HBV Quantification Kit

USER MANUAL

For *in vitro* Diagnostic Use

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Contents

1. PRODUCT DESCRIPTION

Bosphore® HBV Quantification Kit detects and quantitates Hepatitis B Virus DNA in human serum or plasma, encompassing all the HBV genotypes (A-H). The linear range of quantitation is $1x10^1$-$1x10^9$ IU/ml and the analytic sensitivity is 10 IU/ml. A region within the S gene is amplified and fluorescence detection is accomplished using the FAM filter.

An internal control has been integrated into the kit in order to check PCR inhibition. The amplification data of the internal control is detected with the HEX filter. The internal control can be added either during DNA extraction or PCR step.

2. CONTENT

Bosphore® HBV Quantification Kit is composed of Real-Time PCR reagents and quantitation serum standards which have been calibrated against WHO International Standard (NIBSC Code 97/750):

Component	REAGENT	100 Reactions	50 Reactions	25 Reactions
1	dH_2O	(1000 µl)	(500 µl)	(500 µl)
2	PCR Master Mix	(1650 µl)	(825 µl)	(413 µl)
3	Internal Control	(560 µl)	(280 µl)	(140 µl)
4	Positive Control	(44 µl)	(22 µl)	(15 µl)
5	Standard 1 (1×10^6) IU/ml	(880 µl)	(880 µl)	(440 µl)
6	Standard 2 (1×10^5) IU/ml	(880 µl)	(880 µl)	(440 µl)
7	Standard 3 (1×10^4) IU/ml	(880 µl)	(880 µl)	(440 µl)
8	Standard 4 (5×10^2) IU/ml	(880 µl)	(880 µl)	(440 µl)

3. STORAGE

Bosphore® HBV Quantification Kit PCR reagents should be stored at -20°C. Repeated thawing and freezing (>3x) should be avoided since it may reduce sensitivity. If the components are to be used in small amounts, they should be frozen in aliquots.

While preparing the PCR; the components should not be exposed to room temperature for more than 10 min. and the detection mix components should not be exposed to light or air more than necessary, vials must be kept closed except during pipetting. We recommend preparing the PCR on a cooling block and keeping the detection mixes within a closed container.

The components maintain their stability until the expiry dates on the labels, if they are stored at advised conditions.

4. REQUIRED MATERIALS AND DEVICES

- Montania® 483, Montania® 484 or Montania® 4896 Real-Time PCR Instrument (Anatolia Geneworks), or another Real-Time PCR system with FAM and HEX filters (such as iCycler, iQ5–

BioRad, 7500 Real-Time PCR System -ABI, Stratagene Mx3005P, Mx3000P-Agilent, LineGene 9600-Bioer, Rotorgene 6000, Q-Qiagen, LightCycler 480-Roche)

- 0.2 ml Thin-Wall PCR tubes, PCR plates or strips
- Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Viral Nucleic Acid Extraction Kit/ Magrev® 24 stand and Magrev® Viral DNA/RNA Extraction Kit/Magnesia2448 Nucleic Acid Extraction & PCR Setup Robot and Magnesia2448 Viral DNA/RNA Extraction Kit/ Bosphore® Viral DNA Extraction Spin Kit (Anatolia Geneworks) or other high quality viral DNA extraction kits and systems
- Deep freezer (-20°C)
- Desktop centrifuge with rotor for 2 ml. microcentrifuge tubes
- Calibrated adjustable micropipettes
- DNAse, RNAse, pyrogen free micropipette tips with filters
- DNAse, RNAse, pyrogen free 1.5 or 2 ml. microcentrifuge tubes
- Disposable laboratory gloves

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

Important!:

- The product should be delivered on dry ice. Check for presence of dry ice upon arrival.
- Check for the expiry dates on the box and tube labels, upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNAse, RNAse, pyrogen free micropipette tips with filters, and DNAse, RNAse, pyrogen free microcentrifuge tubes should be used.
- Before starting a test procedure, all components should be thoroughly thawed. After thawing, all components should be centrifuged briefly (spin-down for 3-5 seconds), and mixed well to ensure homogeneity prior to use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared, and they should be quickly returned to -20ºC.
- PCR and nucleic acid isolation must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- Pathogen information should be reviewed to be aware of the health related risks.
- Serum and plasma samples should be handled with extreme caution, suitable class microbiological safety cabinet should be used: Physical contact with pathogens should be avoided by; wearing lab coats and gloves, no allowance for eating or drinking within the workspace, prevention of unauthorized individuals' access to the working area.

- All the pathogenic wastes produced during the nucleic acid isolation step; including the serum samples and material contacted with them, should be discarded into medical waste and disposed safely.

6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for in vitro diagnostics.
- This product should be used in accordance with this user manual, by personnel specially trained to perform in vitro diagnostic procedures.

7. PATHOGEN

Causative Agents

Hepatitis B virus (HBV) is one of the smallest enveloped double-stranded DNA viruses, and a member of the Hepadnaviridae family. The replication process of HBV that takes place in liver, is unique among the animal DNA viruses in which reverse transcription is involved. HBV may destroy the liver and cause diseases such as cirrhosis and hepatocellular carcinoma. There are 8 distinctly classified genotypes of hepatitis B virus and further recognized subgenotypes. [1], [2]

Epidemiology

Hepatitis B virus (HBV) infection is a worldwide health problem with the highest burden of disease in Asia, Pacific Islands and Sub-Saharan Africa. There are 2 billion people infected worldwide (one third of world's population), and 400 million suffering from chronic HBV infection (90% of infants and up to 50% of young children infected with hepatitis B will develop chronic infections). HBV infections result in roughly 1 million deaths per year; including the deaths caused by HBV and its complications (HBV-related liver diseases). [3], [4]

Modes of Transmission: Transmission of hepatitis B virus follows the same modes as HIV, but unlike HIV, HBV is 50-100 times more infectious and survives in the open air for at least 7 days. Common modes of transmission are; perinatal (from mothers to infants primarily at birth), early childhood infections (inapparent infection through close contact with infected household), unsafe injection practices, blood transfusions and sexual contact. [4]

8. METHOD

Bosphore® HBV Quantification Kit is based on the Real-Time PCR method. Polymerase chain reaction is a technique that is used for amplification of a DNA region. The reaction occurs by the repeating cycles of heating and cooling. The main components of PCR are primers, dNTPs, Taq polymerase enzyme, buffer solution and template. As a brief explanation, primers are small synthetic DNA those anneal to the specific regions of the template in order to start the synthesis. dNTPs are the building blocks of the amplified products. Taq polymerase amplifies the DNA template. Buffer solution provides the pH adjustment required for the reaction and template, as referred, is the target region for synthesis.

In Real Time PCR technique, in contrast to conventional PCR, PCR product can be monitored during the reaction. Therefore Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, whereby minimizing the risk of contamination. Dual labeled probes employed in the reaction in addition to the conventional PCR reagents, enable detection of the amplified target with increased sensitivity.

The assay utilizes the 5′ exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent probe during the extension phase of PCR. The probe is labeled at the 5′ end with a fluorescent 'reporter' molecule, and at the 3′end with another fluorescent molecule that acts as a 'quencher' for the 'reporter'. When the two fluorophores are in close proximity, and the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step of PCR, Taq Polymerase encounters and cleaves the probe bound to the template. As the reporter is freed from the suppressing effect of the quencher, fluorescence signal can be detected.

The fluorescence generated by the reporter increases as the PCR product is accumulated; the point at which the signal rises above background level and becomes distinguishable, is called the threshold cycle (C_T). There is a linear relationship between the log of the starting amount of a template and its threshold cycle, thus starting amount of unknown templates can be determined using standard curves constructed using C_T values of the known starting amounts of target templates.

Bosphore® HBV Quantification Kit employs multiplex PCR, and an internal control is incorporated into the system in order to control the isolation procedure and to check for possible PCR inhibition. HBV DNA and an internal control are co-amplified in a single reaction, using sequence-specific primers. The fluorescent signal generated by the HBV amplification is detected by a probe labeled at the 3′ end with FAM, through the FAM channel. The fluorescent signal generated by the internal control amplification, is detected by a second probe (labeled at the 5′ end with a different reporter molecule, HEX) through the HEX channel.

9. PROCEDURE

9.1. DNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Viral Nucleic Acid Extraction Kit/ Magrev® 24 stand and Magrev® Viral DNA/RNA Extraction Kit/Magnesia2448 Nucleic Acid Extraction & PCR Setup Robot and Magnesia2448 Viral DNA/RNA Extraction Kit/ Bosphore® Viral DNA Extraction Spin Kit (Anatolia Geneworks) or other high quality viral DNA extraction kits and systems is used with Bosphore® HBV Quantification Kit. The viral DNA isolation should be performed according to the manufacturers' instructions. The amount of internal control that should be used during isolation is 5 µl. The external quantitation standards are provided as serum, so that they undergo the same steps as the patient samples, starting from DNA isolation.

9.2. Kit Components

9.2.1. PCR Master Mix

PCR Master mix contains a highly specific and accurate HotStarTaq DNA Polymerase, the PCR buffer, the dNTP Mix, the HBV-specific forward and reverse primers and a dual-labeled probe, and the internal control-specific forward and reverse primers and a dual-labeled probe.

9.3.2. Internal Control

An internal control is included in the kit to control DNA isolation and PCR inhibition. The internal control is a synthetic DNA molecule. It is added into the serum/plasma, proteinase K and carrier RNA mixture during DNA isolation, to control the isolation efficiency and PCR inhibition. The amount of IC that should be added during isolation is 5 µl per serum sample. Alternatively, the internal control can be added directly into the PCR master mix to control the PCR inhibition exclusively. For this purpose, 0.1 µl of internal control should be added for each reaction into the master mix. Lack of internal control amplification in the FAM negative samples, may indicate a problem in isolation or PCR inhibition. In this case, isolation and PCR should be repeated. In samples that contain a high viral load, internal control can be suppressed and no increase of the signal is detected. Please use the table below for the interpretation of internal control data:

HBV (FAM)	Internal Control (HEX)	Interpretation
+	+	Sample positive
-	+	Sample negative
+	-	Sample positive
-	-	Repeat the test!

9.3.3. Positive Control

The positive control contains HBV DNA. It can be included in the PCR to test the efficiency of the PCR exclusively. The threshold cycle for the positive control is given in the acceptance criteria table (Section 10. Analysis). Threshold cycles higher than the acceptance criteria may indicate an efficiency loss in the reaction.

9.3.4. Quantitation Standards

The quantitation serum standards are calibrated by WHO International Standard (NIBSC Code 97/750).

9.4. Preparing the PCR

All four external quantitation standards should be added into the PCR reaction together with the samples and the negative control. Make sure that all the kit components are thawed before use. Refer to the table below for preparing the PCR.

PCR Master Mix	15 µl
Sample DNA (Standard, Negative/Positive Control)	10 µl
Total Volume	25 µl

Pipette 15 µl of the master mix into the PCR tubes or strips, and add 10 µl of DNA (sample/ standard/positive or negative control). Close the tube cap. Make sure that the solution in each tube is at the bottom of the tube. Centrifuge if necessary.

9.5. Programming the Real-Time PCR Instrument

The thermal protocol for Bosphore® HBV Quantification Kit is composed of an initial denaturation for activation the HotStarTaq DNA Polymerase, a two-step amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle.

Initial denaturation	95°C	14:30 min.	
Denaturation	97°C	00:30 min.	} 50 cycles
Annealing and Synthesis (Data Collection)	54°C	01:30 min.	
Hold	22°C	05:00 min.	

To start a Real-Time PCR reaction using the Bosphore® Kits, the following steps should be completed:

- Choose the filter pairs to be used (FAM and HEX),
- Identify unknown samples, standards, positive and negative controls, assign quantitative values to the standards,
- Select the correct thermal protocol.
- Start the protocol

10. ANALYSIS

By the end of the thermal protocol, the Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold.

Example of an amplification curve is given in Fig. 1.

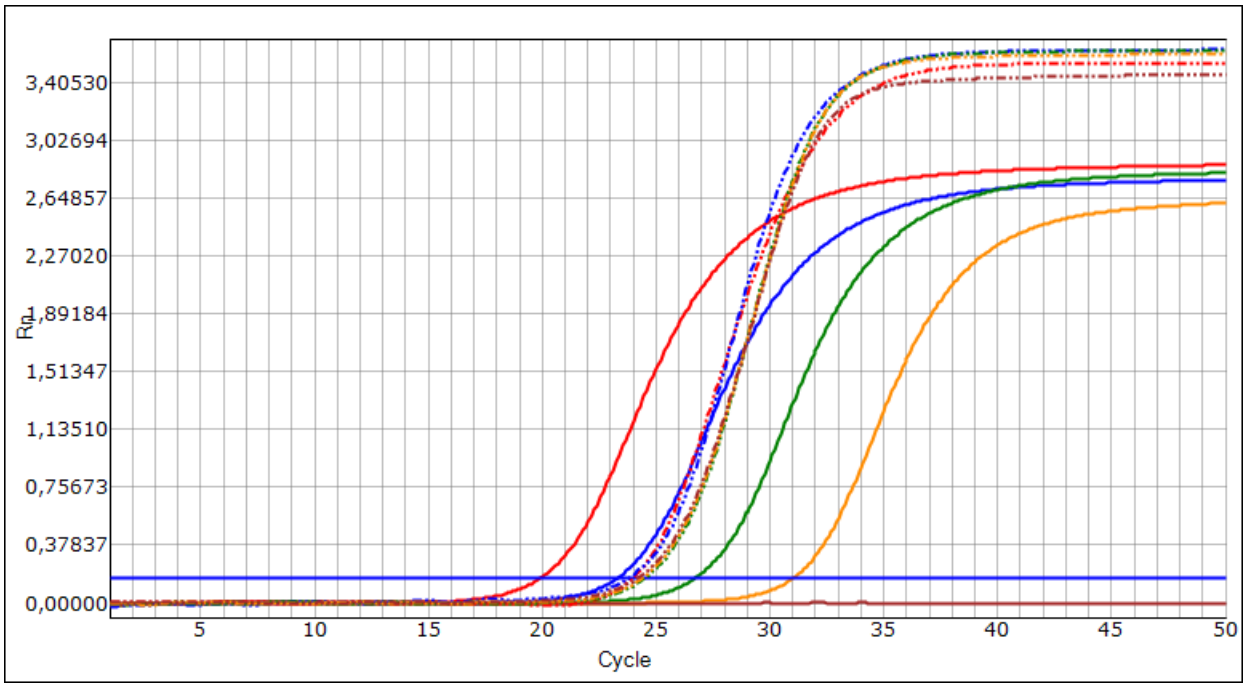

Fig. 1: Amplification Curve of a Bosphore® HBV test

The standard curve is plotted using the data obtained from the defined standards, with the axes Ct-Threshold Cycle and Log Starting Quantity. Example of a standard curve is given in Fig. 2.

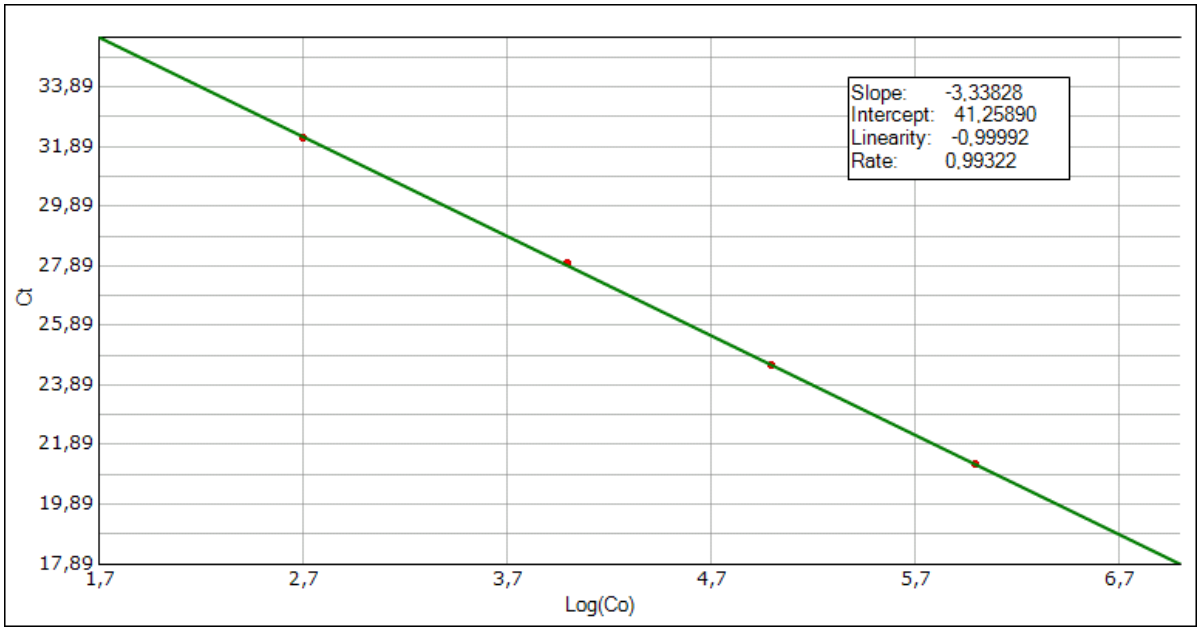

Fig.2: Standard Curve of a Bosphore® HBV test

Analysis of the results should be performed by trained personnel who have received the required training for analyzing Real-Time PCR data. We recommend that the test results must be evaluated by an expert clinician, taking the patient's clinical findings and the results of other tests into consideration.

All analysis is done automatically in routine use. However, when the trained personnel, who have received the required training from manufacturer, consider it as necessary, and if the system's software allows pulling down the threshold as much as possible in order to detect low positive samples, attention should be paid to keep the threshold line above the background and to keep the correlation coefficient at the maximum possible value (and within its acceptance criteria).

The table below displays the acceptance criteria for Bosphore® HBV.

Component/Parameter	Cycle Threshold (C_T)
Standard 1	21.5±2.5
Standard 2	25±2.5
Standard 3	28±2.5
Standard 4	32.5±2.5
Positive Control	28±4
Correlation Coefficient	>0.970
PCR Efficiency*	>%80

Test results should not be reported unless the assay results meet the criteria stated above. Please contact the manufacturer if an impairment in the product's performance is observed (See the last page for contact information).

The samples that cross the threshold in FAM channel are displayed with a calculated starting quantity, samples that do not cut the threshold are displayed as "No Ct". These samples are regarded as negative or

having a viral load below the detection limit of the assay. For these undetectable samples, the HEX data of the internal control should also be checked to avoid false negative results.

The following table shows the possible results and their interpretation:

Signal detected in FAM filter pair	The sample contains HBV DNA, the result is positive	No need to check the internal control since the sample is positive (high positive samples may suppress the signal from the internal control)
No signal in FAM, signal in HEX	The HBV DNA in the sample is not detectable	Signal from HEX filter pair rules out the possibility of PCR inhibition
No signal in FAM and HEX	The diagnosis is inconclusive	No signal in HEX points out to PCR inhibition or to a problem in DNA isolation

In rare cases of PCR inhibition due to medication or other PCR inhibitors in the sample, we recommend to repeat the test of inhibited samples, by freezing and thawing the DNA samples and using them in the PCR after diluting them 1:2 or 1:4 with dH_2O. (Caution: The dilution factor must be taken into account while reporting the Real-Time PCR quantitative results.)

11. SPECIFICATIONS

11.1. Sensitivity

Analytical sensitivity may be expressed as the limit of detection: i.e. the smallest amount of the target marker that can be precisely detected. The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample which can be detected but not necessarily quantitated as an exact value. The analytical sensitivity or detection limit for NAT assays is expressed by the 95% positive cut-off value.

The analytical detection limit for Bosphore® HBV Quantification Kit was found to be $1x10^1$ IU/ml. The sensitivity was determined using serial dilutions of DNA calibrated with the WHO International Standard for HBV DNA NAT assays, (NIBSC Code 97/750). The dilutions were tested in different runs in replicates. The results were analyzed by probit method.

11.2. Genotype Detection

Efficiency of detecting and quantitating different HBV genotypes were ensured both by sequence comparison analysis and Real-Time PCR assays with HBV DNA Genotype Performance Panel PHD 350 (Seracare), and panel members of the QCMD 2010-2015 Hepatitis B virus DNA EQA Programmes, which have been tested with Bosphore® HBV Quantification Kit and were all found positive. These samples contained different HBV genotypes that included HBV A-H. Moreover, 10 positive clinical plasma samples were tested, found positive with Bosphore® HBV Quantification Kit, were sequenced for HBV genome polymerase region and were shown to contain different HBV genotypes.

The following genotypes of the Genotype Performance Panel PHD 350 were tested and found positive:

Panel Member	Genotype	HBV (FAM)
1	D	+

2	D	+
3	F	+
4	C	+
5	A	+
6	E	+
7	E	+
8	E	+
9	C	+
10	F	+
11	G	+
12	H	+
13	A	+
14	B	+
15	B	+

11.3. Linear Range

The linear range of Bosphore® HBV Quantification Kit was determined to be $1x10^1$-$1x10^9$ IU/ml.

In order to assess the linear range, different dilution series including the members of HBV DNA Genotype Performance PHD 350 (Seracare) which has been calibrated against the WHO International Standard for HBV DNA NAT assays, (NIBSC Code 97/750) was analyzed in multiple assays. The standard curve correlation coefficient was found to be 0.999.

11.4. Cross-Reactivity

To eliminate potential cross-reactivity, both assay design evidence and experimental studies were employed. Primer and probe sequences were checked for possible homology to other known pathogen sequences by sequence comparison analysis using database alignment. Samples of CMV, EBV, MTBC, Parvovirus B19, BKV with known high positivity were tested, and found negative.

11.5. Reproducibility

Reproducibility data (on C_T value basis) were obtained by the analysis of one of the quantitation standards of the Bosphore® HBV Quantification Kit. Test was performed in at least 4 replicates by 3 different operators, on multiple days, using 3 different lots. The resulting data is given in Table 1:

Table 1: Reproducibility Data.

HBV (10^4 IU/ml)	**Standard deviation**	**Variance**	**Coefficient of variation [%]**
Intra-assay Variability N=4	0.07	0.005	0.23
Inter-lot Variability N=3	0.10	0.01	0.34
Inter-operator Variability N=3	0.28	0.08	0.91

Total Inter-assay Variability N=5	0.29	0.08	0.93

11.6. Diagnostic Evaluation

The diagnostic evaluation was performed by testing totally 138 HBV negative and 153 HBV positive serum or plasma samples which have been previously analyzed using HBV DNA Amplicor Monitor Test v2.0 Quantitative, COBAS HBV DNA Amplicor Monitor Quantitative, Diasorin ETI-MAK2 HBsAg Plus Assay, Artus® HBV QS-RGQ Kit (Qiagen GmbH), or Abbott RealTime HBV Amplification Reagent Kit (Abbott), were tested with Bosphore® HBV Quantification Kit. All the negative samples were found negative and all the positive samples were found to be positive, including the sample that could only be detected as positive by Diasorin ETI-MAK2 HBsAg Plus Assay, but was noted as below detection limit using HBV DNA Amplicor Monitor Test v2.0 Quantitative, and COBAS HBV DNA Amplicor Monitor Quantitative.

11.7. Calibration Against WHO Standard

HBV Quantitation Standards were calibrated against the WHO International Standards for HBV DNA NAT assays, (NIBSC Code 97/750 and 10/264) by performing multiple assays using various dilutions and testing them in replicates. 1 IU was found to be equal to 4.5 ± 0,2 copies/ml .

12. REFERENCES

1. K. E. Nelson, C. Williams, and N. Graham., Infectious Disease Epidemiology: Theory and Practice, July 15, 2000 , p :907-921
2. Barbara Rehermann and Michelina Nascimbeni, Immunology of Hepatitis B virus and Hepatitis C virus Infection , NATURE REVIEWS , Volume 5, March 2005, p: 215-229
3. Hepatitis B Fact Sheet No. 204. *2008, World* Health Organization.
4. Jinlin Hou, Zhihua Liu, and Fan Gu, Epidemiology and Prevention of Hepatitis B Virus Infection, Int. J. Med. Sci. 2005 2(1),p: 50-57

13. SYMBOLS

 Use-by date

 Batch Code

 Catalogue number

 Temperature limit

 Caution

 Manufacturer

 In Vitro Diagnostic Medical Device

14. ORDERING INFORMATION

Catalog Number: ABHBQ1 (25 rxn/box)
ABHBQ2 (50 rxn/box)
ABHBQ3 (100 rxn/box)

15. CONTACT INFORMATION

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