IVD

CE 1434

bosphore|ultra®

HCV

Quantitation/Detection Kit

User Manual

For *In Vitro* Diagnostic Use

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Anatolia geneworks

Contents

1. PRODUCT DESCRIPTION

Bosphore® Ultra HCV Quantitation/Detection Kit detects and quantitates Hepatitis C Virus RNA in human serum and plasma, encompassing all the 6 HCV genotypes. A region within the 5'UTR 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 and RNA extraction. The amplification data of the internal control is detected with the HEX filter. The internal control can be added either during RNA extraction or PCR step.

2. CONTENT

Bosphore® Ultra HCV Quantitation/Detection Kit is composed of Real-Time RT PCR reagents and quantitation serum standards which have been calibrated against WHO International Standard (NIBSC Code 06/102):

Component	REAGENT	100 Reactions	50 Reactions	25 Reactions
1	dH_2O	(1000 µl)	(1000 µl)	(500 µl)
2	PCR Master Mix	(5280 µl)	(2640 µl)	(1320µl)
3	Internal Control	(560 µl)	(280 µl)	(140 µl)
4	Positive Control 1 ($1x10^6$ IU/ml)	(140 µl)	(70 µl)	(35µl)
5	Positive Control 2 ($1 x 10^5$ IU/ml)	(140 µl)	(70 µl)	(35µl)
6	Positive Control 3 ($2 x10^3$ IU/ml)	(140 µl)	(70 µl)	(35µl)
7	Standard 1 ($1 x 10^7$) IU/ml	(1100 µl)	(1100 µl)	(550 µl)
8	Standard 2 ($1 x 10^6$) IU/ml	(1100 µl)	(1100 µl)	(550 µl)
9	Standard 3 ($1 x 10^5$) IU/ml	(1100 µl)	(1100 µl)	(550 µl)
10	Standard 4 ($1 x 10^4$) IU/ml	(1100 µl)	(1100 µl)	(550 µl)
11	Standard 5 ($2 x 10^3$) IU/ml	(1100 µl)	(1100 µl)	(550 µl)

3. STORAGE

Bosphore® Ultra HCV Quantitation/Detection Kit 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 minutes 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, CFX96–BioRad, LightCycler 480-Roche, 7500 Real-Time PCR System-

ABI, Stratagene Mx3005P, Mx3000P-Agilent, LineGene 9600-Bioer, Rotorgene 6000, Q-Qiagen)

- 0.2 ml Thin-Wall PCR tubes, PCR plates or strips
- Magnesia® 16 Nucleic Acid Extraction System/Magnesia® Viral Nucleic Acid Extraction Kit or Bosphore® Viral RNA Extraction Spin Kit or Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot/Magnesia® 2448 Viral DNA/RNA Extraction Kit or Magrev24/Magrev Viral DNA/RNA Extraction Kit (Anatolia Geneworks) or other high quality viral RNA 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 or plasma samples (including the standards) 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

The hepatitis C virus is a hepacivirus of the Flaviviridae family of viruses that causes Hepatitis C in humans. It is a small, enveloped, 9.6kb single-stranded RNA virus that is classified into six main genotypes (1-6) with more than one hundred different subtypes. [1]

Epidemiology

It is estimated that HCV has a worldwide prevalence of 3% affecting around 180 million people with between 3 to 4 million new infections each year. The vast majority of infected people (70-90%) develop chronic infection. Though chronic infection may be asymptomatic, it is a leading cause of chronic liver diseases, including cirrhosis in between 20 to 50% of patients. Treatment may be effective in 10-50% of patients depending on the applied therapy. [2]

Modes of Transmission:

Hepatitis C is believed to be spread through contact with infected blood. However, unlike many other blood borne viruses, HCV may be transmitted even through indirect sources like a used razor, making HCV more transmissible than other blood borne viruses –including HIV. Common routes of transmission include transfusion of blood products, intravenous and percutaneous drug and needle use, work accidents among healthcare workers and any other blood to blood contacts, such as sexual practices and from mother to newborn (maternal-infant transmission). Statistical studies have revealed no risk factors for HCV transmission in the activities of daily living (sneezing, coughing, hugging, etc.). [2], [3]

8. METHOD

Bosphore® Ultra HCV Quantitation/Detection Kit is based on the Real Time RT PCR method. HCV 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. I

The assay utilizes the 5' exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent hybridization 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® Ultra HCV Quantitation/Detection 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. HCV RNA (cDNA) and an internal control are co-amplified in a single reaction, using sequence-specific primers. The fluorescent signal generated by the HCV amplification is detected by a probe labeled at the 5' 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. RNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System/Magnesia® Viral Nucleic Acid Extraction Kit or Bosphore® Viral RNA Extraction Spin Kit or Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot/Magnesia® 2448 Viral DNA/RNA Extraction Kit or Magrev24/Magrev Viral DNA/RNA Extraction Kit (Anatolia Geneworks) or other high quality viral RNA extraction kits and systems are used with Bosphore® Ultra HCV Quantitation/Detection Kit.

The kit is to be used with serum or plasma samples. We recommend that serum and plasma must be prepared as soon as the blood sample is obtained. After the centrifugation, the upper clear phase must be pipetted carefully not to disturb the red bottom phase, so that only serum or plasma must be subjected to RNA extraction.

The viral RNA isolation should be performed according to the viral RNA extraction kit/system manufacturers' instructions. The quantitation standards are provided as serum and should be processed with the same procedure as the patient samples, starting from RNA isolation.

9.2. Kit Components

9.2.1. PCR Master Mix

PCR Master Mix contains; HotStarTaq DNA Polymerase, RT-PCR Buffer, RT mix, dNTP mix (including dUTP), uracil DNA glycosylase (UNG), the HCV-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 Internal Control

An internal control is included in the kit to control RNA 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 RNA 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, 0.4 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:

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

9.2.3. Positive Control

The positive control 1, 2 and 3 contain previously quantitated HCV RNA with a concentration $1x10^6$ IU/ml, $1x10^5$ IU/ml and of $2x10^3$ IU/ml, respectively. They can be included in the PCR to test the efficiency of the PCR exclusively. The threshold cycle for the positive controls are given in the acceptance criteria table (Section 10. Analysis). Threshold cycles higher than the acceptance criteria may indicate an efficiency loss in the reaction.

9.2.4. Quantitation Standards

The quantitation serum standards are calibrated by WHO International Standard (NIBSC Code 06/102).

9.3. Preparing the RT-PCR

At least three quantitation standards should be added into the reaction together with the samples and the negative control. We strongly recommend the use of four quantitation standards. Make sure that the standards have been subjected to RNA extraction, and all the kit components are thawed before use. Refer to the table below for preparing the PCR.

PCR Master Mix	48 µl
Sample RNA (Standard/ Negative/Positive Control)	32 µl
Total Volume	80 µl

Pipette 48 µl of the master mix into the PCR tubes, plates or strips, and add 32 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® Ultra HCV Quantitation/Detection Kit is composed of an initial reverse transcription step, a denaturation step for activation of the HotStarTaq DNA Polymerase, a three-step amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle.

Optional Step (UNG)	25°C	5:00 min.	
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	0:15 min.	
Hold	22°C	01:00	

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.

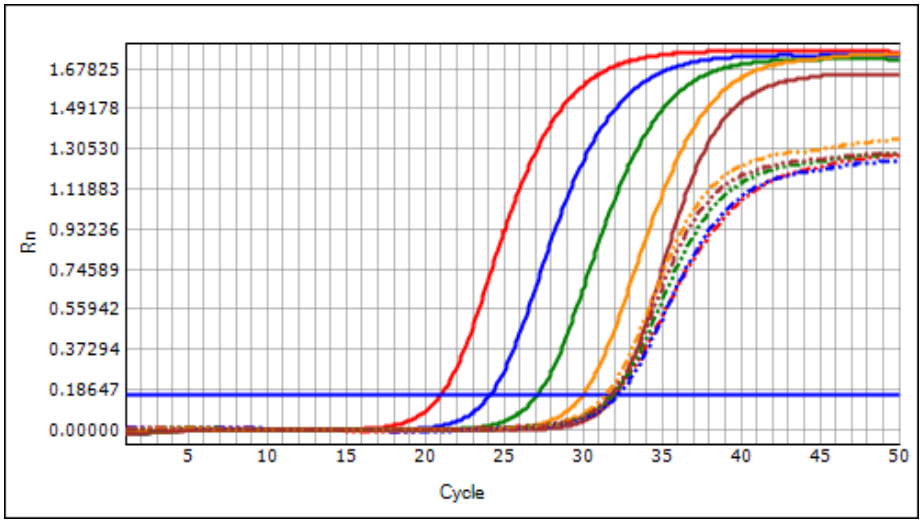

Fig. 1: Amplification Curve of a Bosphore® Ultra HCV test

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.

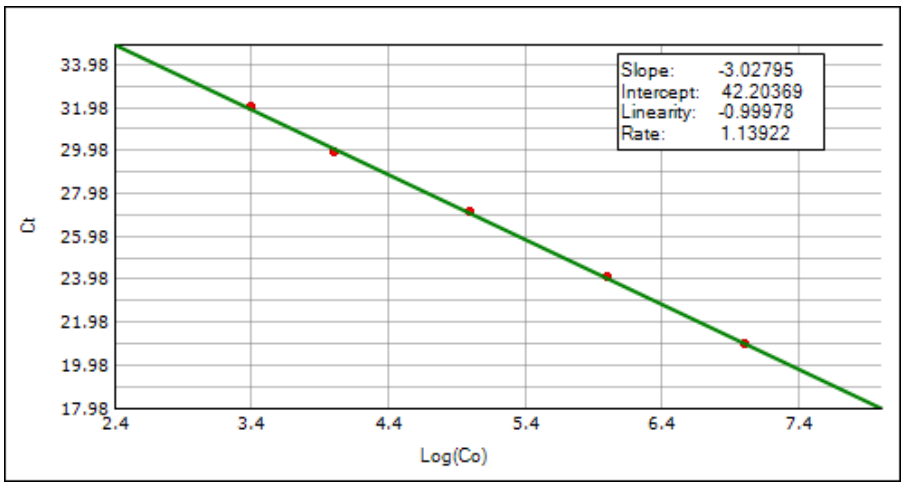

Fig.2: Standard Curve of a Bosphore® Ultra HCV test

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® Ultra HCV Quantitation/Detection Kit:

Component/Parameter	Cycle Threshold (CT)
Standard 1	17±2.5
Standard 2	20.5±2.5
Standard 3	24±2.5
Standard 4	27±2.5
Standard 5	29.5±2.5
Positive Control 1	21±4
Positive Control 2	24±4
Positive Control 3	30±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 quantitative results of the test contain the calculated starting quantities of the unknown samples in each tube. 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 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 HCV 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 HEX	The HCV RNA 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 RNA 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 RNA samples and using them in the PCR after diluting them 1:2 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® Ultra HCV Quantitation/Detection Kit was found to be 8 IU/ml, which was determined using Magrev® Viral DNA/RNA Extraction Kit, extracting serial dilutions of total 90 serum samples calibrated with the WHO International Standard for HCV RNA NAT assays, (NIBSC Code 06/102). The dilutions were tested by Real-Time PCR in different runs and in replicates. The results were analyzed by the probit method.

The sensitivity was also determined using Magnesia 2448® Viral DNA/RNA Extraction Kit with Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot, testing serial dilutions of total 266 serum and samples calibrated with the WHO International Standard for HCV RNA NAT assays, (NIBSC Code 06/102). The dilutions were subjected to RNA isolation and automated PCR setup, and were tested in replicates**.** The detection limit in consideration with the isolation for Bosphore Ultra HCV Quantitation/Detection Kit was found to be 12 IU/ml using the Magnesia 2448® Viral DNA/RNA Extraction Kit with Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot The results were analyzed by the probit method.

11.2. Genotype Detection Efficiency

Efficiency of detecting and quantitating different genotypes were ensured both by sequence comparison analysis and by Real-Time PCR assay using Worldwide HCV RNA Genotype Performance Panel WWHV302(M) (Seracare). The following genotypes were tested and found positive:

Panel Member	Genotype	HCV(FAM)
1	1b	+
2	1a	+
3	1b	+
4	2a/2c	+
6	3b	+
8	3a	+
10	4	+
11	4	+
12	5a	+
14	6a	+

Moreover to demonstrate genotype detection, 8 plasma samples of the QCMD 2015 Hepatitis C virus RNA EQA Programme, 8 plasma samples of the Hepatitis C Virus Genotype 2013 EQA Programme (HCVGT13) and 100 natural patient samples which contained genotypes 1,2,3,4 and 5 have been successfully tested with Bosphore® Ultra HCV Quantitation/Detection Kit.

11.3. Linear Range

The linear range of Bosphore® Ultra HCV Quantitation/Detection Kit was determined to be from $1x10^1$ IU/ml to at least $1x10^9$IU/ml.

In order to assess the linear range, a dilution series of total 36 serum samples that has been calibrated against the WHO International Standard for HCV RNA NAT assays (NIBSC Code 06/102), were analyzed by testing each dilution in duplicates (Fig. 3,4). The standard curve correlation coefficient was found to be 0.9995.

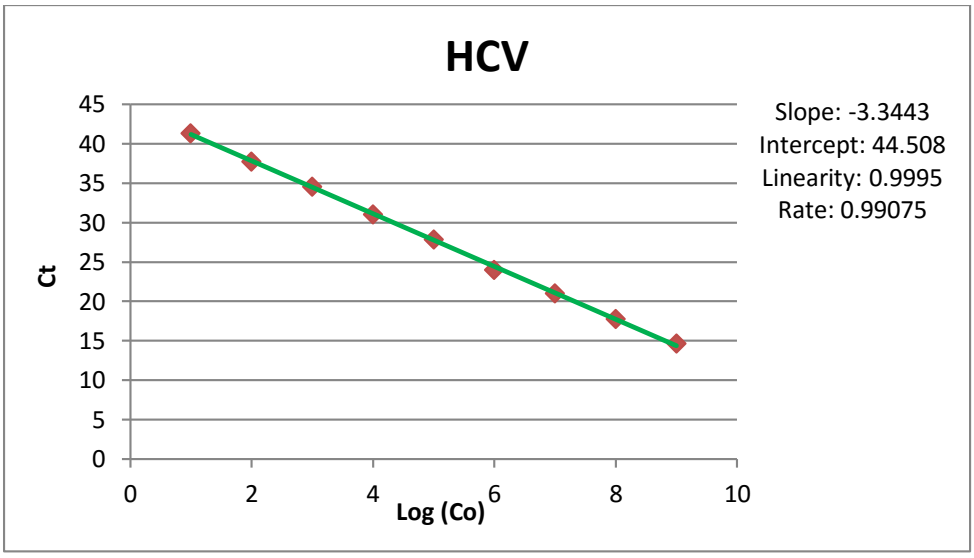

Fig. 3: Linear Range Standard Curve of a Bosphore® Ultra HCV test

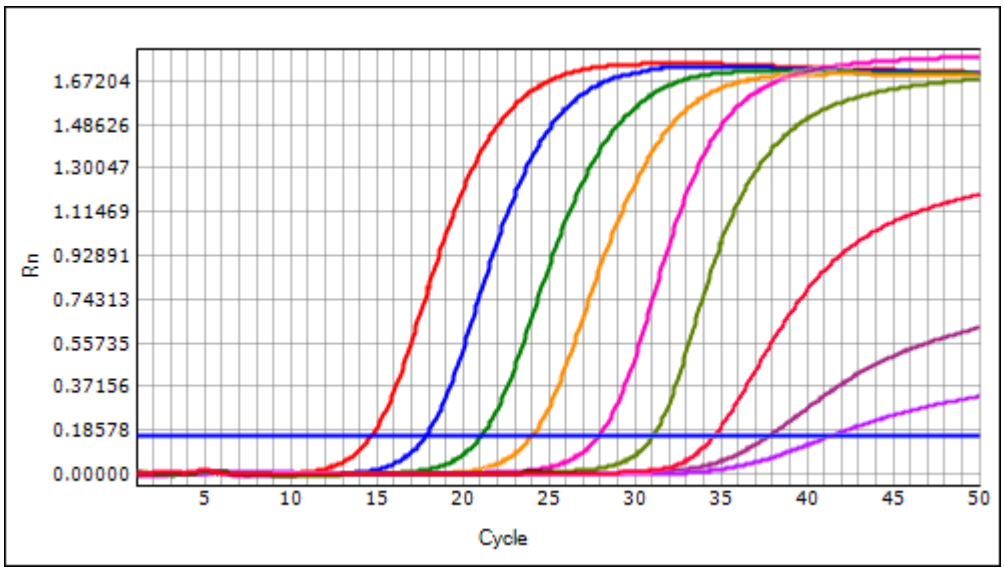

Fig. 4: Linear Range Amplification Curve of a Bosphore® Ultra HCV test

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. A total of 7 serum and plasma samples of HIV, HDV, HBV, West Nile Virus, H1N1, Enterovirus with known high positivity were tested, and found negative.

11.5. Reproducibility and Precision

The ability to duplicate measurements is known as reproducibility, and precision is a measure of the reproducibility of a test. Precision is usually expressed in terms of variability, using standard deviations. Reproducibility data were collected using the third quantitation standard ($1x10^4$ IU/mL). Testing was performed with four replicates, three different lots, on multiple runs and by three operators. Results are given below.

Table 1: Reproducibility Data.

HCV (10000 IU/mL)	Standard deviation	Variance	Coefficient of variation [%]
Intra-assay Variability N=4	0.03	0.001	0.11
Inter-lot Variability N=3	0.05	0.002	0.20
Inter-operator Variability N=3	0.06	0.004	0.23
Total Inter-assay Variability N=5	0.05	0.003	0.21

Table 2: Precision Data

HCV (10000 IU/mL)	Standard deviation	Variance	Coefficient of variation [%]
Intra-assay Variability N=4	312.29	97,527.98	2.80
Inter-lot Variability N=3	426.97	182,303.38	3.91
Inter-operator Variability N=3	466.84	217,939.58	4.40
Total Inter-assay Variability N=5	436.58	190,602.09	4.08

11.6. Diagnostic Evaluation

The diagnostic evaluation was performed by testing 100 HCV negative and 100 HCV positive serum and plasma samples which have been previously analyzed using other established systems with IVD CE mark. All negative samples were found negative, and all of the positive samples were found positive with Bosphore® Ultra HCV Quantitation/Detection Kit.

11.7. Calibration Against WHO Standard

Quantitation Standards were calibrated against the WHO International Standard for HCV RNA NAT assays (NIBSC Code 06/102). 1 IU was found to be equal to 3± 0,2 copies/ml .

11.8. Cross Contamination

To investigate potential carry-over, alternating high positive (Ct values between 17.76± 3) and negative samples were tested in five runs. No indication of cross-contamination was observed.

Positive Results Obtained	Negative Results Obtained
32/32	32/32

12. REFERENCES

1. By K. E. Nelson, C. Williams, and N. Graham., Infectious Disease Epidemiology: Theory and Practice, July 15, 2000 , p :923-926
2. Theodore Sy and M. Mazen Jamal, Epidemiology of Hepatitis C Virus (HCV) Infection, Int J Med Sci. 2006; 3(2), p:41–46
3. Anonymous, *Hepatitis C Fact Sheet No. 164*. 2000, World Health Organization

13. SYMBOLS


Use-by date

Batch Code

Catalogue number


Temperature limit


Caution


Manufacturer

In Vitro Diagnostic Medical Device

14. CONTACT INFORMATION

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