HDV Quantification-Detection Kit v1

USER MANUAL

For *in vitro* Diagnostic Use

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Contents

1. PRODUCT DESCRIPTION

Bosphore® HDV Quantification-Detection Kit v1 detects both qualitatively and quantitatively the Hepatitis D Virus RNA in human plasma or serum, encompassing all HDV genotypes (1-8). The linear range of quantitation is from $1x10^2$ copies/ml to $1x10^8$ copies/ml, and the analytic sensitivity is 45 copies/ml. A region within the structural antigen 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 extraction problems and PCR inhibition. The amplification data of the internal control is detected with the HEX filter. The internal control is added either in the extraction or during RT-PCR step.

2. CONTENT

Bosphore® HDV RNA Quantification-Detection Kit v1 is composed of Real-Time RT PCR reagents and quantitation plasmid standards:

No	Component	100 Reactions	50 Reactions	25 Reactions
1	dH_2O	(500 µl)	(500µl)	(500 µl)
2	PCR Master Mix	(1600 µl)	(800 µl)	(400 µl)
3	RT Mix	(29 µl)	(14.5 µl)	(7.5 µl)
4	Internal Control	(560 µl)	(280 µl)	(140 µl)
5	Positive Control	(44 µl)	(22 µl)	(15 µl)
6	Standard 1 (10.000 copies/µl)*	(88 µl)	(44 µl)	(22 µl)
7	Standard 2 (1.000 copies/µl)*	(88 µl)	(44 µl)	(22 µl)
8	Standard 3 (100 copies/µl)*	(88 µl)	(44 µl)	(22 µl)
9	Standard 4 (20 copies/µl)*	(88 µl)	(44 µl)	(22 µl)

*See section "Analysis" Section for RNA quantitation

3. STORAGE

Bosphore® HDV Quantification-Detection Kit v1 PCR reagents should be stored at -20°C. Repeated thawing and freezing (more than 3 times) 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 and 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 recommended 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 two filters; FAM and HEX filters (such as iCycler, iQ5, CFX–BioRad, LightCycler 480-Roche, 7500 Real-Time PCR System, ABI, Stratagene Mx3005P, Mx3000P-Agilent, ABI, m2000 RealTime System-Abbott Molecular, LineGeneK, LineGene 9600-Bioer, Rotorgene 6000, Q-Qiagen, SmartCycler Real-Time Thermal Cycler- Cepheid)
- Magnesia® 16 Nucleic Acid Extraction System and Magnesia® Viral Nucleic Acid Extraction Kit, or Magrev®24 stand and Magrev® Viral DNA/RNA Extraction Kit, or Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot and Magnesia® 2448 Viral DNA/RNA Extraction Kit, or Bosphore® Viral

RNA Extraction Spin Kit (Anatolia Geneworks), EZ1 Advanced XL and EZ1 Virus Mini Kit v2.0 (Qiagen), NucliSENS® easyMAG® (bioMérieux) or other high quality viral RNA extraction kits and systems

- DNAse, RNAse, pyrogen free 0.2 ml Thin-Wall PCR tubes, PCR strips or PCR plates
- 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 -20°C.
- 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.
- Biological samples should be handled with extreme caution: 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/plasma samples and material contacted with them, should be discarded into medical waste and disposed safely.
- This product is not harmful and pathogenic, the kit contains materials of human origin but not infective.

6. PRODUCT USE LIMITATIONS

- This product should be used in accordance with this user manual.
- This product is to be used by personnel specially trained to perform in vitro diagnostic procedures.

7. PATHOGEN

Causative Agents

The hepatitis D virus (HDV) is classified as Hepatitis delta virus and is known to cause Hepatitis D in humans. It is a small, enveloped virus with a 1.7 kb single-stranded, closed, circular RNA genome and was classified into at least 3 genotypes (1-3), however recent studies showed that there are 8 genotypes of HDV. HDV Genotype 1 is prevalent in all of over the world while other genotypes (HDV genotypes 2-8) are specific to territories. [1-5] HDV does not belong to a viral family and is considered to be satellite virus because of its characteristic to propagate only when Hepatitis B virus (HBV) infection is also present.

Epidemiology

Hepatitis delta virus (HDV) has an epidemiological distribution similar to HBV. It has been found worldwide with unequal distribution rates. There are around 10 million people infected with HDV around the world. It has mostly similar distribution patterns of HBV infection, but with different rates. There is a highest incidence of HDV infection in Southern Italy, the Mediterranean region and in some parts of Africa and Asia. There is an average incidence of HDV infection in Turkey. 10% of the patients with HBV in the west and 15-25% of patients in the central and eastern regions are known to be positive for anti-HDV. [2], [3]

Modes of Transmission:

The modes of HDV transmission are mostly similar to those for HBV, including direct or indirect parenteral exposure to blood or body fluids, sexual and perinatal transmission. Sexual transmission is less efficient than that of HBV. Perinatal transmission occurs seldom since HDV infected mothers are generally anti-HBe positive and thus less infectious. [1]

8. METHOD

Bosphore® HDV Quantification-Detection Kit v1 is based on the Real Time RT PCR method. HDV genetic material is amplified by reverse transcription technique since it is composed of RNA. RT-PCR, which is also referred as RNA PCR, is a two-step reaction. First, complementary DNA is synthesized from RNA by reverse transcription and then complementary DNA is amplified by standard PCR. The primer binds to the target RNA region in RT-PCR and RNA-DNA double strand is synthesized by reverse transcriptase enzyme using the RNA template for complementary DNA. Afterwards, standard PCR continues.

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 addition to these components, in RT PCR reverse transcriptase is added to the reaction and cDNA synthesis from the RNA template is acquired.

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® HDV Quantification-Detection Kit v1 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. HDV RNA (cDNA) and an internal control are co-amplified in a single reaction, using sequence-specific primers. The fluorescent signal generated by the HDV 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).

9. PROCEDURE

While being used in combination with fully automated Magnesia 2448 Nucleic Acid Extraction & PCR Setup Robot all the user has to do is to place the kit within the worktable and start the HDV protocol. No manual processing is neccessary in case of automated use.

9.1. RNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System and Magnesia® Viral Nucleic Acid Extraction Kit, or Magrev®24 stand and Magrev® Viral DNA/RNA Extraction Kit, or Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot and Magnesia® 2448 Viral DNA/RNA Extraction Kit, or Bosphore® Viral RNA Extraction Spin Kit (Anatolia Geneworks), EZ1 Advanced XL and EZ1 Virus Mini Kit v2.0 (Qiagen), NucliSENS® easyMAG® (bioMérieux) or other high quality viral RNA extraction kit and system is used with Bosphore® HDV Quantification-Detection Kit v1. The RNA isolation should be performed according to the manufacturers' instructions. The amount of internal control that should be used during isolation for each sample is 5 µl. If another starting volume or elution volume is used, it should be taken into consideration that a mathematical factor should be applied to the resulting quantitation values of the samples.

9.2. Kit Components

9.2.1. PCR Master Mix

PCR Master Mix contains; HotStarTaq DNA Polymerase, RT-PCR Buffer, dNTP mix (including dUTP), uracil DNA glycosylase (UNG), the HDV-specific forward and reverse primers and its dual-labeled probe, and the internal control-specific forward and reverse primers and its dual-labeled probe.

9.2.2. RT mix

RT Mix contains a unique blend of Reverse Transcriptases, with this enzyme combination it provides highly efficient and sensitive reverse transcription.

9.2.3. Internal Control

An internal control is included in the kit to control PCR inhibition. The internal control is a synthetic DNA molecule totally unrelated with the human or viral genome. It can be added in the sample (for each sample 5ul) before the extraction, or directly into the RT-PCR master mix to control the PCR inhibition exclusively. For this purpose, 0.25 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:

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

9.2.4. Quantitation Standards

The quantitation standards are previously extracted and calibrated standards of 10 000 , 1000, 100 and 20 copies/µl. They are directly included in the PCR reaction, just as the extracted sample RNA. See section "Analysis" Section for RNA quantitation.

9.2.5. Positive Control

The positive control provided in the kit is a previously quantitated HDV nucleic acid sample with a concentration of 200 copies/µl. For quantitation purpose, there is no need to include it in every reaction, but for qualitative purpose (when standards are not used) it must be included in every PCR.

9.3. Preparing the RT-PCR

All four external quantitation standards should be added into the PCR reaction together with the samples and the negative control. The kit can be used as qualitatively if the standards are not included in the reaction, but in this case the Positive Control should be used. Make sure that all the kit components are thawed before use. Refer to the table below for preparing the PCR. It is for only one reaction, multiply these values with the sample number to find the values required for the master mix. While preparing master mixes for more than 5 samples, an extra 10% should be added to the total sample number.

PCR MasterMix	14.5 µl
RT-mix	0.25 µl
Internal Control*	0.25 µl
Sample RNA (Standard, Negative/Positive Control)	10 µl
Total Volume	**25 µl**

*No need to add internal control if it's already added in the extraction step.

Pipette 15 µl of the master mix into the PCR tubes or strips, and add 10 µl of RNA (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.

9.4. Programming the Real-Time PCR Instrument

The thermal protocol for Bosphore® HDV Quantification-Detection Kit v1 is composed of two-steps; firstly a reverse transcription step and secondly Real Time PCR steps; 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.

Reverse Transcription	50°C	30:00 min.	
Initial denaturation	95°C	14:30 min.	
Denaturation	97°C	00:30 min.	50 cycles
Annealing (Data Collection)	55°C	01:20 min.	
Synthesis	72°C	0:15min.	
Hold	22°C	01:00min.	

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

- Choose the filters 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 experiment.

10. ANALYSIS

By the end of the thermal protocol, the software automatically calculates the baseline cycles and the threshold. Example of an amplification curve is given in Fig. 1.

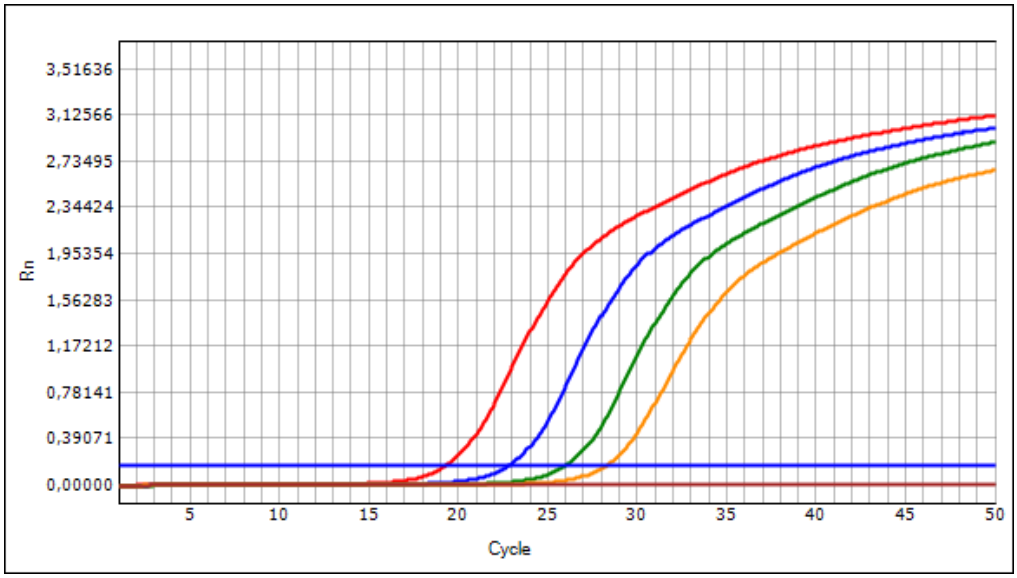


Fig. 1: Amplification Curve of a Bosphore® HDV v1 test

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.

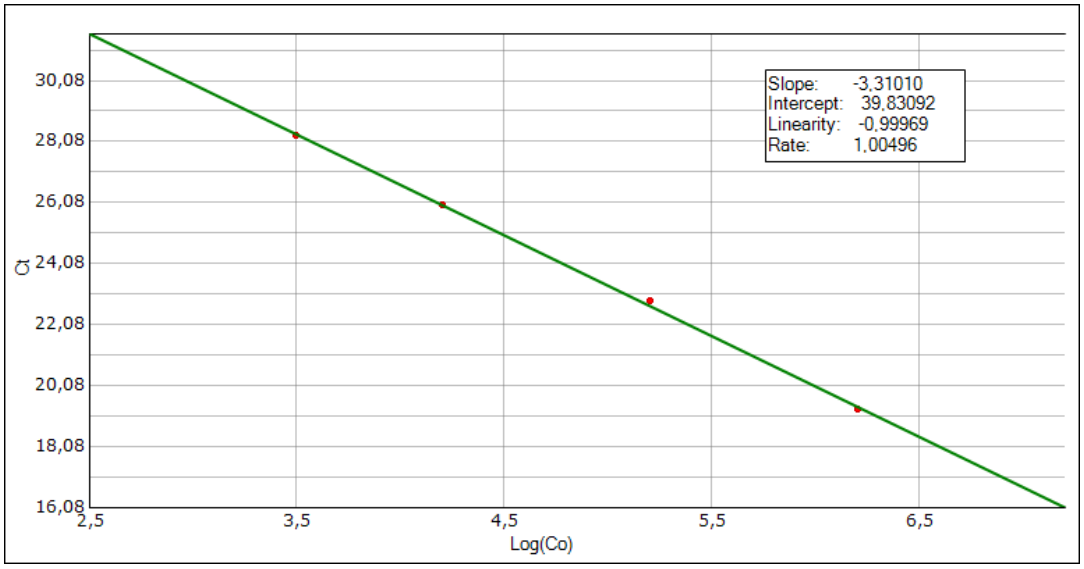


Fig.2: Standard Curve of a Bosphore® HDV v1 test

Caution!: Since the quantitation standards are provided as plasmid samples and as copies/µl, the following formula should be applied to the resulting copies/µl values obtained for the samples, to assess the quantitation values of the samples in copies/ml. This mathematical factor takes the starting volume of RNA extraction and the elution volume into consideration, to ensure correct quantitation of the samples:

$$\frac{\text{(Result in copies/µl) x (Elution Volume in µl)}}{\text{(Starting Extraction Volume in ml)}} = \text{Result in copies/ml}$$

For example, if a sample's result from the automated Standard Curve was calculated as 1000 copies/µl, considering that the starting extraction volume is 400 µl and the elution volume is 60 µl, applying the formula; 1000 x 60 ÷ 0.4 = 150 000 copies/ml is calculated as the HDV RNA that the sample material contains.

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, the trained personnel, who have received the required training from manufacturer, must pay extreme attention that the results are within the acceptance criteria, and must check the internal control amplifications of each patient sample.)

The table below displays the acceptance criteria for Bosphore® HDV v1.

Component/Parameter	Cycle Threshold (C_T)
Standard 1	19.5±2
Standard 2	23±2
Standard 3	26±2
Standard 4	28.5±2
Positive Control	25±4
Correlation Coefficient	>0.970
PCR Efficiency*	>%70

* PCR efficiency is calculated by the following formula: $10^{(-1/\text{slope})}-1$ x100

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, or when the results are inconsistent with clinical findings (See the last page for contact information).

The quantitative results of the test are displayed on the report screen, containing the calculated starting quantities of the unknown samples in each tube. The samples that cross the threshold in Channel 1 (FAM) 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 amplification 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	The sample contains HDV RNA, 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 internal control detection channel	The HDV RNA in the sample is not detectable	Signal from HEX filter rules out the possibility of PCR inhibition
No signal in FAM and internal control detection channel	The diagnosis is inconclusive	No signal in HEX points out to PCR inhibition or to a problem in RNA isolation

11. SPECIFICATIONS

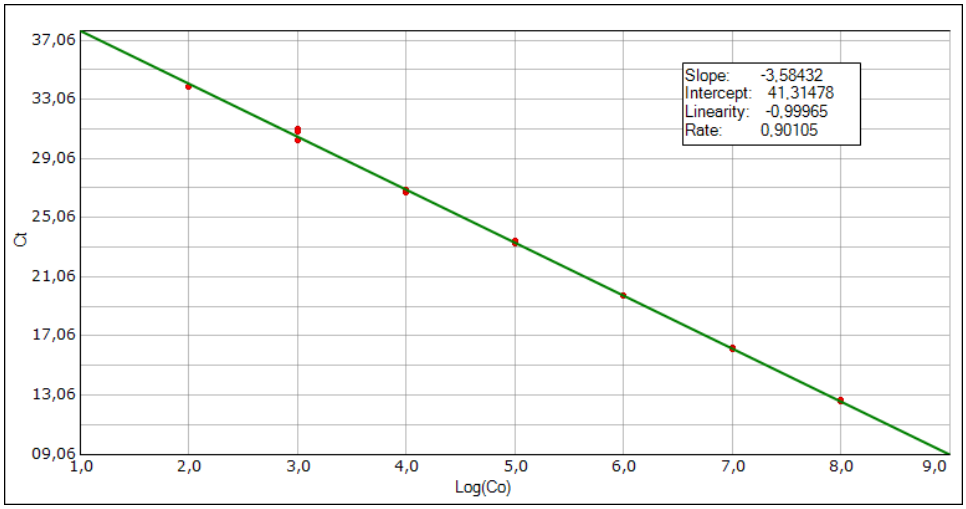
11.1. Sensitivity

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® HDV v1 was found to be 45 copies/ml. The sensitivity was determined using serial dilutions of HDV serum samples previously calibrated against WHO 1st International Standard for Hepatitis D Virus RNA for NAT-based Assays Code 7657/12. The dilutions were tested in different runs in replicates. The results were analyzed by probit analysis method.

11.2. Linear Range

The linear range of Bosphore® HDV Quantification-Detection Kit v1 was determined to be $1x10^2$ copies/ml.to $1x10^8$ copies/ml.



11.3. 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 detailed sequence comparison analyses using many database alignments. Samples of HBV, HCV, HIV, CMV, EBV, BKV, HSV-1, JCV, Adenovirus, Enterovirus and RSV with known high positivity were tested, and found negative.

11.4. Reproducibility

Reproducibility data (on C_T value basis) were obtained by the analysis of one of the quantitation standards of the Bosphore® HDV Quantification-Detection Kit v1. 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 the table below.

HDV (100 copies/ml)	Standard deviation	Variance	Coefficient of variation [%]
Intra-assay Variability N=4	0,12	0,0150	0,38
Inter-lot Variability N=3	0,06	0,0032	0,17
Inter-operator Variability N=3	0,05	0,0026	0,16
Total Inter-assay Variability N=5	0,06	0,0031	0,17

11.5. Calibration Against WHO Standard

HDV Quantitation Standards were calibrated against the WHO 1st International Standard for Hepatitis D Virus RNA for NAT-based Assays Code 7657/12, by performing multiple assays using various dilutions and testing them in replicates. 1 IU was found to be equal to 3.63 copies/ml.

11.6. Whole System Failure

To assess the whole system failure rate, which means the frequency of failures when the entire process is performed as prescribed by the manufacturer; 102 HDV negative plasma samples were spiked with high positive plasma samples to contain 135 copies/ml (three times the 95 % positive cut-off virus concentration) and were subjected to viral RNA extraction and Real-Time PCR. The robustness determined for Bosphore HDV Quantification-Detection Kit v1 resulted in no false-negative results, in accordance with the acceptance criteria (>0.99) positivity were 100% (102/102).

Positive Results Obtained	Negative Results Obtained
102/102	0/102

11.7. Diagnostic Specificity and Clinical Data

Diagnostic specificity was calculated using the frequency of repeatedly reactive (i.e.false positive) results in 100 HDV negative blood donor serum samples provided by an end user hospital.

Positive Results	Negative Results

Obtained	Obtained
0/100	100/100

The clinical data has also been successfully obtained via QCMD 2015 Hepatitis D Virus EQA Pilot Study and QCMD 2016 Hepatitis D Virus EQA Pilot Study and also collected through experiments with the clinical samples obtained from the end users. 33 natural positive serum and plasma samples from two different end user institutions were tested and all the samples were found positive.

12. REFERENCES

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3. Molecular Pathology of Liver Diseases, Molecular Pathology Library, 2011, Volume 5, Part 5, 589-595, DOI: 10.1007/978-1-4419-7107-4_39

4. Molecular Detection of Human Viral Pathogens, Book Edited by Dongyou Liu, 2016.

5. Collaborative Study to Establish a World Health Organization International Standard for Hepatitis D Virus RNA for Nucleic Acid Amplification Technique (NAT)-Based Assays, WHO/BS/2013.2227, World Health Organization 2013

13. SYMBOLS


Use- by date

Batch Code

Catalog number


Temperature limit


Caution


Manufacturer

In Vitro Diagnostic Medical Device

14. ORDERING INFORMATION

Catalog Number: ABHDV1 (25 rxn/box)

ABHDV2 (50 rxn/box)

ABHDV3 (100 rxn/box)

15. CONTACT INFORMATION

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