HBV DNA QUANTITATION (QT)

Quantitative Real-Time PCR for detection of HBV genome

4BShopLab

Rev. 2

HBV DNA

A. INTENDED USE

The **HBV DNA Quantitation** Real-Time PCR kit coded **HBVDNAQT.CE** is intended for quantitative detection of Hepatitis B Virus DNA in human plasma with a simultaneous control of the amplification reaction through an **Internal Control (IC).** The kit is intended for use in conjunction with clinical observation and laboratory markers as an indicator of disease prognosis and for use as an aid in assessing viral response to antiviral treatment.
The kit has been adapted for the use on the Real-Time Thermacyclers ABI 7500 Sequence Detection System® (Applied Biosystems™*) software SDS 1.3.1 or MX3000P® software MxPro 4.01(Stratagene™***).

* Applied Biosystems is a registered trademark and ABI PRISM® is a trademark of Applera Corporation or its subsidiaries in the US and/or certain other countries.
***Stratagene is a registered trademark.

B. INTRODUCTION

The hepatitis B virus is a virus belonging to the Hepadnavirus family. Its genome is a small circular, incomplete ds DNA that became a complete and super-coiled genome (cccDNA) when the virus get into the host hepatocytes. Recently eight genotypes are been identified (A through H). The virus has a high speed replicative and high frequency of mutation. This mutations lead to accumulation in each infected individual of multiple genetic variant called quasispecies. Growing evidence shows that the natural history and treatment response may differ depending on the infecting HBV genotype. However the real correlation between genotype and outcome of disease are not completely understood. Hepatitis B virus infection is a global public health problem concerning 350 million people worldwide. It can evolve to chronicity (CHB) and can be the cause of liver disease or hepatocellular carcinoma (HCC). At the present time some observation showed that the severity of liver injury during the infection, is modulated by the strength of host immune response. According on what It is possible to recognize two type of infection: acute and chronic.
In acute HBV infection hepatitis B *surface* antigen (HBsAg), *core* IgM antigen (HBcIgM), *e* antigen (HBeAg) are detectable in serum but ALT do not increase until the infection is well established. Levels of HBV DNA are generally very high ranging from 200 million UI/ml to 200 billion UI/ml.
Chronic HBV infection may present 5 phases:1) Immune tolerance (high levels of HBeAg, HBsAg and HBV DNA-high rate of viral replication); 2) Immune reactive (HBeAg/HBsAg positive, high or low levels HBV DNA in relation of perinatally or adulthood infection, ALT fluctuating); 3) Inactive carrier state (seroconversion HBeAg vs HBeAb, HBsAg positive, normal ALT, very low or undetectable HBV DNA <3log); 4) HBeAg-negative Chronic Hepatitis (HBeAg negative/HBeAb positive or reversion to HBeAg positive, fluctuating levels of HBV DNA, increased or fluctuating ALT. The lack of HBeAg in some patients, in this phase, is related to a mutation in HBV genome;5) past infection (HBsAb positive, HBsAg negative, normal ALT, negative or very low levels of HBV DNA <2.3log).
The diagnosis and clinical monitoring of HBV infection is based on the detection of immunological, serological markers and circulating viral genome (HBV DNA), as indicated above. Serum or plasma HBV DNA levels reflecting viral replication and potential infectivity, so that quantification for HBV viral load is important in the evaluation and management of patients with chronic HBV infection. The new EASL guidelines recommend HBV viral load to determine which chronic patients should be treated and what therapy to apply (interferon or NUC-nucleosite analogue). The main goal of all therapies is the reduction of HBV DNA below the 2.3log in such a way that the progression to liver cirrhosis or HCC is countered.

C. PRINCIPLE OF THE TEST

The HBVDNAQT.CE Kit uses the Real Time PCR technology to detected and quantify HBV DNA in the clinical plasma samples.

The specificity of the assay is first ensured by the selection of specific primers and probes as well as the selection of stringent reaction conditions.

HBV DNA, recovered from the plasma sample is extracted and amplified together with the unrelated sequence (**IC**) introduced into the each specimen at the beginning of sample preparation. This IC serves to demonstrate that the whole process proceeds properly for each one.
The amplified product is detected and quantified, against the standard curve, using a fluorescent reporter dye probe specific for HBV sequence. The construction of standard curve allow the determination of the viral load.
The assay is standardized against the World Health organization (WHO) 2nd International Standard for Hepatitis B virus (NIBSC Code 97/750). Results are reported in Units per millilitre (UI/ml) or copies/ml.
This assay is not intended for use as a screening test for HBV or as a diagnostic test to confirm the presence of HBV infection.

D. COMPONENTS

The standard format of the product code HBVDNAQT.CE contains reagents for 50 tests.

Component	Labelling and Contents	HBVDNAQT.CE 50 Reactions
A **CODED: ALL/MM** **Colour code : blue**	Master mix	N° 2 / 0.40 ml
B **CODED: HBV/CB** **Colour code : yellow**	Lyophilised Primers/Probes	N° 2 (Dissolve with the ALL/C indicated on the vial label)
C **CODED: ALL/C** **Colour code : red**	MG Water	N° 3 vials/1.5 ml
NTC **CODED: ALL/NTC** **Colour code : white**	Negative control	N° 1 vials /1.5 ml
STD **CODED: HBV/STD** **Colour code : red**	Lyophilised Standard Curve Calibrator (2.0E+05 UI/µl)	N° 4 (Dissolve with the ALL/C indicated on the vial label)
I.C. **CODED: ALL/IC** **Colour code: green**	Lyophilized Internal Control	N° 2 (Dissolve with the ALL/C indicated on the vial label
Package Insert	Instruction for Use	N° 1

Important note: Upon request, Dia.Pro can supply reagents for 25, 100, 150 tests, as reported below **:**

Component A	n°1 vial/0.4 ml	n°4 vials/0.4 ml	n°6 vials/0.4 ml
Component B	n°1 vial	n°4 vials	n°6 vials
Component C	n°2 vial/1.5 ml	n°3 vials/1.5 ml	n°5vials/1.5 ml
NTC	n°1 vial/1.5ml	n°1 vials/1.5ml	n°1 vials/1.5ml
IC	n°1 vial	n°4 vials	n°6 vials
STD	n°2 vial	n°4 vials	n°6 vials
Pack. insert	n° 1	n° 1	n° 1
Number of tests	**25**	**100**	**150**
Code	**HBVDNAQT.CE.25**	**HBVDNAQT.CE.100**	**HBVDNAQT.CE.150**

E. STORAGE STABILITY

The kit code HBVDNAQT.CE must be stored at +2…..+8°C.
Once dissolve Component B (coded HBV/CB) and Component I.C. (coded ALL/IC) are stable for 30 days at -20°C. Instead Component STD (Coded HBV/STD) is stable for 15 days at -20°C. Thaw only one time components dissolved.

F.MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (0,5 µl <volume<1000 µl)
2. DNA extraction kit
3. MG EtOH
4. Thermal Block
5. Thermo-Shaker
6. Microcentrifuge
7. Tube and racks for 2 ml and 1.5 ml of volume
8. Sterile filtered tips with aerosol barrier
9. 0,2 ml Microtubes recommended from the Real-Time PCR instruments manufacturers
10. Disposable gloves, powder-free
11. Real-Time PCR Thermalcycler (*)
12. Absorbent paper tissues.
13. Vortex or similar mixing tools.

(*) Attention: A valid calibration of the pure dyes (Pure Spectra Component File) and of the background (Background Component File) is necessary when putting the instruments into operation.

G. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
2. The technical personnel must be deeply trained in the use of Real-Time thermalcyclers, in the manipulation of Molecular Biology reagents and skilled in the Real-Time PCR amplification protocols.
3. The kit has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
4. All the personnel involved in performing the assay have to wear protective laboratory clothes, talc-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
5. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
6. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents, when opening kit vials and the Components and when performing the test.
7. Component A and B are light sensitive. Protect them from strong light exposition
8. Pay **special attention** during the dissolution of the tubes lyophilized observing that the powder present on the walls of the tube, in the case in which the centrifugation was not sufficient, is included in the volume of the water used for dissolution
9. Incorrect dissolution of the lyophilized tube could compromise the result.
10. Avoid vibration of the bench surface where the test is undertaken
11. Upon receipt, store the kit at 2..8°C into a temperature controlled refrigerator or cold room.
12. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.
13. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
14. The extraction step is very important to obtain the correct result. Carefully follow the instruction recommended in the user guide
15. During the extraction some steps are crucial. A key step in the purification procedure is the centrifugation. It is important to obtain the pellet and the supernatant should be clear. The next complete resuspension of the pellet is vital to ensure the maximum nucleic acid recovery.
16. Incorrect centrifugation speed can cause the formation of the pellet which will be difficult to resuspend. The incorrect dissolution of the pellet can cause a mistake in the quantification. In this case refer to "optimization of centrifugation" indicated in the user manual.
17. Caution should be taken to avoid cross-contamination between samples by using disposable tips and changing them after each sample.
18. Prevent cross-contamination between kit reagents by using disposable tips and changing them every-time.
19. Do not use the kit after the expiration date stated on the external container and internal (vials) labels.
20. Treat all specimens as potentially infective. All human plasma specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
21. Store and extract positive materials (specimens, controls and amplicons) separately from the other reagents and use a separate room for their handling
22. Dissolve the lyophilised reagents with the correct amount, stated in the labels, of Molecular Grade water (component C coded:ALL/C) supplied in the kit
23. Carry on all the working operations as quickly as possible
24. Workflow in the laboratory must proceed in an unidirectional way, beginning in the Extraction Area and moving to the Amplification and Data Analysis Area. Do not return samples, equipment and reagents to the area where previous steps have been performed.
25. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.
26. Waste produced during the use of the kit have to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infective material and inactivated before . Do not put in contact the extraction waste with blench.
27. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
28. Other waste materials generated from the use of the kit (example: tips used for samples) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.
29. Monitoring the laboratory for the presence of amplification product. It is recommended to monitor laboratory surface and equipment for contamination.

H. SPECIMEN: PREPARATION AND RECOMMENDATIONS

1.Blood is drawn aseptically by venepuncture and plasma is prepared using standard techniques of preparation of samples for clinical laboratory analysis.
2. The whole peripheral blood samples for DNA extraction must be collected in EDTA according to laboratory procedure, transported and stored at +2/+8°C for a maximum period of 3 days. Do not freeze the whole peripheral blood samples to avoid cell lysis and loss.viral load.
3. No influence has been observed in the preparation of the sample with citrate, EDTA.
Attention: Heparin (≥10 IU/ml) affects the PCR reactions.
Samples, which has been collected in tubes containing heparin as an anticoagulant should not be used. Also, samples of heparinised patients must not be used.
4. Avoid any addition of preservatives to samples.
5. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results.
6.Haemolysed (red) and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
7. Plasma samples with high amount of lipid can cause little or no recovery of nucleic acid. In this case the pellet will be small or not present at all. Take a fresh sample and follow the troubleshooting guide present in the user manual.
8. Plasma, if not used immediately, must be aliquoted and stored at -20°..-80°C after collection. Samples can be stored frozen at 20°..-80°C for several months. Any frozen samples should not be frozen/thawed more than once as this may affect the test result.
9.The plasma samples for DNA extraction must be collected according to the common laboratory procedures, transported and stored at +2/+8°C for a maximum period of 4 hours. The plasma samples can be stored frozen at -20°C for a maximum period of 30 days or -70°C for long periods Avoid repeated freezing/thawing cycles
10.We recommend you, for optimal storage samples, to split them in several aliquots (minimum volume 500 µl, what is necessary for a single session of extraction) and store them frozen at -20°C for a maximum period of 30 days or -70°C for long periods.
11.**Remember that there is a pre-analytical treatment of the plasma sample. All samples must be diluted 1:2 in Phosphate Buffer Solution (500 µl sample + 500 µl PBS) before extraction**.
12.The lack of the step indicated above causes an error of quantification.

13.When using frozen samples, thaw the samples just immediately before the extraction in order to avoid possible causes of nucleic acid degradation.

I. PREPARATION OF COMPONENTS AND WARNINGS

Master Mix:

Component A. Ready to use. Mix well on vortex before use.
WARNING: Component A is light sensitive. Protect it from strong light exposition

Primers/Probes:

Component B.

- Centrifuge the vial for 1 min at 12000 rpm
- Open carefully the vial cap avoiding powder dispersion
- Dissolve homogenously the Lyophilized Component B with the volume of water (Component C coded: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 15 min at room temperature (15°C<RT<25°C)
- Briefly vortex it avoiding the formation of foam

WARNING: Component B is light sensitive. Protect it from strong light exposition

MG Water:

Component C. ready to use

NTC or Negative Control :

ALL/NTC. Ready to use.

Standard Curve:

Component STD.

- Centrifuge the vial for 1 min at 12000 rpm
- Open carefully the vial cap avoiding powder dispersion
- Dissolve homogenously the Lyophilized Component HBV/ STD with the correct volume of water (Component C coded: ALL/C) as indicated on the vial label.
- Keep it dissolve on the benchtop for at least 15 min at room temperature (15°C<RT<25°C)
- Briefly vortex it avoiding the formation of foam
- Prepare 5 Nuclease Free tubes for the preparation of the Standard Curve
- Set up a 1:10 serial dilution in **Component C** (MG water) to obtain the standard curve as table below:

Standard curve preparation		
STD	Calibrator 200000 (UI/µl)	Volume of Component C (MG water) as written on the vial label
STD 1	20000 (UI/µl)	10 µl (STD) + 90 µl Component C (MG water**)**
STD 2	2000 (UI/µl)	10 µl (STD) + 90 µl Component C (MG water**)**
STD 3	200 (UI/µl)	10 µl (STD) + 90 µl Component C (MG water**)**
STD 4	20 (UI/µl)	10 µl (STD) + 90 µl Component C (MG water**)**
STD 5	2 UI/µl	10 µl (STD) + 90 µl Component C (MG water**)**

Internal Control:

Component I.C..

- Centrifuge the vial for 1 min at 12000 rpm
- Open carefully the vial cap avoiding powder dispersion
- Dissolve homogenously the Lyophilized Component IC with the volume of water (Component C coded: ALL/C) indicated on the vial label .
- Keep it dissolve on the benchtop for at least 15 min at room temperature (15°C<RT<25°C)
- Briefly vortex it avoiding the formation of foam

L. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. **Micropipettes:** have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/- 5%. Decontamination of spills or residues of kit components should also be carried out regularly.
2. **Extraction Device:** The HBVDNAQT.CE Kit is intended for the use in combination with QIAamp UltraSense virus Code.53706 (QIAGEN).The end users must strictly follow the Instruction for use supplied by the manufacturer.
3. **Real-Time Thermalcyclers:** The HBVNAQT.CE Kit is intended for the use in combination only with the Real Time Thermal cyclers ABI PRISM 7500 (Applied Biosystems) software SDS version 1.3.1, MX3000P® software MxPro version 4.01 (Stratagene™). The end users must strictly follow the Instruments Instruction for use supplied by the manufacturers.

M. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that the vials of the Lyophilized components is present a well formed aggregate. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box.
3. Dissolve Lyophilized Components with the appropriate amount of Component C (Molecular Grade water) as written on the vial label.
4. Turn the Thermalcycler on, check settings and be sure to use the right assay protocol.
5. Follow strictly the Instruments Manuals supplied by the manufacturers for the correct setting of the Real-Time Thermalcyclers.
6. Check that the micropipettes are set to the required volume.
7. Check that all the other equipment is available and ready to use.
8. In case of problems, do not proceed further with the test and advise the supervisor.

N. ASSAY PROCEDURE

The assay has to be carried out according to what reported here below.

N.1 Viral DNA extraction

The extraction step of the HBV genomic DNA has to be carried out exclusively in combination with the following kit:

Material	Description	Kit code	manufacturer
Plasma	QIAamp UltrSense virus®	53706	Qiagen™

1. Before you begin, setting up as many tubes as there are samples to be extracted
2. Add 500 µl of sterile Phosphate Buffer Solution (PBS) in each tube
3. Add 500 µl of the sample. So that the total volume is 1 ml
4. Add 10 µl of ALL/IC. The IC is added in this step to evaluate if the DNA extraction is performed properly.
5. Proceed with the extraction as indicated in the user guide.
6. The elution volume recommended is 70 µl
7. The DNA isolation must be carried out according to the Manufacturer's instructions and all of our advice.(see Section H)

In the table below we summarize the pre-analytical process

Pre-analytical process				
Sample code	**PBS volume**	**Sample volume**	**ALL/IC volume**	**Total volume**
Assigned defined code	500 µl	500 µl	10 µl	1010 µl

The DNA extracted from the sample and not used in the run has to be stored frozen (-20°C…-80°C).

N.2 Setting up of the reaction

HBVDNAQT.CE kit is intended to be used exclusively in combination with ABI PRISM 7500 standard (Applied Biosystem)) software SDS version 1.3.1, MX3000P® software MxPro version 4.01 (Stratagene™).

N.2.1 Preparing the PCR

Important: *An example of dispensation scheme is reported in Section O. Please, refer to it before starting to read the instructions here below.*

- Prepare the components as described in Section I;
- Prepare the required number of reaction tubes or a 96-well reaction plate for the samples under evaluation and for the Standard curve (prepared as described in section I).

Important note: *Use only optical tubes or microplates suggested by the Real-Time thermalcyclers manufacturers.*

- Consider that the samples , if possible, should be tested in duplicate or triplicate;
- Include at least 1 tube for the NTC (negative control)
- Prepare the **Amplification Mix** for **Samples, NTC and standard curve** as indicated below:

Tab 1-Internal Control (IC) as Amplification control

Preparation of the Amplification Mix

Number of Reactions		1 rxn	10 rxn
A	Master mix	12,5 µl	125 µl
B	Primers/probes	2 µl	20 µl
I.C.	Internal Control	0,5 µl	5 µl
Tot vol.		**15 µl**	**150 µl**

Tab 2-.Internal Control (IC) as Extraction and Amplification control

Preparation of the Amplification Mix

Number of Reactions		1 rxn	10 rxn
A	Master mix	12,5 µl	125 µl
B	Primers/probes	2 µl	20 µl
ALL/C	MG water	0,5 µl	5 µl
Tot vol.		**15 µl**	**150 µl**

In this case (Tab 2) the internal control (IC) is included yet in the sample before extracting.

N.2.2 Amplification procedure

- dispense 15 µl of the amplification mix in each reaction tube or microplate well
- add 10 µl of the **Samples, NTC and Standard curve** to the reaction tubes
- centrifuge briefly reaction tubes at 2000 rpm
- don't leave reaction tubes at room temperature (RT) for more than 30 min and at light exposure
- cover strictly the tubes
- load the tubes in the Real-Time Thermacycler Thermoblock Holder
- after the setting operation described in the section N3 (Instrument Programming) start the Thermoclycler run.

Important note: Component Lyophilized after dissolution in component C (MG water) are stable no more the 3 hours at 2°C….8°C.

At the end of the working day discard adequately the material leftover of the STD Dilution Points.

The not used volume of Component B, STD and IC can be split in aliquots and kept frozen at -20°C.The aliquots must be thaw only one time and must be used as indicated in **Section E**.

N.3 Instrument programming

Programming tools, refers to instruction manuals (Absolute Quantitation Guidelines) provided by the manufacturers

Important note: *For Mx3000P (Stratagene) pay attention to set "filter set gain" as indicated below;*

ROX =x1;FAM=x8;JOE=x1

N.3.1 Thermal Profile

The thermal profile is reported in the table below:

Step	Cycle	Temp.	Time
1	1	50°C	2 min
1	1	95°C	15 min
2	45	95°C	15 sec
		60°C(*)	1 min

IMPORTANT NOTE :(*) step for the real time data collection

Warning*: keep attention to set up the Real-Time Thermalcycler with correct Thermal Profile following the instruments manual supplied by the manufacturer*

N.3.2 Selection of the Detectors

Following the Instruction manuals of the Real-Time thermo cyclers suggested (ABI 7500, and MX3000P®) select the Detectors reported in the table here below:

Detection	Reporter	Quencher
HBV	FAM	Non Present
Internal Control (I.C.)	JOE	Non Present
Passive Reference	ROX	Non Present

O. ASSAY SCHEME

An example of dispensation scheme for Quantitative Analysis is reported below:

Microplate/Tubes

	1	2	3	.	.
A	**STD 1**				
B	**STD 2**				
C	**STD 3**				
D	**STD 4**				
E	**STD 5**				
F	NTC				
G	Sample 1				
H	Sample 2				

Legenda: NTC = Negative Control STD 1,2,3,4,5 = HBV DNA Standard Curve, Sample 1,2, = Samples under evaluation.

P. INTERNAL QUALITY CONTROL

P.1 Pre- Analysis setting

Before starting the interpretation of the data:

- Set the **"Baseline"** (the background fluorescence level) as reported in the table below:

"Baseline"	
ABI ™ PRISM 7500SDS	**Manual Baseline:** Start cycler=3 End cycler=10
MX3000P® (Stratagene™)	**Adaptive baseline** Important note: *Do not use Mx4000 v1.00 to v3.00 algorithm*

- Set manually the FAM/JOE fluorescence **"Threshold"**

"Threshold"	FAM	JOE
ABI ™ PRISM 7500SDS	0.15	0.1
MX3000P® (Stratagene™)	0.15	0.03

P.2 Data Analysis

A check is carried out on the STD 1 calibrator any time the kit is used in order to verify whether its Ct value is as expected and reported in the table below.

Check FAM	ABI7500SDS; Mx3000P
STD1 (20000 UI/µl)	19≤C(Threshold Cycle)≤22

Moreover the Slope and R^2 values are checked in order to verify the quality of the run. The following requirements must be fulfilled.

Check FAM	Requirements
Slope	-3.1 < Slope <-3.9

Check FAM	Requirements
Efficiency	R^2 > 0.98

Q. INTERPRETATION OF RESULTS AND TROUBLESHOOTING

For each samples FAM fluorescence (positive/negative Ct value) and Internal Control JOE fluorescence are assumed to validate HBV detection as described in the table below:

HBV FAM	Internal control JOE	Assay result
SAMPLE POSITIVE	+	CORRECT
	-	CORRECT**
SAMPLE NEGATIVE	Ct<42	CORRECT
	Ct>42 or undetermined	INVALID***

IMPORTANT NOTE:
(**) High initial concentration of HBV DNA in the sample (positive FAM) can lead to REDUCE or ABSENT Fluorescence Signal of Internal Control IC due to the reagent competition
(***) In this case problems have occurred during the amplification step (inefficient or absent amplification) or during the extraction step (presence of inhibitors) which may lead to incorrect result and false negatives. So that it is necessary to repeat the extraction with a new fresh sample.

For each positive sample undergone to the extraction and detected by kit code HBVDNAQT.CE a correct Quantification of the HBV viral load can be applied as reported in the table below:

ABI™PRISM® 7500 SDS - STRATAGENE™ Mx3000P®	
Sample HBV run data (UI/ml)	**HBV viral load (UI/ml)**
Quantity > 2.0E+07	HBV viral load >2.0E+07
5.0E+01< Quantity < 2.0E+07	***QUANTITATION***
Quantity < 5.0E+01	HBV viral load < 5.0E+01

IMPORTANT NOTE: *For samples quantification refer to section R*

The result obtained with this product must be interpreted with consideration of clinical presentation and other laboratory markers inherent to the patient.
The following results are possible:

Troubleshooting table

	FAM	JOE	Result	CHECK
SAMPLE unknown	+	+/-	CORRECT RESULT *Positive*	***IMPORTANT***: High Initial concentration of HBV DNA (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents competition.
SAMPLE unknown	-	-	ATTENTION ! POSSIBILITY OF: Inhibition, error in the procedure or no functioning of the Instruments	1. that components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected FAM for the HBV detection and JOE for the I.C. detection; 4. that the analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitor have been contaminated the tube 7. that the extraction procedures have been executed correctly as indicated **Section N**
SAMPLE unknown	-	+	CORRECT RESULT *Negative*	
STD	+	+/-	CORRECT RESULT	***IMPORTANT***: High Initial concentration of HBV DNA (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents competition.

STD	-	-	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. that the selected detection dyes are corrected FAM for the HBV detection and JOE for the I.C. detection; 4. that the analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitor have been contaminated the tube.
STD	-	+	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. that the selected detection dyes are corrected FAM for the HBV detection and JOE for the I.C. detection; 4. that the analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly.
NTC	-	+	CORRECT RESULT	
NTC	+	+	ATTENTION ! POSSIBILITY OF: Contamination	1. that components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the work space and Instruments are decontaminated at regular intervals; 4.that the kit has been stored correctly;
NTC	+	-	ATTENTION ! POSSIBILITY OF: Contamination	1. that components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. that the work space and Instruments are decontaminated at regular intervals; 4.;that the kit has been stored correctly;

If the results of the test match the *CORRECT RESULT* requirements stated above, proceed to the next section.
If one or more of the problems described in the table above happens, after checking, report any residual problem to the supervisor for further actions.

R. QUANTITATION

The STD calibrators are treated as purified samples and the same volume is used 10 µl.
To generate a standard curve, all five standard calibrators should be used and defined as standard with a specific concentration.
All STD calibrators are defined as UI/µl.
The following equation has to be apply to calculate the **initial concentration** of the samples undergone to the assay:

$$\text{Result (UI/ml)} = \frac{\text{Result obtained (UI/µl) x Elution volume (µl)}}{\text{sample volume (ml)}}$$

Important note:
**as indicated in the Sections H and N the initial sample volume is 0.5 ml.*
Samples quantification can be reported both in UI/ml and copies/ml. **The conversion factor is 2.5 (1UI/ml=2.5 copies/ml).

Example:
Sample volume used to the extraction= 0.5 ml
Result obtained (UI/µl)= 20
Elution volume=70 µl

$$\text{Result (UI/ml)} = \frac{20\ (\text{UI/µl}) \times 70\ (\text{µl})}{0.5\ (\text{ml})}$$

Result (UI/ml)= 2800

Result (copies/ml) = Result (UI/ml) x 2.5 = 2800 x 2.5 = 7000

Important notes:
1. *Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.*
2. *When test results are transmitted from the laboratory to an informatics centre, attention has to be done to avoid erroneous data transfer.*

S. PERFORMANCES

Evaluation of Performances has been conducted in accordance to what reported in the Common Technical Specifications or CTS (art. 5, Chapter 3 of IVD Directive 98/79/EC).
The performance evaluation was carried out in DiaPro's laboratories on materials supplied by the Fondazione Irccs Cà Granda-Ospedale Ospedale Maggiore Policlinico Milano Italy.

S.1 ANALYTICAL SENSITIVITY

Analytical sensitivity of a quantitative molecular method refers to the smallest amount of the target marker that can be properly detected.
In the context of the CTS it may be expressed as: **limit of detection** or **limit of quantification:**
Limit of detection (LOD): it is the lowest **concentration** of the target that can be detected with a 95% of probability.
In the kit code HBVDNAQT.CE the **LOD** it was determined by testing serial dilution of standard curve at limiting concentration which were prepared in HBV negative plasma.
Each dilution was extracted and amplified. A total of 24 replicates, for each one, were tested in three different runs. The results were analyzed by PriProbit program ver 1.63 to obtaining the HBV DNA concentration detected with a **95%** of probability. As the HBVDNAQT.CE can be used in combination with different instruments, we define the LOD for each one. Data are reported below:

Detection Limit (LOD)	
ABI™PRISM® 7500 SDS	**10 UI/ml**
(Stratagene™) MX3000P®	**30 UI/ml**

S.1.1 Dynamic Range and Linearity

Limit of quantification is determined by **Linearity** or **Dynamic range**. **Linearity** is the measure of the degree to which a standard curve approximate a straight line and it is represent by a **Slope** value.
Dynamic range is the span of standard points concentrations for which the final output value (Ct threshold cycle) of system is directly proportional to the concentration for each one.
The boundaries of the measuring range are the lower and upper limits of quantification **(Limit of quantification)**
In the kit code HBVDNAQT.CE the linearity (analytical measurement) was determined by testing in three replicate different concentrations of standard curve ranging from 2.0E+08 UI/µl to 6.24E-0.2 UI/µl. The dilution series has been calibrated against the 2nd WHO International Standard for hepatitis B virus (code:97/750). Date for each instrument are reported in the table 1:

Tab 1

Limit of quantification (analytical measurement)		
instrument	**upper**	**lower**
ABI™PRISM® 7500 SDS	**2.0+08 UI/ µl**	**2.5E-0.1 UI/ µl**
(Stratagene™) MX3000P®	**2.0+08 UI/ µl**	**2.5E-0.1 UI/ µl**

In addition the upper and lower limit of quantification were determined in consideration of the purification step using QIAamp Ultrasense virus Kit. At first were prepared serial dilution points of standard curve in a pool of negative plasma sample ranging from 2.0E+07 UI/ml to 5.0E+01 UI/ml, each dilution was analyzed with two lots. Secondly we analyzed HBV DNA Quantification panel (Acrometrix) in the same way.

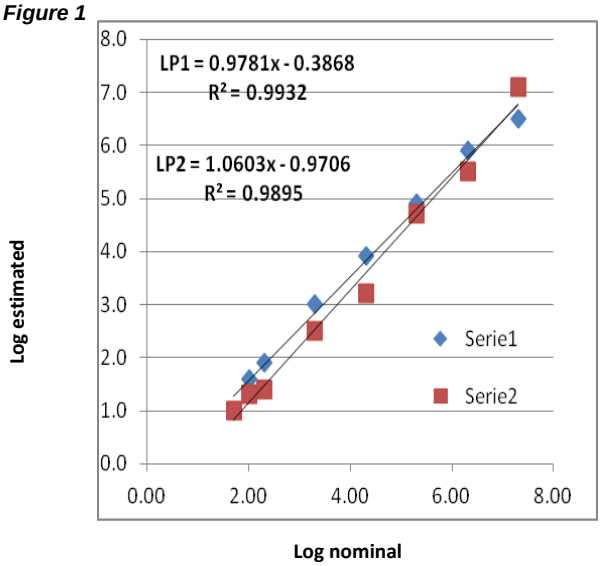
Limits of quantification are reported in the table 2:

Tab 2

Limit of quantification		
Instrument	**Upper limit**	**Lower limit**
ABI™PRISM® 7500 SDS	**2.0+07 UI/ ml**	**5.0E+01 UI/ml**
(Stratagene™) MX3000P®		

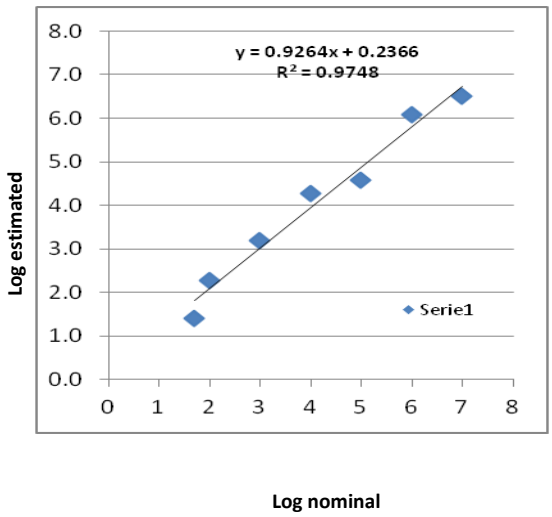
The figure 1 reported below refers to ABI 7500SDS and the **standard curve in negative plasma sample**:

Figure 1



The figure 2 reported below i referes to Mx3000P and to **HBV DNA Quantification panel (Acrometrix).**

Figure 2



The coefficient R^2 show a good correlation. Similar result were obtained by the other instrument.
The upper **limit of quantification for positive samples** is 7.3log (2.0+07 UI/ml) and the lower limit of quantification is 1.69log (5.0+01 UI/ml).

S.2 ANALITYCAL SPECIFICITY

Analytical specificity is the ability of a method to detect and quantify only the target marker.
The analytical specificity of HBV DNA assay has been studied as follow:

1. The primer/probe Set has been choose analysing the genome target sequence with an appropriate software (Primer Express v.3.0" supplied by Applied Biosystem Inc.).
2. The primer/probe Set and the target genome sequence has been controlled by the "BLAST" software, in order to check if any of the nucleotide sequences deposited in the worldwide genomic banks has any homology with HBV, and by the "ClustalX" software, in order to compare the genome target sequences of the different genotypes of HBV.
3. The specificity was improved through the selection of stringent reaction conditions.
4. Plasmas from patients infected with organisms potentially interfering were obtained from Fondazione Irccs Cà Granda-Ospedale Ospedale Maggiore Policlinico Milano –Italy and tested

The results are reported in the following table:

Number of samples	Organism	Result
12	HIV	negative
10	HCV	negative
7	CMV	negative
1	Enterovirus	negative
1	VZV	negative
1	HH6	negative
1	HSV1	negative
1	HSV2	negative
1	EBV	negative

S.3 DIAGNOSTIC SPECIFICITY AND SENSITIVITY

S.3.1 Diagnostic Specificity:
Diagnostic specificity is the probability that the device gives a negative result in the absence of the target marker. So that **true negative** sample is a specimen known to be negative for the target marker and correctly classified by the device

HBV DNA Negative sample

TRUE NEGATIVES	100
FALSE POSITIVES	0
TOTAL SAMPLES	100
SPECIFICITY %	**100**

On the basis of the results obtained Diagnostic Specificity for HBVDNAQT.CE is 100%, so that the system satisfies the acceptance criteria (≥99.5%.)

S.3.2 Diagnostic Sensitivity

Diagnostic sensitivity is the probability that the device gives a positive result in the presence of the target marker. So that **true positive** sample is a specimen known to be positive for the target marker and correctly classified by the device.
In the kit code HBVDNAQT.CE this parameter was studied by examining HBV DNA positive plasma samples supplied by the Fondazione Irccs Cà Granda-Ospedale Ospedale Maggiore Policlinico Milano-Italy, in the same run and then it was been calculated the percentage (%) of positive samples .
The positive samples were tested positive with the kit in use, **Abbott Real Time HBV**, in the laboratory that provided them.

HBV DNA Positive samples

TRUE POSITIVES	100
FALSE NEGATIVES	0
TOTAL SAMPLES	100
SENSITIVITY %	**100**

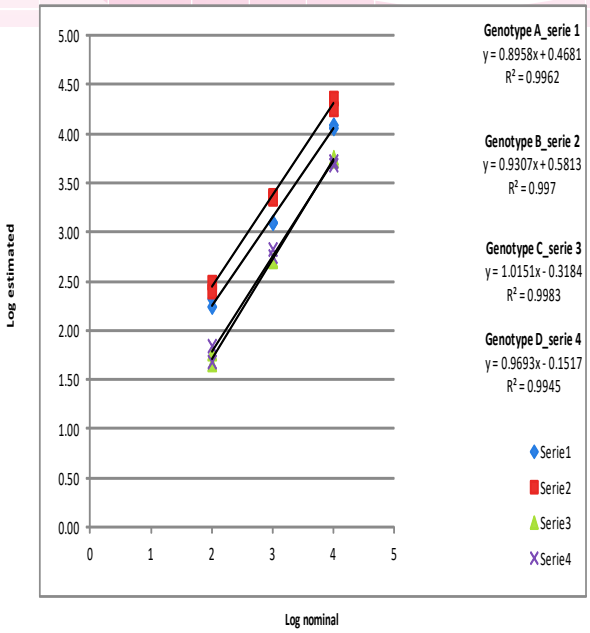
Moreover the Diagnostic Sensitivity of the assay was tested using QCMD Panel (HBVDNA08, HBVDNA10A, HBVDNA11A) in duplicates in different runs. Results obtained were consistent with the expected.
On the basis of the results obtained Diagnostic Sensitivity of the system has been calculated in the 100%.

Diagnostic Sensitivity	100 %
Diagnostic Specificity	≥99.5 %

T. DETECTION AND QUANTIFICATION OF HBV RELEVANT GENOTYPE

According to CTS the kit code HBVDNAQT.CE was evaluated for genotypes detection and quantification efficiency. For the analysis were tested genotypes included in the QCMD Genotyping Panel 2010 (HBVGT10) and 2011 (HBVGT11).
The kit code HBVDNAQT.CE is able to detected all genotypes included in the panels except **Genotype H** (it was detected with less efficiency than expected).

The kit code HBVDNAQT.CE was also evaluated for diluting of relevant genotypes (A, B, C, and D) from 10000 copies/ml (4000 IU/ml) to 100 copies/ml (40 IU/ml).
In the graph is shown regression analysis with the results.



As showed the correlation coefficient ranged from 0.994 to 0.998.

U. PRECISION
Precision shows the degree of the system's reliability. Every measurement procedure has an inherent random variation called "random error". Random error does not have a number value but it is determined by dispersion of measurement as standard deviation (DevST) and coefficient variation (CV%). Usually precision of an assay refers to the agreement between replicate measurements of the same material.
In the kit code HBVDNAQT.CE, **precision** was expressed as intra-assay variability and inter-assay variability. Were tested in the same run (intra-assay) and in three different runs (inter-assay) all 5 standard points curve in 8 replicates for each one.
Intra and inter-assay variability were then calculated.
In absence of an established parameters in the European IVD Directive CTS we have identified the following value of acceptability for the HBV DNA:
Intra-Assay Coefficient Variation (CV%) ≤ 10%.
Inter-Assay Coefficient Variation (CV%) ≤ 10%.

V. LIMITATIONS

1. For in vitro diagnostic use only.
2. Before using this kit is recommended to read carefully and understand the package insert.
3. Strict adherence to the protocol is necessary in order to obtain reliable test results.
4. Optimal performance of this test requires appropriate specimen collection, storage, transport and handling of samples and reagents.
5. The kit is intended to be use only for plasma samples according to our advice
6. Environment laboratory, equipment, instrumentation and reagents must be controlled through good laboratory practise to avoid contamination during all steps starting from extraction to amplification. Low level positive result may occur from cross contamination during processing of specimen with high DNA copy number. In any case per treatment guidelines a 1 log increase is need in order to impact patient management.
7. In addition, treatment guidelines require two consecutive elevated measurements to occur before changing patient management.
8. Follow out a workflow both an accurate pipetting of samples/reagents and appropriate setting of thermocycler instrument.
9. Be careful when entering standards concentration during the set up of the assay. It is advisable to express the

concentrations of standards in UI/µl as indicated in the **Section I**

10. A positive result has extensive medical, social, psychological and economic implications.
11. It is recommended that confidentiality, appropriate counselling and medical evaluation must be considered as an essential aspect of the testing sequence.

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Z.1 SYMBOLS

LEGENDA			
REF	Product code		Storage temperature
IVD	In Vitro Diagnostic Device		See use instructions
LOT	Lot number		Manufacturer
	Expiry date		Number of tests
CE	CE conformity mark		Date of manufacturing

All the IVD Products manufactured by the company are under the control of a certified Quality Management System approved by an EC Notified Body. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

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