determined by probit analysis. The analytical detection limit of the RoboGene HBV DNA Quantification Kit (Rotor-Gene kit version) in consideration of DNA purified with the INSTANT Virus DNA Kit and the "QIAamp DSP Virus Kit was 50 and 17 IU/ml, respectively (Figure 3 A1 and A2). The analytical detection limit of the kit (ABI PRISM 7000 SDS and 7500 Fast/LP [0.1 ml] kit version) in subject to DNA purified with the INSTANT Virus DNA Kit were 95 IU/ml and 52 IU/ml, respectively (Figure 3 B and C).

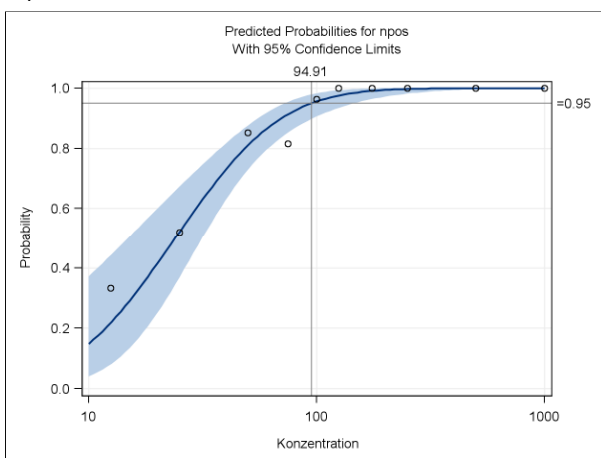
A1)



A2)



B)



C)

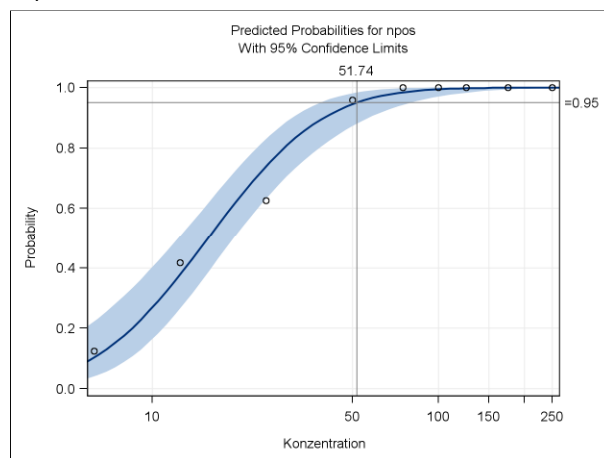


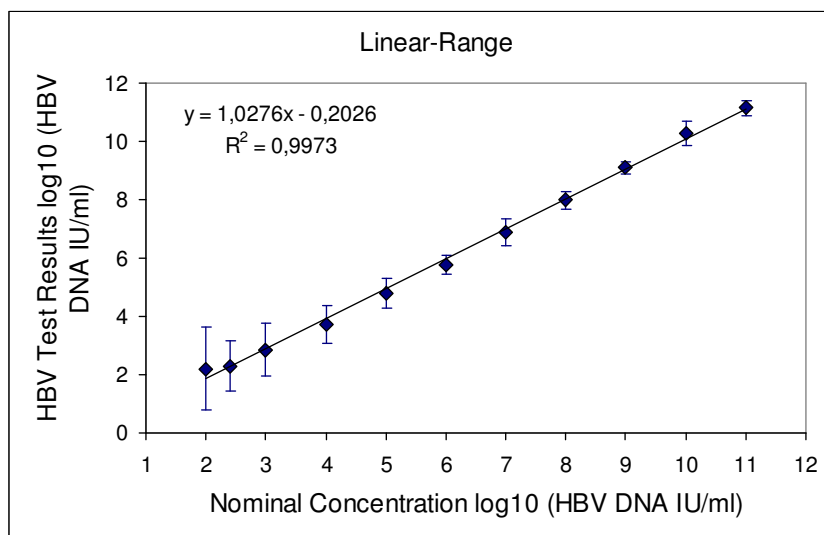
Figure 3: Analytical sensitivity of the RoboGene® HBV DNA Quantification Kit is subject to the real time PCR instrument and purification kit in use. Probit analysis: A) Rotor-Gene 3000, B) ABI PRISM 7000 SDS, C) 7500 Fast System. A1), B) and C): Sample purification performed with the INSTANT Virus DNA Kit.; A2) Sample purification performed with the QIAamp DSP Virus Kit.

Linear range

The linear range of the RoboGene® HBV DNA Quantification Kit was determined by analyzing a dilution series of a HBV quantification standard ranging from 1×10^2 IU/ml to 1×10^{11} IU/ml on both Rotor-Gene 3000 and ABI PRISM 7000 SDS. Each dilution was tested in replicates ($n=10$ for each concentration). The dilution series was calibrated against PEI HBV reference plasma.

The linear range of the RoboGene HBV DNA Quantification Kit was determined to cover concentrations from 1×10^2 to 1×10^{11} IU/ml independently from the real time PCR instrument used.

A)



B)

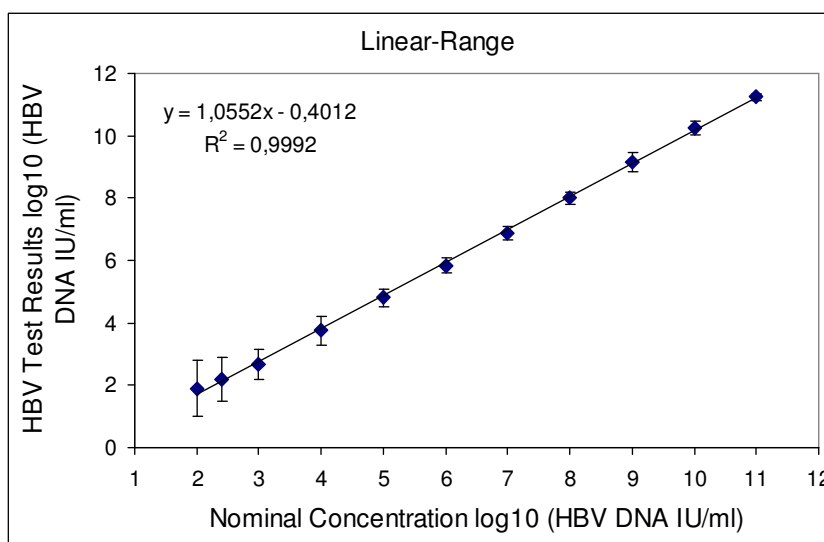


Figure 4: Linearity of the RoboGene® HBV DNA Quantification Kit. The study was performed with synthetic HBV DNA specimen calibrated against PEI HBV reference plasma and 10 replicates at each level on A) Rotor-Gene 3000, and B) ABI PRISM 7000 SDS. The linearity of the assay was >9 logs as determined by a linear regression of the log₁₀ calculated with the log₁₀ nominal concentrations for both used real time PCR instruments. The equation of the respective regression lines is included in the figures.

Specificity

HBV genotype testing

Eight HBV genotypes (A-H) have been defined based on nucleotide divergence within the complete genome (2). Most genotypes have specific geographical distributions and implications on the response to antiviral therapy (3, 4). The performance of the RoboGene® HBV DNA Quantification Kit on HBV genotypes was evaluated with Hepatitis B Virus genotype reference panel 1.1 (Institut für Medizinische Virologie, Justus- Liebig-Universität Giessen, Nationales Konsiliarlabor für Hepatitis B and D), HBV reference plasma, 80.000 IU/ml (Lot #3620/05, Genotyp D, Subtyp ayw2/3, Paul-Ehrlich-Institut, Langen), and genotype H sample was obtained from Teragenix

Corporation, Florida, USA. The study demonstrated that all HBV genotype samples under study were correctly detected (Table 12) and were amplified with similar efficiency thus allowing similar diagnostic evaluation results independent from the analyzed genotype sample (Figure 5).

Table 12: Specificity testing of relevant HBV genotypes

Genotype	Source	Given HBV DNA [IU/ml]	HBV (FAM)	IC-DNA (JOE/VIC)
A	panel 1.1	462,963	+	+
B	panel 1.1	29,630	+	+
C	panel 1.1	88,888	+	+
D	panel 1.1	240,741	+	+
E	panel 1.1	1,203,704	+	+
F	panel 1.1	277,778	+	+
G	panel 1.1	5,370,370	+	+
D	PEI	80,000	+	+
H	Teragenix	128	+	+

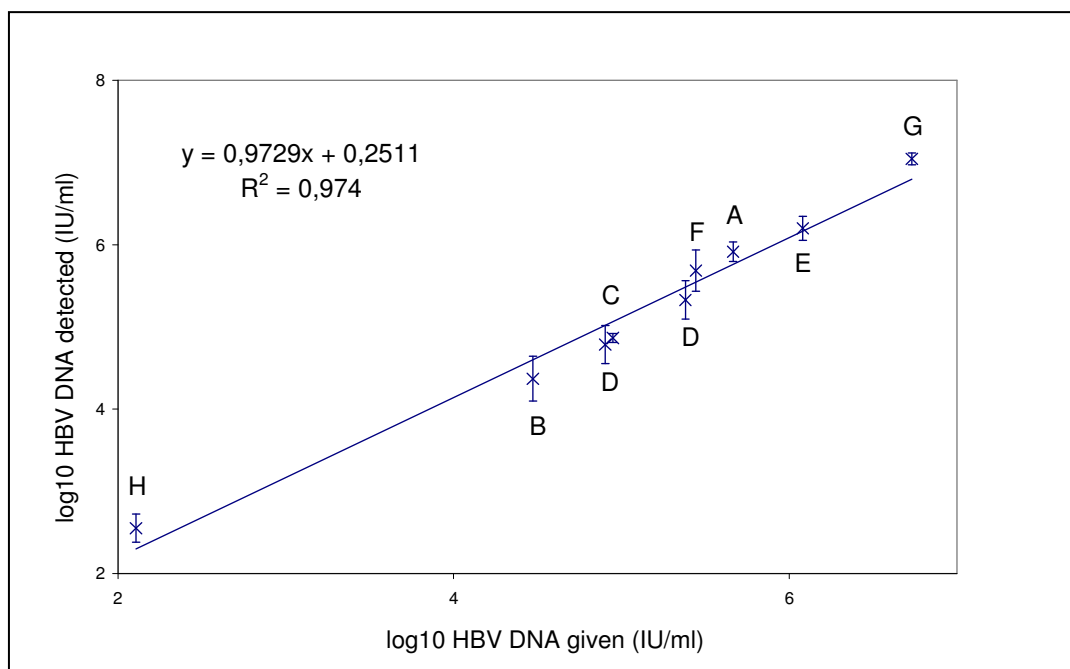


Figure 5. Quantitative detection of relevant HBV genotypes. The RoboGene® HBV DNA Quantification Kit allows correct detection of all tested genotypes as measured on Rotor-Gene 3000. The linearity of the assay was given by inclusion of all genotype samples as determined by a linear regression of the \log_{10} calculated with the \log_{10} nominal concentrations. The equation of the respective regression lines is included in the figure.

Analytical and diagnostic specificity

HBV-negative plasma samples were analyzed to determine the specificity of the RoboGene® HBV DNA Quantification Kit, which is expressed as negative result in absence of the target. The analytical specificity was evaluated by analyzing 16 non-HBV-positive specimens (see Table 13). Furthermore, 108 plasma samples from blood donors which have been tested negative for HBV DNA using the Cobas TaqMan HBV kit were analysed to determine the diagnostic specificity (Table 14). The RoboGene® HBV DNA Quantification Kit had a perfect analytical and diagnostic specificity. None of the analyzed samples gave positive test results for HBV DNA.

Table 13: Pathogen samples used for analysis of analytical specificity

Control group	HBV (FAM)	IC-DNA (JOE/VIC)
DNA virus		
Herpes simplex virus 1 (HSV 1), n=1	-	+
Herpes simplex virus 2 (HSV 2), n=1	-	+
Human cytomegalovirus (HCMV), n=7	-	+
Parvovirus B19 (PVB19), n=1	-	+
RNA virus		
Hepatitis A virus (HAV), n=1	-	+
Hepatitis C virus (HCV), n=4	-	+
Hepatitis D virus (HDV), n=1	-	+
Human immunodeficiency virus 1 (HIV-1), n=1	-	+

Table 14: Diagnostic specificity of the RoboGene® HBV DNA Quantification Kit

Analyzed samples	HBV positive	IC-DNA positive
HBV negative plasma (n=108)	0	108

Precision

The precision data reported here represents the complete test procedure, i.e. plasma samples have been purified with the INSTANT Virus DNA kit and quantified for HBV DNA using the RoboGene® HBV DNA Quantification Kit. Dilution series consisting of 3 different viral load levels, i.e. low (630-800 IU per ml), intermediate ($6.3 - 8.0 \times 10^4$ IU per ml), and high ($6.3 - 8.0 \times 10^6$ IU per ml) were measured by at least 8 replicates of each diluted sample.

Table 15: Inter-assay variability of the RoboGene® HBV DNA Quantification Kit on basis of quantitative results

Factor	HBV DNA	S.D. in log	% CV	within acceptance interval*
different experiments	high	5.98	18.0	yes
	intermediate	4.13	16.1	yes
	low	2.26	20.4	yes
different days of measurement	high	6.24	24.7	yes
	intermediate	4.05	12.6	yes
	low	2.10	11.6	yes
different lots	high	5.99	16.8	yes
	intermediate	3.79	7.4	yes
	low	2.31	23.2	yes
different laboratories and operators	high	6.14	22.3	yes
	intermediate	4.41	47.2	yes
	low	2.51	45.4	yes
different lots in different laboratories	high	5.49	5.4	yes
	intermediate	4.11	24.8	yes
	low	2.24	29.3	yes
different days of measurement in different laboratories	high	6.24	29.9	yes
	intermediate	4.25	36.1	yes
	low	2.32	34.9	yes

* defined as +/- 0.5 log of set point

Robustness

The robustness expresses the total failure rate of the RoboGene® HBV DNA Quantification Kit and was tested for the complete test procedure using the INSTANT Virus DNA kit for sample preparation. Reference plasma diluted to the 3-fold virus concentration of the 95% cut-off value of the test was analyzed with 100 replicates on Rotor-Gene 3000 instrument. Data shows that all tested samples were positive for both HBV DNA and IC-DNA, respectively. Thus the failure rate of the test was 0% (data not shown).

Diagnostic evaluation

The diagnostic sensitivity and linearity of the RoboGene® HBV DNA Quantification Kit was analyzed with 101 HBV DNA positive patient samples and quantitative data were compared with results obtained with the Cobas TaqMan HBV kit (Roche Diagnostics). The linear regression analysis showed that the results are in the linear range and exhibit a high degree of correlation (Figure 6). By consideration of both the methods inherent imprecision and variability of individual data points (Deming regression analysis) it was shown that all analyzed data points were within the default ± 1 log acceptance interval (not shown).

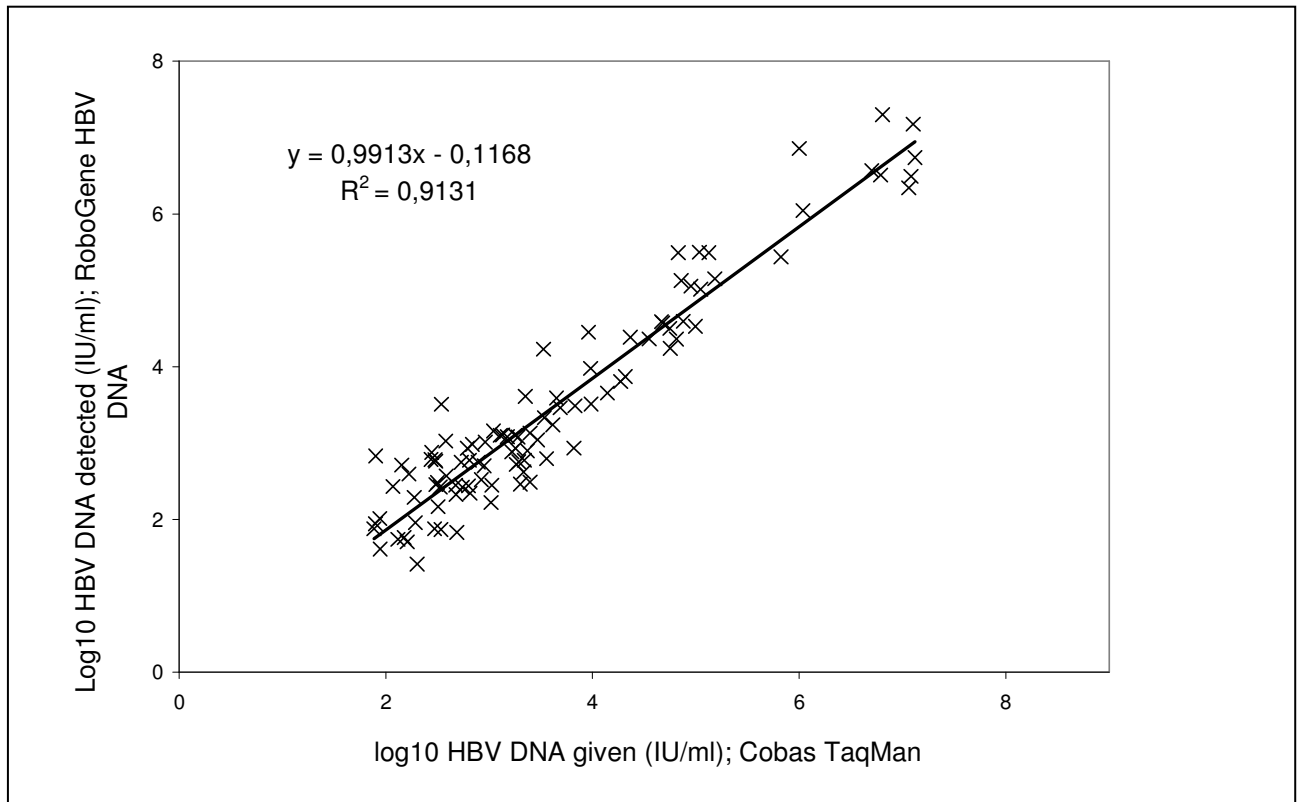


Figure 6: Diagnostic evaluation: comparison of the RoboGene[®] HBV DNA Quantification Kit (sample purification with the INSTANT Virus DNA Kit) with the the Cobas TaqMan HBV kit. The correlation of quantitative results from both tests (n=101) was analyzed by linear regression. The equation of the respective regression lines is included in the figure.

References

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Notes

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