IVD CE 1434

bosphore

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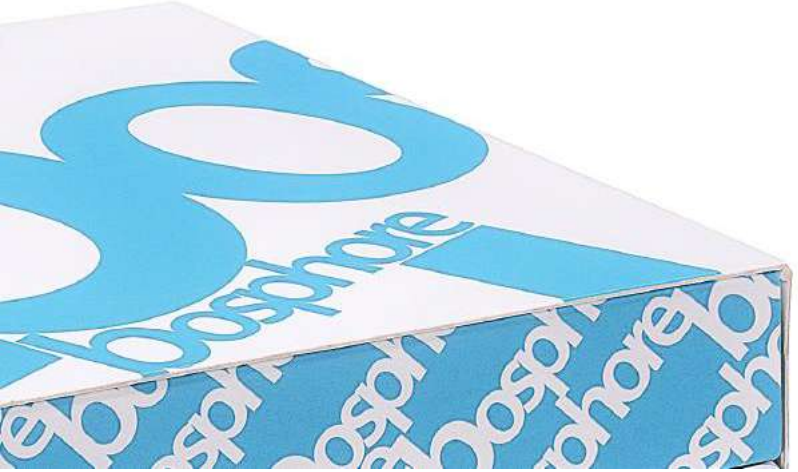
USER MANUAL

HBV
Quantification Kit

For *In Vitro* Diagnostic Use

MB198v8f
30th November 2023



Anatolia geneworks

CONTENTS

1. PRODUCT DESCRIPTION

Bosphore HBV Quantification Kit is a Real-Time PCR kit for *in vitro* diagnostics that detects and characterizes the region within the S gene of HBV genotypes (A-J) from serum and plasma samples. Fluorescence detection is performed using FAM and HEX filters. HBV DNA is amplified and fluorescence detection is performed using the FAM filter.

Component	FAM	HEX
PCR Master Mix	HBV Genotypes (A-J)	Internal Control

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

2. CONTENT

Bosphore HBV Quantification Kit consists of the following dH2O, Real-Time PCR Master Mix, positive control, internal control, and quantitation serum standards which have been calibrated against WHO International Standard (NIBSC Code 10/264).

Component	Reagent	100 Reactions	50 Reactions	25 Reactions
1	dH_2O	(1000 µL)	(1000 µL)	(1000 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

The PCR reagents for the Bosphore HBV Quantification Kit 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 minutes, and the PCR master 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 PCR master mix in a closed container. If the components are stored according to the recommended conditions, they will remain stable until the expiry dates on the labels.

4. REQUIRED MATERIALS AND DEVICES

- Montania 4896 Real-Time PCR Instrument – Anatolia Geneworks, CFX96 Real-Time PCR Detection System - Bio-Rad, QuantStudio 5 Real-Time PCR System – ThermoFisher, LightCycler 480 Instrument II – Roche, Q qPCR Cycler - Quantabio, Rotor-Gene Q – QIAGEN or another Real-Time PCR system with FAM and HEX filters*
- 0.2 mL thin-wall PCR tubes, PCR plates or strips
- UNIO B24 Extraction System and UNIO Viral DNA/RNA Extraction Kit 600 µl, Magrev 24 Stand and Magrev Nucleic Acid Versatile Extraction Kit or Magrev Viral DNA/RNA Extraction Kit, UNIO 96 Extraction and PCR Setup System and UNIO 96 Nucleic Acid Extraction Versatile Kit, Bosphore Viral DNA Extraction Spin Kit, Bosphore Viral DNA/RNA Extraction Spin Kit or Bosphore Nucleic Acid Extraction Versatile Spin Kit or Magnesia 16 Nucleic Acid Extraction System and Magnesia Viral Nucleic Acid Extraction Kit (Anatolia Geneworks) or other high-quality DNA extraction kits and systems
- Deep freezer (-20 °C)
- Desktop centrifuge with rotor for 2 mL or 1.5 mL microcentrifuge tubes
- DNase, RNase, pyrogen-free 1.5 mL or 2 mL microcentrifuge tubes
- Calibrated, adjustable micropipettes
- DNase, RNase, pyrogen-free micropipette tips with filters
- Disposable laboratory gloves

* For other Real-Time PCR devices that can be used with the Bosphore HBV Quantification Kit, please contact Anatolia Geneworks from the information in Section 15.

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

- The product should be delivered on dry ice. Check for the presence of dry ice upon arrival.
- Check for the expiration dates on the box and tube labels upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNase, RNase, and pyrogen-free micropipette tips with filters, and DNase, RNase, and 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 before use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared and quickly returned to -20 ºC.
- PCR and nucleic acid extraction 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 and in a microbiological safety cabinet of the appropriate class. Physical contact with pathogens should be avoided by wearing lab coats and gloves, making no allowance for eating or drinking within the workspace and preventing unauthorized individuals' access to the working area.
- All the pathogenic wastes produced during the nucleic acid extraction step, including the serum and plasma samples and materials in contact with them, should be discarded into medical waste and disposed of safely.

6. PRODUCT USE LIMITATIONS

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

7. INFECTION

Hepatitis B is an infection of the liver caused by the hepatitis B virus. The infection can be acute (short and severe) or chronic (long-term). Hepatitis B can cause a chronic infection and puts people at high risk of death from cirrhosis and liver cancer.

It can spread through contact with infected body fluids like blood, saliva, vaginal fluids and semen. It can also be passed from a mother to her baby. Hepatitis B can be prevented with a safe and effective vaccine. The vaccine is usually given soon after birth with boosters a few weeks later. It offers nearly 100% protection against the virus.

Hepatitis B is a major global health problem. The burden of infection is highest in the WHO Western Pacific Region and the WHO African Region, where 116 million and 81 million people, respectively, are chronically infected. Sixty million people are infected in the WHO Eastern Mediterranean Region, 18 million in the WHO South-East Asia Region, 14 million in the WHO European Region, and 5 million in the WHO Region of the Americas (WHO, 2023)

8. METHOD

Bosphore HBV Quantification Kit is based on the Real-Time PCR method. The polymerase chain reaction is a technique that is used for the amplification of a DNA region. The reaction occurs through repeated cycles of heating and cooling. The main components of PCR are primers, dNTPs, *Taq* DNA Polymerase (with hot-start property), buffer solutions, and templates. As a brief explanation, primers are small synthetic DNA that anneals to the specific regions of the template to start the synthesis, dNTPs are the building blocks of the amplified products, and *Taq* DNA Polymerase amplifies the DNA template. Finally, buffer solutions provide the pH adjustment required for the reaction, and the template, as referred to, is the target region for synthesis.

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

The assay utilizes the 5' exonuclease activity of *Taq* DNA Polymerase to cleave a dual-labeled fluorescent hydrolysis 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 nearby, and the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step of PCR, *Taq* DNA Polymerase encounters and cleaves the probe bound to the template. As the reporter is freed from the suppressing effect of the quencher, a 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 the 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.

Bosphore HBV Quantification Kit uses multiplex qPCR and internal control is included in the system to control the extraction procedure, PCR inhibition, and application issues.

The reaction is performed in one PCR tube containing PCR Master Mix.

In PCR Master Mix, HBV genome amplification is screened using the FAM filter, and the fluorescent signal produced by the internal control amplification is detected in PCR Master Mix via the HEX filter.

9. PROCEDURE

9.1. Specimen Collection and Storage

Bosphore HBV Quantification Kit is to be used with serum or plasma samples. Blood samples can be collected in serum-separating tubes or tubes containing anticoagulants such as citrate or EDTA, but we recommend that serum and plasma must be prepared as soon as the blood sample is obtained; within 1 hour if the blood is stored at room temperature, or within 24 hours if the blood is stored between +2 °C and +8 °C. After the centrifugation, the upper clear phase must be pipetted carefully into a sterile polypropylene tube without disturbing the red bottom phase, so that only serum or plasma must be subjected to DNA extraction. Plasma and serum samples can be stored for up to 2 days at 4 °C. The serum and plasma samples can be stored at -20 °C (between -20 °C to -90 °C) for 6 weeks. During this period, freeze and thaw of the sample more than 3 times should be avoided in order not to lose DNA.

9.2. DNA Extraction

We recommend that the UNIO B24 Extraction System and UNIO Viral DNA/RNA Extraction Kit 600 µl, Magrev 24 Stand and Magrev Nucleic Acid Versatile Extraction Kit or Magrev Viral DNA/RNA Extraction Kit, UNIO 96 Extraction and PCR Setup System and UNIO 96 Nucleic Acid Extraction Versatile Kit, Bosphore Viral DNA Extraction Spin Kit, Bosphore Viral DNA/RNA Extraction Spin Kit or Bosphore Nucleic Acid Extraction Versatile Spin Kit or Magnesia 16 Nucleic Acid Extraction System and Magnesia Viral Nucleic Acid Extraction Kit (Anatolia Geneworks) or other high-quality extraction kits and systems are used with Bosphore HBV Quantification Kit. The DNA extraction should be performed according to the manufacturer's instructions.

9.3. Kit Components

9.3.1. PCR Master Mix

PCR Master Mix contains a highly specific and accurate *Taq* DNA Polymerase (with hot-start property), PCR buffers, and dNTPs mix. PCR Master Mix also contains forward and reverse primers and dual-labeled probes specific for HBV and internal control.

9.3.2. Internal Control

The internal control included in the kit to check for DNA extraction, PCR inhibition, and application errors is a synthetic DNA molecule. The internal control is added to the mixture of sample and proteinase K at the beginning of DNA extraction to check extraction efficiency and application errors. If the internal control is to be added during nucleic acid extraction, 5 µL is added per sample; and when added directly to the PCR Master Mix to control PCR inhibition, 0.1 µL is added. We recommend adding an internal control to the negative control to evaluate the efficiency of the extraction system.

Caution! It is not necessary to include the internal control in the PCR Master Mix if it has already been added during the extraction step. The absence of internal control amplification in the HEX channel in negative samples may indicate a problem in extraction or application, or that the PCR reaction is inhibited. In this case, extraction and PCR should be repeated. In samples with high viral load (>10^5 IU/mL), including the positive control, the internal control may be suppressed and an increase in fluorescent signal may not be detected. Therefore, internal control amplification should be evaluated according to the table in Section 10.

9.3.3. Positive Control

The kit contains 1 positive control containing synthetic HBV DNA. It must be included in the PCR reaction to test the reaction efficiency.

Positive Control: HBV DNA

The threshold C_T value of the positive control is given in the acceptance criteria table (Section 10). A threshold C_T value of the positive control greater than the upper limit of the range in the table may indicate a loss of yield in the reaction.

9.3.4. Quantitation Standards

The quantitation serum standards are calibrated by the WHO International Standard (NIBSC Code: 10/264).

9.4. Preparing the PCR

The standards, positive and negative controls should be added into the PCR reaction together with the samples. Ensure that all kit components are dissolved before use and refer to the table below to prepare the PCR reaction mix. The volumes given are for one reaction only, multiply these volumes by the number of samples to find the volumes required for the master mix. When preparing the mix for more than 5 samples, 10% should be added to the total number of samples.

Components	Volume
PCR Master Mix	15 µL
Internal Control*	0.2 µL*
Sample DNA (Negative / Positive Control / Standard)	10 µL
Total Volume	25 µL

*Internal control should not be added to the reaction if it has already been added during the extraction step.

Pipette 15 µL of the PCR Master Mix into the PCR tubes or strips and add 10 µL of template (sample/ positive or negative control / standard). Close the tube cap. Make sure that the mix 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 the Bosphore HBV Quantification Kit consists of initial denaturation for activation of the *Taq* DNA Polymerase (with hot-start property), a two-step amplification cycle, and a terminal hold. The Real-Time data is collected at the second step of the amplification cycle.

The thermal protocol to be applied for the reaction is indicated below:

Steps	Temperature	Time	
Initial Denaturation	95 °C	14:30 min	
Denaturation	97 °C	00:30 min	50 Cycles
Annealing (Data Collection)	54 °C	01:30 min	
Hold	32 °C	01:00 min	

Before starting to work with the Bosphore HBV Quantification Kit, the following steps must be completed and checked:

- Choose all the filters to be used (FAM and HEX),
- Identify unknown samples, positive and negative controls, standards,
- Select the correct thermal protocol,
- Start the experiment.

10. ANALYSIS

By the end of the thermal protocol, the Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold. Analysis of the results should be performed by trained personnel who have received the required training for analyzing Real-Time PCR data. An example of an amplification curve is given in Figure 1.

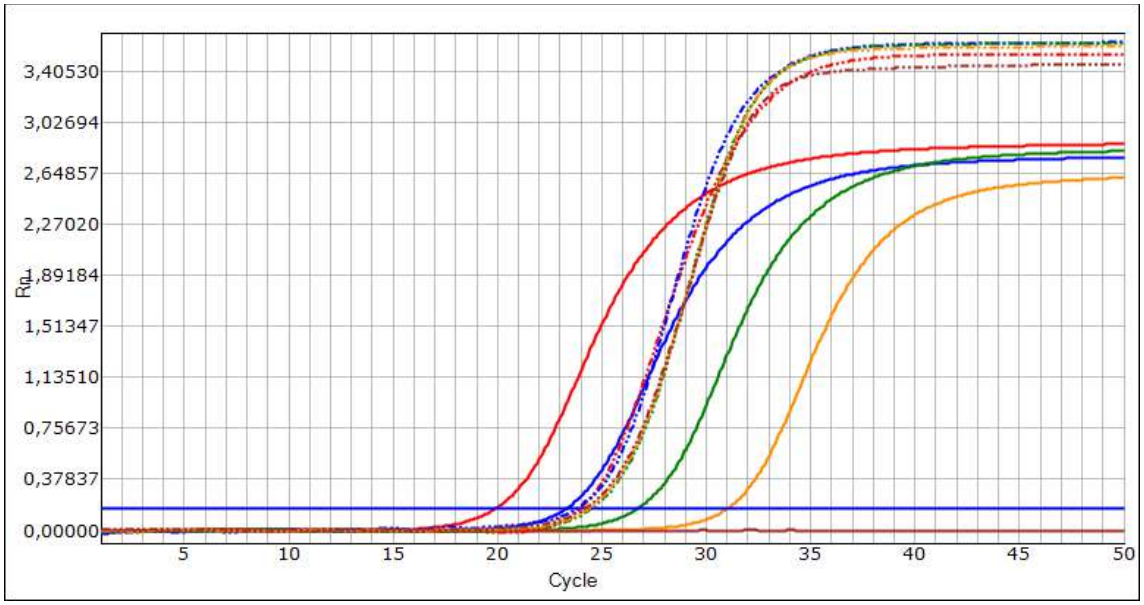


Figure 1: Amplification curve of Bosphore HBV Quantification Kit

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

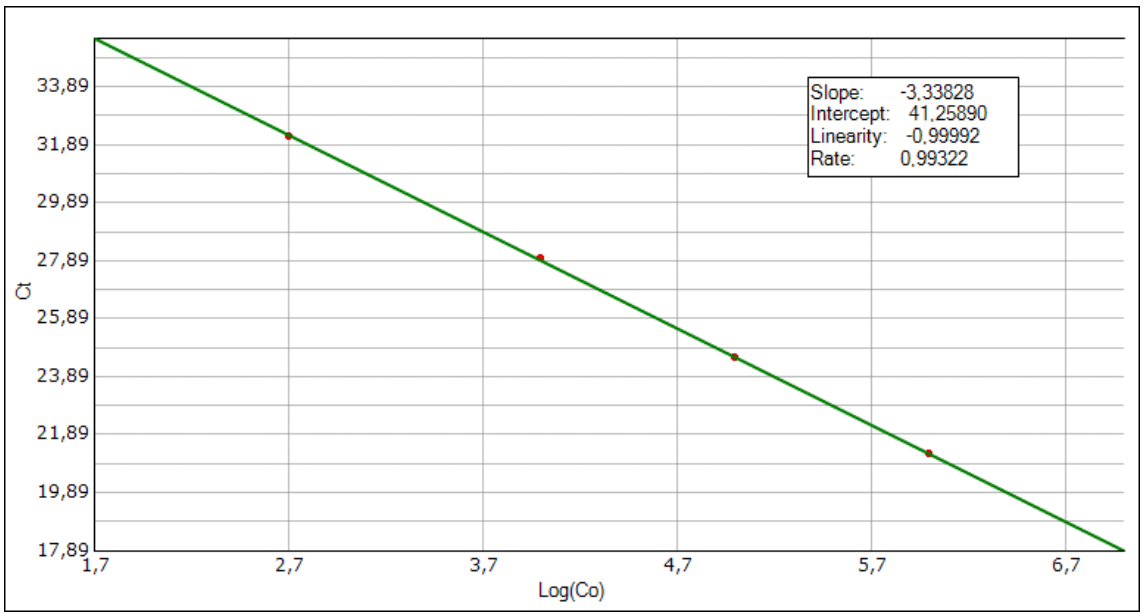


Figure 2: Standard curve of Bosphore HBV Quantification Kit

We recommend that the test results 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 the manufacturer, consider it necessary if the system allows pulling down the threshold as much as possible to detect low amplifications, attention should be paid to keep the threshold line above the background.

The negative control is essential for accurate result analysis. Please check the negative control and ensure it shows no amplification outside the associated internal control filter. If the negative control has a signal outside the filter associated with the internal control, please do not report the results. Repeat the experiment after taking the necessary precautions against contamination.

If the same result is encountered again, please contact the manufacturer.

Internal control, standards, and positive control of the Bosphore HBV Quantification Kit are essential for accurate result analysis. The cycle threshold acceptance criteria for the internal control, standards, and positive control are listed below:

Component / Parameter	Threshold Value (C_T)*
Standard 1	21.5±4
Standard 2	25±4
Standard 3	28±4
Standard 4	32.5±4
Internal Control	≤32
Positive Control	28±4
Correlation Coefficient	>0.970
PCR Efficiency**	>80%

*Cycle thresholds of the standards change depending on the starting volume and the efficiency of the extraction system used.

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

In quantitative test results; examples that cross the threshold in the HEX channel; it is evaluated as "Positive", samples that do not cross the threshold are shown as "No C_T" or "Negative". These samples are considered to have a negative or viral load below the detection limit of the assay.

The internal control data in the HEX channel of these "undetected" samples should also be checked to avoid false negative results.

The delayed amplification of the internal control may indicate a problem in nucleic acid extraction / PCR inhibition or application failure. In this case, extraction and PCR should be repeated. Please consider that in the samples that contain high viral concentration, internal control can be suppressed therefore delayed or no increase in internal control signal may be detected.

Please note that this product only provides testing pathogens shown below. Experimental results from the tube must be considered when providing a result for the patients, also in consideration of the patient's clinical findings, and the guidelines of the relevant health authorities. The table below shows the possible results and their interpretation:

PCR Master Mix	HBV (FAM)	Internal Control (HEX)	X (Texas RED)	X (Cy5)	Result
	+	+/-	X	X	The sample is HBV positive
	-	+	X	X	Sample is negative
	-	-	X	X	The test should be repeated!

In rare cases of PCR inhibition due to medication or other PCR inhibitors in the sample, we recommend repeating the test of inhibited samples, by freezing and thawing the DNA 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 the Bosphore HBV Quantification Kit was found to be 10 IU/ml. The sensitivity was determined using serial dilutions of DNA calibrated with the WHO International Standard for HBV DNA NAT assays (NIBSC Code 10/264) and extracted with Magnesia 16 Nucleic Acid Extraction System and Magnesia Viral Nucleic Acid Extraction Kit using 400 µL starting volume and 60 µL elution volume. The dilutions were tested in different runs in replicates. The results were analyzed by the probit method.

11.2. Genotype Detection

The efficiency of detecting and quantitating different HBV genotypes was 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 2010-2017 Hepatitis B virus DNA EQA Programmes, which have been tested with Bosphore HBV Quantification Kit and were all found positive, and the variance was within 0.5 log.

These samples contained different HBV genotypes that included HBV A-H. Moreover, 15 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 (A, B, D, E, H). The genotypes of the Genotype Performance Panel PHD 350 were tested in replicates and found positive, and with consistent quantitation with repeat to the other systems. Genotypes I and J were tested using plasmid samples, as no reference material could be found.

11.3. Linear Range

The linear range of the Bosphore HBV Quantification Kit was determined to be from 10 IU/mL to at least $1x10^9$ 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 10/264) 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. To eliminate the risk of cross-reactivity; CMV, EBV, MTBC, Parvovirus B19, and BKV samples with known high positivity were tested and found negative. The experimental results indicated that the kit detects specifically and only the HBV pathogen that it intends to detect, but not the others.

11.5. Reproducibility

Reproducibility data (on a C_T value basis) were obtained by the analysis of one of the quantitation standards (for $1x10^4$ IU/mL) and also at 2 other plasma samples with different concentration levels ($1x10^3$ IU/mL and $1x10^5$ IU/mL). Foreach concentration, test was performed 4 replicates by 3 different operators, on multiple days, using 3 different lots. The resulting data is given below.

Variability ($1x10^3$ IU/mL)	**Standard Deviation**	**Variance**	**Coefficient of Variation [%]**
Intra-assay (n=4)	0.0838	0.0070	0.2691
Inter-lot (n=3)	0.0603	0.0036	0.1934
Inter-operator (n=3)	0.1016	0.0103	0.3255
Total Inter-assay (n=5)	0.0776	0.0060	0.2485

Variability ($1x10^4$ IU/mL)	Standard Deviation	Variance	Coefficient of Variation [%]
Intra-assay (n=4)	0.07	0.005	0.23
Inter-lot (n=3)	0.10	0.01	0.34
Inter-operator (n=3)	0.28	0.08	0.91
Total Inter-assay (n=5)	0.29	0.08	0.93
Variability ($1x10^5$ IU/mL)	**Standard Deviation**	**Variance**	**Coefficient of Variation [%]**
Intra-assay (n=4)	0.0271	0.0007	0.1109
Inter-lot (n=3)	0.0639	0.0041	0.2614
Inter-operator (n=3)	0.0743	0.0055	0.3039
Total Inter-assay (n=5)	0.0682	0.0046	0.2790

11.6. Diagnostic Specificity

Diagnostic specificity was calculated using the frequency of repeatedly reactive (i.e., false positive) results in 100 HBV negative blood donor serum samples. The HBV surface Antigen detection was carried out using Elecsys HbsAg II Kit, and HBV DNA was tested with Cobas Amplicor HBV Monitor Test (Roche Diagnostics), and with both systems the test results were reported as negative. In order to validate the diagnostic specificity of the Bosphore HBV Quantification Kit, these samples were tested and shown to be HBV DNA negative.

Positive Results Obtained	Negative Results Obtained
0/100	100/100

To further test and demonstrate diagnostic specificity. Accurun 803 Nucleic Acid Negative Quality Control sample was also tested and found negative.

11.7. Calibration Against WHO Standard

Quantitation standards were calibrated against the WHO HBV DNA International Standard (NIBSC Code: 97/750 and 10/264). 1 IU was found to be equal to 4.5±0.2 copies/mL.

12. REFERENCES

1. https://www.who.int/news-room/fact-sheets/detail/hepatitis-b

13. SYMBOLS

	Use-by Date		Caution, consult accompanying documents.
	Batch Code		Manufacturer
	Catalog Number		*In vitro* Diagnostic Device

14. ORDERING INFORMATION

Catalog Number:

ABHBQ3 (100 rxn/box)
ABHBQ2 (50 rxn/box)
ABHBQ1 (25 rxn/box)

15. CONTACT INFORMATION

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V3	03	06th May 2012	The general content and type check
V4	04	05th January 2013	The content has been updated and checked
V5	05	04th May 2014	The general content and type check
V6	06	13rd August 2016	The content has been updated and checked
V7	07	04th August 2019	The general content and type check
V8	08	30th November 2023	Partial content correction

IVD CE

bosphore®

HCV

Genotyping Kit v7

User Manual

For *in vitro* Diagnostic Use

MB403v1f
January 2020

Anatolia geneworks

CONTENTS

1. PRODUCT DESCRIPTION

Bosphore® HCV Genotyping Kit v7 detects and characterizes the genotype of Hepatitis C Virus in human serum or plasma, encompassing 6 major and most predominant HCV genotypes (1,1a,1b,2,3,3a,3b,4,5,6). The analytic sensitivity is 100 IU/ml. A region within the NS5B is amplified and fluorescence detection is accomplished using the FAM and HEX filters.

An internal control has been integrated into the kit in order to check nucleic acid extraction, PCR inhibition and application errors. The amplification data of the internal control is detected with the Cy5 filter. The internal control can be added either during RNA extraction or PCR step.

2. CONTENT

Bosphore® HCV Genotyping Kit v7 is composed of Real-Time RT PCR reagents and positive and negative controls:

Component	REAGENT	100 Tests	50 Tests	25 Tests
1	dH_2O	(1000 µl)	(1000 µl)	(500 µl)
2	PCR Master Mix 1	(2860 µl)	(1430 µl)	(715 µl)
3	PCR Master Mix 2	(2860 µl)	(1430 µl)	(715 µl)
4	PCR Master Mix 3	(2860 µl)	(1430 µl)	(715 µl)
5	PCR Master Mix 4	(2860 µl)	(1430 µl)	(715 µl)
6	PCR Master Mix 5	(2860 µl)	(1430 µl)	(715 µl)
7	PCR Master Mix 6	(2860 µl)	(1430 µl)	(715 µl)
8	Internal Control	(560 µl)	(280 µl)	(140 µl)
9	Positive Control 1	(120 µl)	(60 µl)	(30 µl)
10	Positive Control 2	(120 µl)	(60 µl)	(30 µl)
11	Positive Control 3	(120 µl)	(60 µl)	(30 µl)
12	Positive Control 4	(120 µl)	(60 µl)	(30 µl)
13	Positive Control 5	(120 µl)	(60 µl)	(30 µl)
14	Positive Control 6	(120 µl)	(60 µl)	(30 µl)

3. STORAGE

Bosphore® HCV Genotyping Kit v7 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 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 components 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, HEX and Cy5 filters (Anatolia Geneworks), or another Real-Time PCR system with FAM, HEX and Cy5 filters (iCycler, iQ5, CFX–BioRad, 7500 Real-Time PCR System,–ABI, LightCycler 480-Roche, Stratagene Mx3005P, Mx3000P-Agilent, LineGeneK, 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 / Magrev®24 and Magrev® Viral DNA/RNA Extraction Kit / Bosphore Viral RNA Spin 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 -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, 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; biological 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.
- Bosphore HCV Genotyping Kit v7 is not intended to be used for HCV detection or for HCV quantitation.

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)

Genotype 1 is the most common one and is the one with the least response to therapy. Since HCV has a high tendency to mutate, and doesn't initiate a severe response in human T- lymphocytes of the immune system (a white blood cell type), it results in a high rate of chronic infection. The genetic heterogeneity of this virus, which cannot be

grown by cell culture, makes the diagnosis difficult, lowers the response to treatment and also impedes the development of the vaccine against the disease(4,5). It has been observed that different HCV genotypes show different responses to antiviral therapy. The duration and success rate of HCV medication (PEG-IFN and ribavirin) mostly depend on the virus genotype. The response rate to treatment in genotype 2 and 3 is higher than the one in genotype 1 and 4 (70-80% against 40-50% in long term). Moreover, the successful treatment of genotype 2 and 3 takes approximately 6 month, while it is 1 year for genotype 1 and 4 (6,7,8). It has been reported that the response to treatment is 0-3%, and ceasing the treatment should be considered, if the HCV RNA level of the patients has not revealed at least 2 log decrease after the 12 weeks of treatment (9, 10). It has been observed that duration of the treatment of acute infection of genotype 1 is shorter and the success rate is higher than the chronic infection of genotype 1 (11, 12, 13). HCV genotype 6 treatment is better with interferon-based therapy compared with HCV genotype 1. However patient clinical characteristics and side effect profiles are similar between HCV genotype 6 and other HCV genotypes (17).

The nucleotide sequences of genotypes differ around 31-34% from each other, the subtypes differ around 20 to 23%. Though the genotypes first appeared endemically in geographically distinct regions, currently they are spread all over the world. As the Genotypes 1, 2 and 3 are widely seen all over the world, genotype 4 and 5 are predominant in Africa and prevalence of hepatitis C virus (HCV) genotype 6 may be as high as 50% in parts of Southeast Asia. For instance, in the U.S., approximately, 75% of all cases are caused by genotype 1, 15% by genotype 2, 5% by genotype 3 and 1% by genotype 4. Genotype 6 is typical to Southeast Asia, genotype 1 is prevalent in Western Europe and U.S, genotype 3 is very common in the UK. (14, 15, 16, 17)

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® HCV Genotyping Kit v7 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.

The assay utilizes the 5′ exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent hydrolysis 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® HCV Genotyping Kit v7 employs multiplex PCR, and an internal control is incorporated into the system in order to control the isolation procedure, check for possible PCR inhibition and application errors. 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 3′ end with FAM/HEX, through the FAM/HEX 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, Cy5) through the Cy5 channel.

9. PROCEDURE

9.1. RNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Viral Nucleic Acid Extraction Kit / Magrev®24 and Magrev® Viral DNA/RNA Extraction Kit / Bosphore Viral RNA Spin Kit (Anatolia Geneworks) isolation system is used with Bosphore® HCV Genotyping Kit v7. The RNA isolation should be performed according to the manufacturers' instructions. The recommended starting volume is 400 µl and the elution volume is 60 µl. The amount of internal control that should be used during isolation for each system is 5 µl.

9.2. Kit Components

9.2.1. PCR Master Mix 1

PCR Master Mix contains DNA polymerase, various salts and additives and dNTP mix. DNA polymerase is ultra pure, stable at higher temperatures, and highly resistant to PCR inhibitors.

PCR master mix also contains a unique reverse transcriptase that has improved activity and stability at higher temperatures.

PCR master mix also contains forward and reverse primers and dual-labeled probes specific for HCV genotypes 1b, 4 and for internal control.

9.2.2. PCR Master Mix 2

PCR Master Mix contains DNA polymerase, various salts and additives and dNTP mix. DNA polymerase is ultra pure, stable at higher temperatures, and highly resistant to PCR inhibitors.

PCR master mix also contains a unique reverse transcriptase that has improved activity and stability at higher temperatures.

PCR master mix also contains forward and reverse primers and dual-labeled probes specific for HCV genotypes 1a, 2 and for internal control.

9.2.3. PCR Master Mix 3

PCR Master Mix contains DNA polymerase, various salts and additives and dNTP mix. DNA polymerase is ultra pure, stable at higher temperatures, and highly resistant to PCR inhibitors.

PCR master mix also contains a unique reverse transcriptase that has improved activity and stability at higher temperatures.

PCR master mix also contains forward and reverse primers and dual-labeled probes specific for HCV genotype 3 and for internal control.

9.2.4. PCR Master Mix 4

PCR Master Mix contains DNA polymerase, various salts and additives and dNTP mix. DNA polymerase is ultra pure, stable at higher temperatures, and highly resistant to PCR inhibitors.

PCR master mix also contains a unique reverse transcriptase that has improved activity and stability at higher temperatures.

PCR master mix also contains forward and reverse primers and dual-labeled probes specific for HCV genotype 1(1 c, d, e, f, g, h, i, j, k) and for internal control. (Please see Analysis section*).

9.2.5. PCR Master Mix 5

PCR Master Mix contains DNA polymerase, various salts and additives and dNTP mix. DNA polymerase is ultra pure, stable at higher temperatures, and highly resistant to PCR inhibitors.

PCR master mix also contains a unique reverse transcriptase that has improved activity and stability at higher temperatures.

PCR master mix also contains forward and reverse primers and dual-labeled probes specific for HCV genotypes 5, 6 and for internal control.

9.2.6. PCR Master Mix 6

PCR Master Mix contains DNA polymerase, various salts and additives and dNTP mix. DNA polymerase is ultra pure, stable at higher temperatures, and highly resistant to PCR inhibitors.

PCR master mix also contains a unique reverse transcriptase that has improved activity and stability at higher temperatures.

PCR master mix also contains forward and reverse primers and dual-labeled probes specific for HCV genotypes 3a, 3b and for internal control.

9.2.7. 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 sample, proteinase K and carrier RNA mixture during RNA isolation to control the isolation efficiency, PCR inhibition and application errors. The amount of IC that should be added during isolation is 5 µl per 400 µl sample. We recommend adding the IC to the negative control at the extraction. 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. **Caution: It is not necessary to include the internal control in the reaction if it has already been added during the extraction step.** Lack of internal control amplification in the FAM/HEX 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.

9.2.8. Positive Control

The kit includes 6 positive controls;

Positive control 1: HCV 1b

Positive control 2: HCV 1a

Positive control 3: HCV 3

Positive control 4: HCV 1 (c, d, e, f, g, h, i, j, k)

Positive control 5: HCV 6

Positive control 6: HCV 3a

Each positive control should be used with its specific PCR Master Mix. They must be included in the PCR to test the efficiency of the PCR exclusively.

9.3. Preparing the RT-PCR

Make sure that all the kit components are thawed before use. Refer to the table below for preparing the PCR.

PCR Master Mix	26 µl
Internal Control *	0.4 µl
Sample RNA (Negative/Positive Control)	14 µl
Total Volume	40 µl

* Internal control should not be added in the reaction if it has already been added during the extraction step

Pipette 26 µl of the master mix into the PCR tubes or strips, and add 14 µl of RNA (sample/ 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® HCV Genotyping Kit v7 is composed of two-steps; first a reverse transcription step and secondly Real Time PCR steps; an initial denaturation for activation of Taq DNA Polymerase (with hot-start property), a two-step amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle.

The thermal protocol required to run with the PCR Master Mix 1, 2, 3 and 5 is in the table below:

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)	54°C	01:20 min.	
Synthesis	72°C	00:15 min.	
Hold	32°C	01:00 min.	

The thermal protocol required to run with the PCR Master Mix 4 and 6 is in the table below:

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)	58°C	01:10 min.	
Hold	32°C	01:00 min.	

Before starting a Real-Time PCR reaction using the Bosphore® HCV Genotyping Kit v7, the following steps should be completed:

- Choose the filters to be used (FAM,HEX and Cy5),
- Identify unknown samples, positive and negative controls,
- Select the correct thermal protocol.
- Start the experiment.

10. ANALYSIS

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

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, if the system allows pulling down the threshold as much as possible in order to detect slight amplifications, attention should be paid to keep the threshold line above the background.

Test results should not be reported unless there is amplification of the internal control in negative samples. 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 and HEX Channel are displayed with their positive/negative results, 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 Cy5 data of the internal control should also be checked to avoid false negative results.

The table below shows the possible results and their interpretation:

PCR Master Mix No	FAM Channel	HEX Channel	Cy5 Channel (Internal control)	Result
1	+	-	-/+	Sample is HCV 4
1	-	+	-/+	Sample is HCV 1b
1	-	-	+	Sample is negative for PCR Master Mix 1 (Genotype 4 and 1b)

1	-	-	-	Test must be repeated
2	+	-	-/+	Sample is HCV 1a
2	-	+	-/+	Sample is HCV 2
2	-	-	+	Sample is negative for PCR Master Mix 2 (Genotype 1a and 2)
2	-	-	-	Test must be repeated
3	None	+	-/+	Sample is HCV 3
3	None	-	+	Sample is negative for PCR Master Mix 3 (Genotype 3)
3	None	-	-	Test must be repeated
4	+	None	-/+	Sample is HCV 1 (HCV 1c,d,e,f,g,h,I,j,k if PCR Master Mix 1 HEX signal and PCR Master Mix 2 FAM signal are negative)*
4	-	None	+	Sample is negative for PCR Master Mix 4 (Genotype 1c,d,e,f,g,h,I,j,k)
4	-	None	-	Test must be repeated
5	-	+	-/+	Sample is HCV 6
5	+	-	-/+	Sample is HCV 5
5	-	-	+	Sample is negative for PCR Master Mix 5 (Genotype 5 and 6)
5	-	-	-	Test must be repeated
6	+	-	-/+	Sample is HCV 3a positive
6	-	+	-/+	Sample is HCV 3b positive
6	-	-	+	Sample is negative for PCR Master Mix 6 (Genotype 3a and 3b)
6	-	-	-	Test must be repeated

*Some 1a and 1b genotypes may also amplify with PCR Master Mix 4. So 1a and 1b genotype interpretation must exclusively performed according to the PCR Master Mix 1 and 2 data.

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® HCV Genotyping Kit v7 was found to be $1x10^2$ IU/ml (p=0.05). The sensitivity was determined using serial dilutions of RNA calibrated with the WHO International Standard for HCV RNA NAT assays, (NIBSC Code 06/100). 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 genotypes were ensured both by sequence comparison analysis and a Real-Time PCR assay using Worldwide HCV Performance Panel WWHV302(M) (Seracare). The following genotypes were tested and found positive for the following genotypes:

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

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 sequence comparison analysis using database alignment. Samples of HIV, HDV, HBV with known high positivity were tested, and found negative.

12. REFERENCES

1. 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.
4. Simmonds et al, Hepatology 21: 570-83, 1995
5. Klenerman et al (2009) PloS Med 6(6): e1000096. doi:10.1371/journal.pmed.1000096
6. Sizmann D, Boeck C, Boelter J, Fischer D, Miethke M, Nicolaus S, Zadak M, Babiel R. J Clin Virol 2007, 38:326-33.
7. J Viral Hepat. 2007 14: 213-20
8. Zeuzem et al J Viral Hepat 16:75-90
9. Strader et al Hepatology 2004, 39, 1147-1171.
10. Davis et al Hepatology 2003, 38, 645-652.
11. Fried, M. W. et al. 2002. N. Engl. J. Med. 347:975–982.
12. Manns, M. P., J. G. McHutchison, S. C. Gordon, V. K. Rustgi, M. Shiffman, R. Reindollar, Z. D. Goodman, K. Koury, M. Ling, and J. K. Albrecht. 2001. Lancet 358:958–965.
13. Kamal et al Gastroeneterology 2006, 130, 632-638.
14. Lau et al J Infect Dis 1995, 171, 281-289.
15. Simmonds et al Hepatology 2005, 42, 962-73.
16. Mellor et al J. Clin. Microbiol. 34, 417-423.
17. Chao D.T., Abe K. and Nguyen M.H., Alimentary Pharmacology & Therapeutics, Volume 34, Issue 3, pages 286–296, 2011.

13. SYMBOLS

 Use-by date

 Batch Code

 Catalogue number

 Caution

 Manufacturer

 *In vitro* Diagnostic Medical Device

14. ORDERING INFORMATION

Catalog Number: **ABHCGJ (25 rxn/box)**

ABHCGK (50 rxn/box)

ABHCGL (100 rxn/box)

15. CONTACT INFORMATION

Anatolia Tanı ve Biyoteknoloji Ürünleri Ar-Ge San. Ve Tic. A.S.

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IVD CE 1434

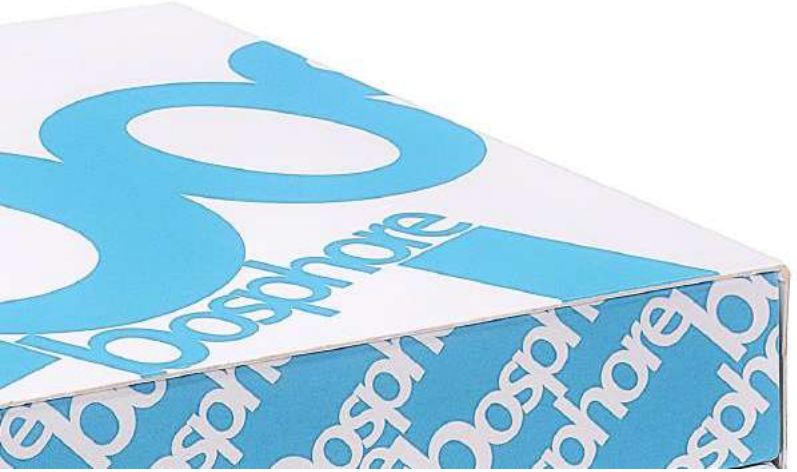
bosphore

INSTRUCTIONS FOR USE

HCV
Quantification Kit

For *In Vitro* Diagnostic Use

MB197v10f
05th February 2025



Anatolia geneworks

CONTENTS

1. PRODUCT DESCRIPTION

Bosphore HCV Quantification Kit is a Real-Time PCR kit for *in vitro* diagnostics that detects and characterizes the 5' UTR region of HCV genotypes (1-8) from serum and plasma samples. Fluorescence detection is performed using FAM, and HEX filters. HCV RNA is amplified, and fluorescence detection is performed using the FAM filter.

Component	FAM	HEX
PCR Master Mix	HCV Genotypes (1-8)*	Internal Control

*Although some scientific articles mentioned the existence of genotypes 7 and 8; Genotype 7 has not been formally disclosed or recognized yet by the WHO, Genotype 8 has not been formally disclosed or recognized yet by the WHO, EASL, and CDC. Bosphore HCV Quantification Kit does not detect genotypes separately.

Internal control has been integrated into the kit to check RNA extraction, PCR inhibition, or application problems. The amplification data of the internal control is detected with the HEX filter. The internal control can be added either during RNA extraction or the PCR step.

2. CONTENT

Bosphore HCV Quantification Kit consists of the following dH_2O, Real-Time PCR Master Mix, positive control, internal control, 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	(1,000 µL)	(1,000 µL)	(500 µL)
2	PCR Master Mix	(2,640 µL)	(1320 µL)	(660 µL)
3	Internal Control	(560 µL)	(280 µL)	(140 µL)
4	Positive Control	(70 µL)	(35 µL)	(18 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 (2×10^3) IU/mL	(880 µL)	(880 µL)	(440 µL)

3. STORAGE

PCR reagents for Bosphore HCV Quantification Kit 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 minutes, and the PCR master 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 PCR master mix in a closed container. If the components are stored according to the recommended conditions, they will remain stable until the expiry dates on the labels.

4. REQUIRED MATERIALS AND DEVICES

- Montania 4896 Real-Time PCR Instrument – Anatolia Geneworks, CFX96 Real-Time PCR Detection System - Bio-Rad, QuantStudio 5 Real-Time PCR System – ThermoFisher, LightCycler 480 Instrument II – Roche, Q qPCR Cycler - Quantabio, Rotor-Gene Q – QIAGEN or another Real-Time PCR system with FAM, and HEX filters*
- 0.1 mL or 0.2 mL thin-wall PCR tubes, PCR plates or strips
- Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Viral DNA/RNA Extraction Kit 600 µl, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit, Unio M32 Extraction System and Unio M32 Viral DNA/RNA Extraction Kit, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Viral DNA/RNA Extraction Kit, Bosphore Viral RNA Extraction Spin Kit, Bosphore Viral DNA/RNA Extraction Spin Kit or Bosphore Nucleic Acid Extraction Versatile Spin Kit or Magnesia 16 Nucleic Acid Extraction Instrument and Magnesia Viral DNA/RNA Extraction Kit EP (Anatolia Geneworks) or other high-quality RNA extraction kits and systems
- Deep freezer (-20 °C)
- Desktop centrifuge with rotor for 2 mL or 1.5 mL microcentrifuge tubes
- DNase, RNase, pyrogen-free 1.5 mL or 2 mL microcentrifuge tubes
- Calibrated, adjustable micropipettes
- DNase, RNase, pyrogen-free micropipette tips with filters
- Disposable laboratory gloves, coats and caps

* For other Real-Time PCR devices that can be used with Bosphore HCV Quantification Kit, please contact Anatolia Geneworks from the information in Section 15.

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

- The product should be delivered on dry ice. Check for the presence of dry ice upon arrival.
- Check for the expiration dates on the box and tube labels upon arrival. Do not use expired products or components.

- Calibrated or verified micropipettes, DNase, RNase, and pyrogen-free micropipette tips with filters, and DNase, RNase, and 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 before use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared and quickly returned to -20 ºC.
- PCR and nucleic acid extraction 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 and in a microbiological safety cabinet of the appropriate class. Physical contact with pathogens should be avoided by wearing lab coats and gloves, making no allowance for eating or drinking within the workspace, and preventing unauthorized individuals' access to the working area.
- After working biological samples, all pathogenic wastes produced during the nucleic acid extraction step, including materials contacted with them, should be discarded into medical waste, and disposed of safely.

6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for *in vitro* diagnostics.
- This product should be used by this user manual.
- The assay is intended to be used as an aid in the management of patients infected with HCV under antiviral treatment; to measure HCV RNA levels at baseline and during treatment to assess response to treatment. It is not intended for screening or confirmation of the presence of HCV RNA in blood or blood products.
- This product is to be used by personnel specially trained to perform *in vitro* diagnostic procedures.
- The product has been validated only for use with serum samples or plasma samples containing EDTA or citrate anticoagulant.

7. INFECTION

Hepatitis C is a liver infection caused by the hepatitis C virus (HCV). Hepatitis C is spread through contact with blood from an infected person. Today, most people become infected with the hepatitis C virus by sharing needles or other equipment used to prepare and inject drugs.

For some people, hepatitis C is a short-term illness, but for more than half of people who become infected with the hepatitis C virus, it becomes a long-term, chronic infection. Chronic hepatitis C can result in serious, even life-threatening health problems like cirrhosis and liver cancer.

People with chronic hepatitis C can often have no symptoms and don't feel sick. When symptoms appear, they often are a sign of advanced liver disease. There is no vaccine for hepatitis C. The best way to prevent hepatitis C is by avoiding behaviors that can spread the disease, especially injecting drugs. Getting tested for hepatitis C is important because treatments can cure most people with hepatitis C in 8 to 12 weeks (CDC, 2023).

8. METHOD

Bosphore HCV Quantification Kit is based on the Real-Time PCR method. The polymerase chain reaction is a technique that is used for the amplification of an DNA region. The reaction occurs through repeated cycles of heating and cooling. The main components of PCR are primers, dNTPs, *Taq* DNA Polymerase (with hot-start property), buffer solutions, and templates. As a brief explanation, primers are small synthetic DNA that anneals to the specific regions of the template to start the synthesis, dNTPs are the building blocks of the amplified products, and *Taq* DNA Polymerase amplifies the DNA template. Finally, buffer solutions provide the pH adjustment required for the reaction, and the template, as referred to, 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 the Real-Time PCR technique, in contrast to conventional PCR, PCR products can be monitored during the reaction. Therefore, Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, thereby minimizing the risk of contamination. Dual-labeled probes employed in the reaction, in addition to the conventional PCR reagents, enable the detection of the amplified target with increased sensitivity. The assay utilizes the 5' exonuclease activity of *Taq* DNA Polymerase to cleave a dual-labeled fluorescent hydrolysis 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 nearby, and the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step of PCR, *Taq* DNA Polymerase encounters and cleaves the probe bound to the template. As the reporter is freed from the suppressing effect of the quencher, a 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 the 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 HCV Quantification Kit uses multiplex qPCR and internal control is included in the system to control the extraction procedure, PCR inhibition, and application issues.

The reaction is performed in one PCR tube containing PCR Master Mix. In PCR Master Mix, HCV genome amplification is screened using the FAM filter, and the fluorescent signal produced by the internal control amplification is detected in PCR Master Mix via the HEX filter.

9. PROCEDURE

9.1. Specimen Collection & Storage

Bosphore HCV Quantification Kit is to be used with serum or plasma samples. Blood samples can be collected in serum separating tubes or tubes containing anticoagulant as citrate or EDTA, but we recommend that serum and plasma must be prepared as soon as the blood sample is obtained; within 1 hour if the blood is stored at room temperature, or within 24 hours if the blood is stored between 2 °C and 8 °C. After the centrifugation (for serum 10-20 min at 1100-1300 x g, for plasma 10-20 min at 1000-2000 g is recommended), the upper clear phase must be pipetted carefully into a sterile polypropylene tube without disturbing the red bottom phase, so that only serum or plasma must be subjected to RNA extraction.

Plasma and serum samples can be stored for up to 2 days at +4 °C. The serum and plasma samples can be stored at -20 °C (between -20 °C to 90 °C) for 6 weeks. During this period, freeze and thaw of the sample more than 3 times should be avoided in order not to lose RNA.

9.2. RNA Extraction

We recommend that Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Viral DNA/RNA Extraction Kit 600 µl, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit, Unio M32 Extraction System and Unio M32 Viral DNA/RNA Extraction Kit, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Viral DNA/RNA Extraction Kit, Bosphore Viral RNA Extraction Spin Kit, Bosphore Viral DNA/RNA Extraction Spin Kit or Bosphore Nucleic Acid Extraction Versatile Spin Kit or Magnesia 16 Nucleic Acid Extraction Instrument and Magnesia Viral DNA/RNA Extraction Kit EP (Anatolia Geneworks) or other high-quality RNA extraction kits and systems are used with Bosphore HCV Quantification Kit. The RNA extraction should be performed according to the manufacturer's instructions.

9.3. Kit Components

9.3.1. Negative Control

The negative control is an essential component of Bosphore HCV Quantification Kit for Real-Time PCR. It consists of distilled water (dH_2O) and serves as a reference sample to ensure accurate results by detecting any contamination or errors that could lead to false-positive outcomes. Including the negative control and carefully monitoring it will enable you to confidently interpret your data.

9.3.2. PCR Master Mix

PCR Master Mix contains a highly specific and accurate *Taq* DNA Polymerase (with hot-start property), reverse transcriptase, PCR buffers, and dNTPs mix. PCR Master Mix also contains forward and reverse primers and dual-labeled probes specific for HCV and internal control.

9.3.3. Internal Control

The internal control included in the kit to check for RNA extraction, PCR inhibition, and application errors, internal control is a synthetic DNA molecule. The internal control is added to the mixture of sample, proteinase K, and Carrier RNA at the beginning of RNA extraction to check extraction efficiency and application errors. If the internal control is to be added during nucleic acid extraction, 5 µL is added per sample; and when added directly to the PCR Master Mix to control PCR inhibition, 0.2 µL is added. We recommend adding an internal control to the negative control to evaluate the efficiency of the extraction system. The external quantitation standards are provided as serum, so that they undergo the same steps as the patient samples, starting from RNA extraction.

Caution! It is not necessary to include the internal control in the PCR Master Mix if it has already been added during the extraction step. The absence of internal control amplification in the HEX filter in negative samples may indicate a problem in extraction or application, or that the PCR reaction is inhibited. In this case, extraction and PCR should be repeated. In samples with high viral load, including the positive control, the internal control may be suppressed and an increase in fluorescent signal may not be detected. Therefore, internal control amplification should be evaluated according to the table in Section 10.

9.3.4. Positive Control

The kit contains 1 positive control containing synthetic HCV DNA. It must be included in the PCR reaction to test the reaction efficiency.

Positive Control: Synthetic HCV DNA

The threshold C_T value of the positive control is given in the acceptance criteria table (Section 10). A threshold C_T value of the positive control greater than the upper limit of the range in the table may indicate a loss of yield in the reaction.

9.3.5. Quantitation Standards

The quantitation serum standards are calibrated by the WHO International Standard (NIBSC Code: 06/100).

9.4. Preparing the PCR

The standards, positive and negative controls should be added into the PCR reaction together with the samples. Ensure that all kit components are dissolved before use and refer to the table below to prepare the PCR reaction mix. The volumes given are for one reaction only, multiply these volumes by the number of samples to find the volumes required for the master mix. When preparing the mix for more than 5 samples, 10% should be added to the total number of samples.

Components	Volume
PCR Master Mix	24 µL
Internal Control*	0.2 µL*
Sample RNA (Negative / Positive Control /Standards)	16 µL
Total Volume	40 µL

*Internal control should not be added to the reaction if it has already been added during the extraction step.

Pipette 24 µL of the PCR Master Mix into the PCR tubes or strips and add 16 µL of template (sample/ positive or negative control/ standards). Close the tube cap. Make sure that the mix 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 HCV Quantification Kit consists of reverse transcription, initial denaturation for activation of the *Taq* DNA Polymerase (with hot-start property), a three-step amplification cycle, and a terminal hold. The Real-Time data is collected at the second step of the amplification cycle. The thermal protocol to be applied for the reaction is indicated on the next page.

Steps	Temperature	Time	
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	00:15 min	
Hold	32 °C	01:00 min	

Before starting to work with Bosphore HCV Quantification Kit, the following steps must be completed and checked:

- Choose all the filters to be used (FAM and HEX),
- Identify unknown samples, positive and negative controls, standards,
- Select the correct thermal protocol,
- Start the experiment.

10. ANALYSIS

By the end of the thermal protocol, the Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold. An example of an amplification curve is given in Figure 1.

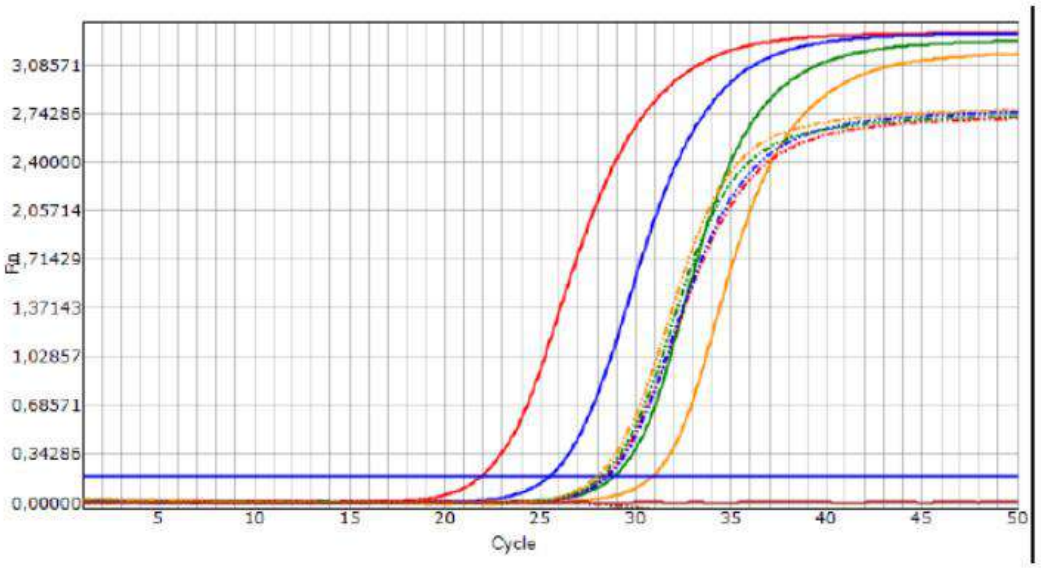


Figure 1: Amplification curve of Bosphore HCV Quantification Kit

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

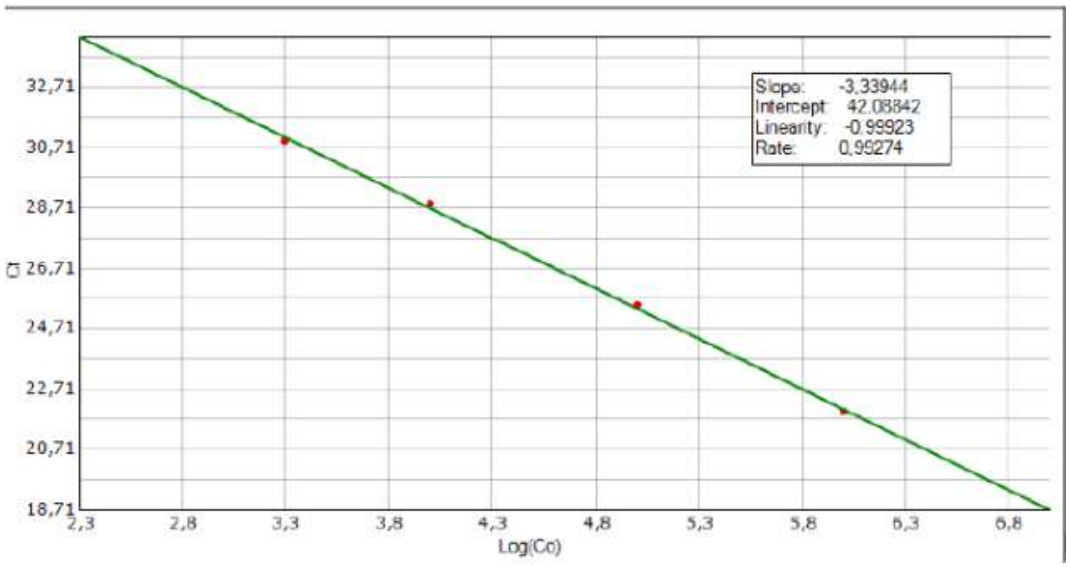


Figure 2: Standard curve of Bosphore HCV Quantification Kit

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 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 the manufacturer, consider it necessary if the system allows pulling down the threshold as much as possible to detect low amplifications, attention should be paid to keep the threshold line above the background.

The negative control is essential for accurate result analysis. Please check the negative control and ensure it shows no amplification outside the associated internal control filter. If the negative control has a signal outside the filter associated with the internal control, please do not report the results. Repeat the experiment after taking the necessary precautions against contamination. If the same result is encountered again, please contact the manufacturer.

Internal control, standards, and positive control of Bosphore HCV Quantification Kit are essential for accurate result analysis. The cycle threshold acceptance criteria for the internal control, standards, and positive control are listed below:

Component / Parameter	Threshold Value (C_T)*
Standard 1	22±4
Standard 2	25±4

Standard 3	28.5±4
Standard 4	31±4
Internal Control	≤32
Positive Control	30±4
Correlation Coefficient	>0.970
PCR Efficiency**	>80%

*Cycle thresholds of the standards change depending on the starting volume and the efficiency of the extraction system used.

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

In quantitative test results; samples that cross the threshold in the FAM filter; it is evaluated as "Positive", samples that do not cross the threshold are shown as "No C_T" or "Negative". These samples are considered to have a negative or viral load below the detection limit of the assay. The internal control data in the HEX filter of these "undetected" samples should also be checked to avoid false negative results.

The delayed amplification of the internal control may indicate a problem in nucleic acid extraction / PCR inhibition or application failure. In this case, extraction and PCR should be repeated. Please consider that in the samples that contain high viral concentration, internal control can be suppressed therefore delayed or no increase in internal control signal may be detected.

Please note that this product only provides testing pathogens shown below. Experimental results from the tube must be considered when providing a result for the patients, also in consideration of the patient's clinical findings, and the guidelines of the relevant health authorities. The table below shows the possible results and their interpretation:

	HCV (FAM)	Internal Control (HEX)	Result
PCR Master Mix	+	+/-	The sample is HCV positive
	-	+	Sample is negative
	-	-	The test should be repeated!

In rare cases of PCR inhibition due to medication or other PCR inhibitors in the sample, we recommend repeating 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 HCV Quantification Kit was found to be 12 IU/mL. The sensitivity was determined using Magrev 24 Stand and Magrev Viral DNA/RNA Extraction Kit using 1000 µL starting volume and 60 µL elution volume (Anatolia Geneworks) and by testing serial dilutions of serum samples calibrated with the WHO International Standard Hepatitis C Virus for Nucleic Acid Amplification Techniques (4th WHO International Standard (NIBSC Code: 06/102). The dilutions were subjected to viral RNA Extraction and Real-Time PCR in different runs and replicates. The results were analyzed by the probit method.

The detection limit in consideration with the extraction for Bosphore HCV Quantification Kit was found to be 19.5 IU/mL using the Magnesia 2448 Viral DNA/RNA Extraction Kit and Magnesia 2448 Nucleic Acid Extraction & PCR Setup Robot using 400 µL starting volume and 60 µL elution volume (Anatolia Geneworks).

The detection limit in consideration in consideration with the extraction for HCV Quantification Kit was found to be 25 IU/mL using the Magnesia 16 Nucleic Acid Extraction Instrument and Magnesia Viral DNA/RNA Extraction Kit EP using 400 µL starting volume and 60 µL elution volume (Anatolia Geneworks).

11.2. Genotype Detection

The efficiency of detecting and quantitating different genotypes was ensured both by sequence comparison analysis and Real-Time PCR assays using QCMD Hepatitis C virus RNA EQA Programme 2011-2017 panel members and natural clinical samples (containing samples with HCV genotypes 1a, 1b, and 3a) and the Worldwide HCV Performance Panel WWHV302(M) (Seracare). The following genotypes of the genotype panel were tested and found positive.

WWHV302(M) 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, 16 plasma samples of the QCMD (2015-2017) Hepatitis C virus RNA EQA Programmes, 24 plasma samples of Hepatitis C Virus Genotype 2013, 2015, and 2016 QCMD EQA Programmes having various HCV genotypes, and 100 natural patient samples which contained genotypes 1,2,3,4,5, and 6, have been successfully tested with Bosphore HCV Quantification Kit.

Additionally, subsequent to the fixed genotypes as per the QCMD report, further investigation was conducted to confirm the detectability of HCV genotypes 7 and 8. Through meticulous in-silico analysis, it was ascertained that these genotypes can be reliably detected using Bosphore HCV Quantification Kit, strengthening the assay's capability to identify and quantify a broad spectrum of HCV genotypes, including the rare genotypes 7 and 8.

11.3. Linear Range

The linear range of Bosphore HCV Quantification Kit was determined to be from 12 IU/mL to at least $1x10^9$ IU/mL. To assess the linear range, a serum dilution series which has been calibrated against the WHO International Standard for HCV RNA NAT assays (NIBSC Code: 06/100) was analyzed by viral RNA extraction and Real-Time PCR. The standard curve correlation coefficient was found to be 0.995.

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. To eliminate the risk of cross-reactivity; HIV, HDV, HBV, West Nile Virus, and Dengue Virus samples with known high positivity were tested and found negative. The experimental results indicated that the kit detects specifically and only HCV pathogens that it intends to detect, but not the others.

11.5. Reproducibility and Precision

Reproducibility and Precision data (on a C_T value basis) were obtained by the analysis of the previously quantitated HCV positive samples. The test was performed in at least 4 replicates by 3 different operators, on multiple days, using 3 different lots. The resulting data is given on the next page for 10^4 IU/mL.

Reproducibility Variability (HCV)	Standard Deviation	Variance	Coefficient of Variation [%]
Intra-assay (n=4)	0.03	0.001	0.11
Inter-lot (n=3)	0.29	0.08	0.92
Inter-operator (n=3)	0.26	0.08	0.92
Total Inter-assay (n=5)	0.25	0.06	0.81

Precision Variability (HCV)	Measured Quantity (MQ) IU/mL	Standard Deviation (MQ)	Coefficient of Variation [%] (MQ)	Threshold Cycle (C_T)	Standard Deviation (C_T)
Intra-assay (n=4)	10852.5	267.87	2.46	31.24	0.03
Inter-lot (n=3)	11072.17	1074.97	9.70	31.41	0.29
Inter-operator (n=3)	9290.58	1354.50	14.57	31.53	0.26
Total Inter-assay (n=5)	10047.15	1596.95	15.89	31.52	0.25

11.6. Diagnostic Evaluation

The diagnostic evaluation was initially performed by testing a total of 213 HCV negative and 71 HCV positive serum and plasma samples which have been previously analyzed using Roche Diagnostics Elecsys 2010, Roche COBAS Amplicor HCV RNA Monitor v2.0, Bayer Versant HCV RNA v3.0, Abbott HCV RNA m2000, Roche HCV RNA Taqman and Artus HCV RG RT-PCR Kit. All of the negative samples were found negative, and all of the positive samples were found positive with Bosphore HCV Quantification Kit.

11.7. Calibration Against WHO Standard

Quantitation standards were calibrated against the WHO HCV RNA International Standard (NIBSC Code: 06/100 and 06/102). 1 IU was found to be equal to 3±0.2 copies/mL.

12. REFERENCES

1. https://www.cdc.gov/hepatitis/hcv/index.htm

13. SYMBOLS

	Use-by Date		Caution, consult accompanying documents.
	Batch Code		Manufacturer
	Catalog Number		*In vitro* Diagnostic Device

14. ORDERING INFORMATION

Catalog Number:

ABHCQ3 (100 rxn/box)
ABHCQ2 (50 rxn/box)
ABHCQ1 (25 rxn/box)

15. CONTACT INFORMATION

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Document Revision History

Document Version No	Revision No	Date	Description
V1	01	04th December 2010	First Publishing
V2	02	12th January 2011	Partial content correction
V3	03	06th May 2012	The general content and type check
V4	04	05th January 2013	The content has been updated and checked
V5	05	04th May 2014	The general content and type check
V6	06	13th August 2016	The content has been updated and checked
V7	07	04th August 2019	The general content and type check
V8	08	30th November 2023	Partial content correction
V9	09	19th April 2024	Added Specimen Collection & Storage section and sensitivity for Magnesia 16 Nucleic Acid Extraction Instrument

IVD CE 1434

bosphore

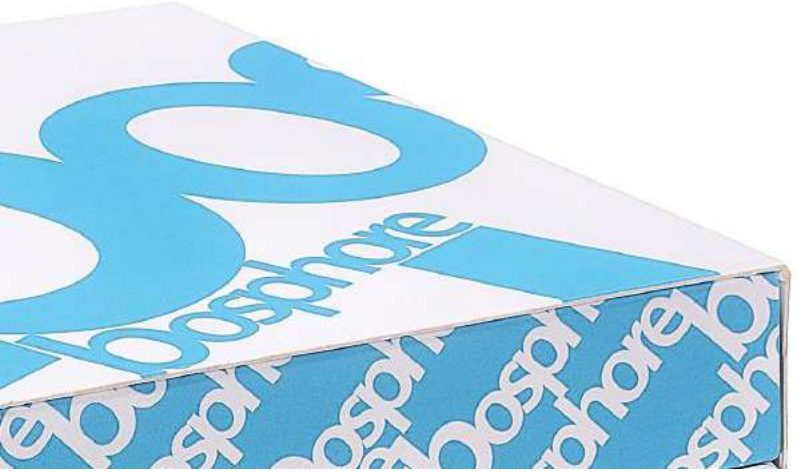
INSTRUCTIONS FOR USE

HDV

Quantification-Detection Kit v1

For *In Vitro* Diagnostic Use

MB18v15f
14th April 2024



Anatolia geneworks

CONTENTS

1. PRODUCT DESCRIPTION

Bosphore HDV Quantification-Detection Kit v1 is a Real-Time PCR kit for *in vitro* diagnostics that 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 quantification is from $1x10^2$ copies/mL to $1x10^8$ copies/mL, and the analytical sensitivity is 45 copies/mL. A region within the structural antigen gene is amplified and fluorescence detection is accomplished using the FAM filter.

Component	**FAM** (Gene)	**HEX**
PCR Master Mix	HDV (Genotypes 1-8)*	Internal Control

*Bosphore HDV Quantification-Detection Kit v1 does not detect genotypes separately.

Internal control has been integrated into the kit to check RNA extraction, PCR inhibition, or application problems. The amplification data of the internal control is detected with the HEX filter. The internal control can be added either during RNA extraction or the PCR step.

2. CONTENT

Bosphore HDV Quantification-Detection Kit v1 consists of the following dH_2O, Real-Time PCR Master Mix, RT Mix, positive control, standards, and internal control.

Component	**Reagent**	**100 Reactions**	**50 Reactions**	**25 Reactions**
1	dH_2O	(1,000 µL)	(1,000 µL)	(1,000 µL)
2	PCR Master Mix	(1,660 µL)	(800 µL)	(400 µL)
3	RT Mix	(28 µL)	(14 µL)	(7 µL)
4	Internal Control	(550 µL)	(275 µL)	(275 µL)
5	Positive Control	(44 µL)	(22 µL)	(22 µL)
6	Standard 1 (10,000 copies/µL)*	(88 µL)	(44 µL)	(44 µL)
7	Standard 2 (1,000 copies/µL)*	(88 µL)	(44 µL)	(44 µL)
8	Standard 3 (100 copies/µL)*	(88 µL)	(44 µL)	(44 µL)
9	Standard 4 (20 copies/µL)*	(88 µL)	(44 µL)	(44 µL)

*See "Analysis" section for RNA quantitation

3. STORAGE

PCR reagents for Bosphore HDV Quantification-Detection Kit v1 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 minutes, and the PCR master 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 PCR master mix in a closed container.

If the components are stored according to the recommended conditions, they will remain stable until the expiry dates on the labels.

4. REQUIRED MATERIALS AND DEVICES

- Montania 4896 Real-Time PCR Instrument – Anatolia Geneworks, CFX96 Real-Time PCR Detection System - Bio-Rad, QuantStudio 5 Real-Time PCR System – ThermoFisher, LightCycler 480 Instrument II – Roche, Q qPCR Cycler - Quantabio, Rotor-Gene Q - QIAGEN or another Real-Time PCR system with FAM and HEX filters*
- 0.1 mL or 0.2 mL thin-wall PCR tubes, PCR plates or strips
- Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Viral DNA/RNA Extraction Kit 600 µl, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit, Unio M32 Extraction System and Unio M32 Viral DNA/RNA Extraction Kit, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Viral DNA/RNA Extraction Kit, Bosphore Viral RNA Extraction Spin Kit, Bosphore Viral DNA/RNA Extraction Spin Kit or Bosphore Nucleic Acid Extraction Versatile Spin Kit or Magnesia 16 Nucleic Acid Extraction Instrument and Magnesia Viral DNA/RNA Extraction Kit EP (Anatolia Geneworks) or other high-quality RNA extraction kits and systems
- Deep freezer (-20 °C)
- Desktop centrifuge with rotor for 2 mL or 1.5 mL microcentrifuge tubes
- DNase, RNase, pyrogen-free 1.5 mL or 2 mL microcentrifuge tubes
- Calibrated, adjustable micropipettes
- DNase, RNase, pyrogen-free micropipette tips with filters
- Disposable laboratory gloves, coats and caps

*For other Real-Time PCR devices that can be used with Bosphore HDV Quantification-Detection Kit v1, please contact Anatolia Geneworks from the information in Section 15

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

- The product should be delivered on dry ice. Check for the presence of dry ice upon arrival.
- Check for the expiration dates on the box and tube labels upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNase, RNase, and pyrogen-free micropipette tips with filters, and DNase, RNase, and 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 before use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared and quickly returned to -20 ºC.
- PCR and nucleic acid extraction 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 and in a microbiological safety cabinet of the appropriate class. Physical contact with pathogens should be avoided by wearing lab coats and gloves, making no allowance for eating or drinking within the workspace, and preventing unauthorized individuals' access to the working area.
- After working biological samples, all pathogenic wastes produced during the nucleic acid extraction step, including materials contacted with them, should be discarded into medical waste, and disposed of safely.

6. PRODUCT USE LIMITATIONS

- This product is a NAT test intended to be used for in vitro diagnostics.
- The assay is intended to be used as an aid in the management of patients infected with HDV under antiviral treatment; to measure HDV RNA levels at baseline and during treatment to assess response to treatment. It is not intended for screening or confirmation of the presence of HDV RNA in blood or blood products.
- This product should be used by this user manual, by personnel specially trained to perform in vitro diagnostic procedures.
- The product has been validated only for use with serum samples or plasma containing EDTA or citrate anticoagulant.

7. INFECTION

Hepatitis D virus (HDV) is a virus that requires Hepatitis B virus (HBV) for its replication. Hepatitis D virus (HDV) affects globally nearly 5% of people who have a chronic infection with Hepatitis B virus (HBV). HDV infection occurs when people become infected with both Hepatitis B and D simultaneously (co-infection) or get hepatitis D after first being infected with hepatitis B (super-infection). Populations that are more likely to have HBV and HDV co-infection include recipients of hemodialysis and people who inject drugs. Worldwide, the number of HDV infections has decreased since the 1980s, due mainly to a successful global HBV vaccination programme. The combination of HDV and HBV infection is considered the most severe form of chronic viral hepatitis due to more rapid progression towards liver-related death and hepatocellular carcinoma. Hepatitis D infection can be prevented by hepatitis B immunization, but treatment success rates are low (WHO, 2023).

8. METHOD

Bosphore HDV Quantification-Detection Kit v1 is based on the Real-Time PCR method. The polymerase chain reaction is a technique that is used for the amplification of an DNA region. The reaction occurs through repeated cycles of heating and cooling. The main components of PCR are primers, dNTPs, *Taq* DNA Polymerase (with hot-start property), buffer solutions, and templates. As a brief explanation, primers are small synthetic DNA that anneals to the specific regions of the template to start the synthesis, dNTPs are the building blocks of the amplified products, and *Taq* DNA Polymerase amplifies the DNA template. Finally, buffer solutions provide the pH adjustment required for the reaction, and the template, as referred to, 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 the Real-Time PCR technique, in contrast to conventional PCR, PCR products can be monitored during the reaction. Therefore, Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, thereby minimizing the risk of contamination. Dual-labeled probes employed in the reaction, in addition to the conventional PCR reagents, enable the detection of the amplified target with increased sensitivity.

The assay utilizes the 5’ exonuclease activity of *Taq* DNA Polymerase to cleave a dual-labeled fluorescent hydrolysis 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 nearby, and the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step of PCR, *Taq* DNA Polymerase encounters and cleaves the probe bound to the template. As the reporter is freed from the suppressing effect of the quencher, a 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 the 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, the 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 uses multiplex qPCR and internal control is included in the system to control the extraction procedure, PCR inhibition, and application issues.

The reaction is performed in one PCR tube containing PCR Master Mix.

In PCR Master Mix, HDV RNA amplification is screened using the FAM filter, and internal control amplification is screened using the HEX filter.

9. PROCEDURE

9.1. Specimen Collection, Storage, and RNA Extraction

Bosphore HDV Quantification-Detection Kit v1 is to be used with serum or plasma samples. Blood samples can be collected in serum separating tubes or tubes containing anticoagulant as citrate or EDTA, but we recommend that serum and plasma must be prepared as soon as the blood sample is obtained; within 1 hour if the blood is stored at room temperature, or within 24 hours if the blood is stored between 2 °C and 8 °C. After the centrifugation (for serum 10-20 min at 1,100-1,300 x g, for plasma 10-20 min at 1,000-2,000 g is recommended), the upper clear phase must be pipetted carefully into a sterile polypropylene tube without disturbing the red bottom phase, so that only serum or plasma must be subjected to RNA extraction. Plasma and serum samples can be stored for up to 2 days at +4 °C. The serum and plasma samples can be stored at -20 °C (between -20 °C to -90 °C) for 6 weeks. During this period, the freeze and thaw of the sample more than 3 times should be avoided in order not to lose RNA.

We recommend that Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Viral DNA/RNA Extraction Kit 600 µl, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit, Unio M32 Extraction System and Unio M32 Viral DNA/RNA Extraction Kit, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Viral DNA/RNA Extraction Kit, Bosphore Viral RNA Extraction Spin Kit, Bosphore Viral DNA/RNA Extraction Spin Kit or Bosphore Nucleic Acid Extraction Versatile Spin Kit or Magnesia 16 Nucleic Acid Extraction Instrument and Magnesia Viral DNA/RNA Extraction Kit EP (Anatolia Geneworks) are used with Bosphore HDV Quantification-Detection Kit v1. The RNA extraction should be performed according to the manufacturer's instructions.

The amount of internal control that should be used during extraction 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. Negative Control

The negative control is an essential component of Bosphore HDV Quantification-Detection Kit v1 for Real-Time PCR. It consists of distilled water (dH_2O) and serves as a reference sample to ensure accurate results by detecting any contamination or errors that could lead to false-positive outcomes. Including the negative control and carefully monitoring it will enable you to confidently interpret your data.

9.2.2. PCR Master Mix

PCR Master Mix contains a highly specific and accurate *Taq* DNA Polymerase (with hot-start property), PCR buffers, and dNTPs mix. PCR Master Mix also contains forward and reverse primers and dual-labeled probes specific for HDV and internal control.

9.2.3. RT Mix

RT Mix contains a high quality and performance reverse transcriptase and RNase inhibitor mix.

9.2.4. Internal Control

The internal control included in the kit to check for RNA extraction, PCR inhibition, and application errors, the internal control is a synthetic DNA molecule. The internal control is added to the mixture of sample, proteinase K, and Carrier RNA at the beginning of RNA extraction to check extraction efficiency and application errors. If the internal control is to be added during nucleic acid extraction, 5 µL is added per sample; and when added directly to the PCR Master Mix to control PCR inhibition, 0.25 µL is added. We recommend adding an internal control to the negative control to evaluate the efficiency of the extraction system.

Caution! It is not necessary to include the internal control in the PCR Master Mix if it has already been added during the extraction step. The absence of internal control amplification in the HEX filter in negative samples may indicate a problem in extraction or application, or that the PCR reaction is inhibited. In this case, extraction and PCR should be repeated. In samples with high viral load, including the positive control, the internal control may be suppressed and an increase in fluorescent signal may not be detected. Therefore, internal control amplification should be evaluated according to the table in Section 10.

9.2.5. Quantitation Standards

The quantitation standards are previously extracted and calibrated standards of 10,000, 1,000, 100, and 20 copies/µL. They are directly included in the PCR reaction, just as the extracted sample RNA. See the "Analysis" Section for RNA quantitation.

9.2.6. 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 purposes, there is no need to include it in every reaction, but for qualitative purposes (when standards are not used) it must be included in every PCR.

9.3. Preparing the PCR

The standards, positive and negative controls should be added into the PCR reaction together with the samples. Ensure that all kit components are dissolved before use and refer to the table below to prepare the PCR reaction mix. The volumes given are for one reaction only, multiply these volumes by the number of samples to find the volumes required for the master mix. When preparing the mix for more than 5 samples, 10% should be added to the total number of samples.

Components	Volume
PCR Master Mix	14.5 µL
RT Mix	0.25 µL
Internal Control*	0.25 µL*
Sample RNA (Negative / Positive Control / Standards)	10 µL
Total Volume	25 µL

*Internal control should not be added to the reaction if it has already been added during the extraction step.

Pipette 15 µL of the PCR Master Mix (PCR Master Mix – RT Mix mixture) into the PCR tubes or strips and add 10 µL of template (sample/ positive or negative control/standards). Close the tube cap. Make sure that the mix 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 consists of reverse transcription, initial denaturation for activation of the *Taq* DNA Polymerase (with hot-start property), a two-step amplification cycle, and a terminal hold.

The Real-Time data is collected at the second step of the amplification cycle. The thermal protocol to be applied for the reaction is indicated on below:

Steps	Temperature	Time	
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	00:15 min	
Hold	22 °C	01:00 min	

Before starting to work with Bosphore HDV Quantification-Detection Kit v1, the following steps must be completed and checked:

- Choose all the filters to be used (FAM and HEX),
- Identify unknown samples, positive and negative controls, standards,
- Select the correct thermal protocol,
- Start the experiment.

10. ANALYSIS

By the end of the thermal protocol, the Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold. Analysis of the results should be performed by trained personnel who have received the required training for analyzing Real-Time PCR data. An example of an amplification curve is given in Figure 1.

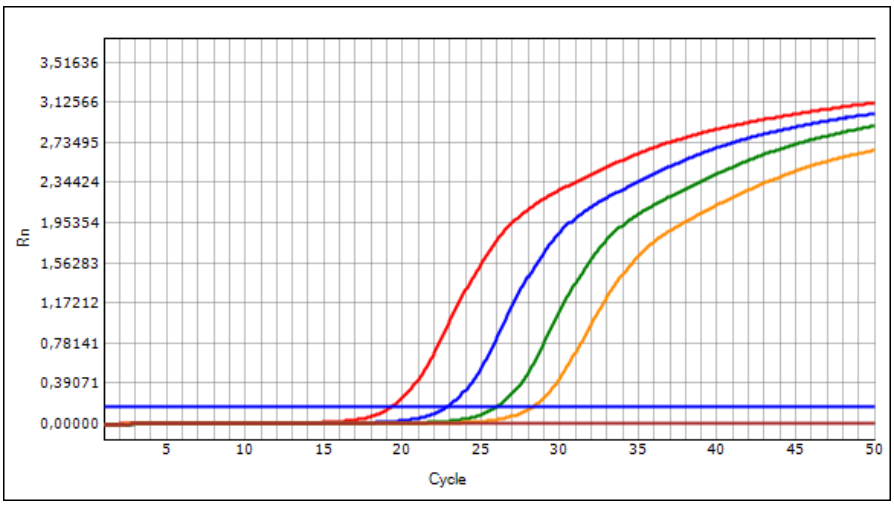


Figure 1: Amplification Curve of a Bosphore HDV Quantification-Detection Kit v1

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

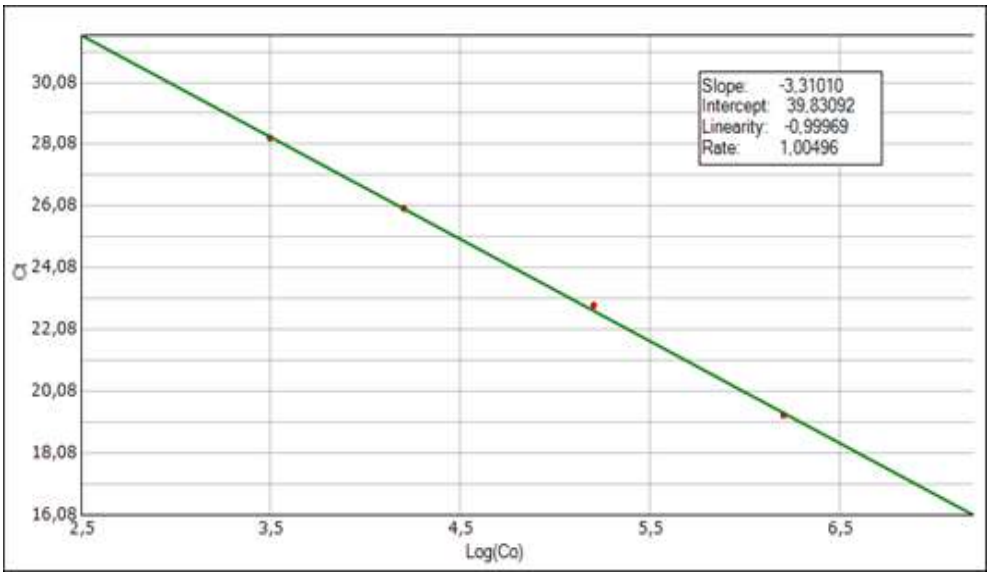


Figure 2: Standard Curve of a Bosphore HDV Quantification-Detection Kit v1

Caution!: Since the quantitation standards are provided as plasmid samples and as copies/ul, the following formula should be applied to the resulting copies/ul 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 the 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 1,000 copies/µL, considering that the starting extraction volume is 400 ul and the elution volume is 60 ul, applying the formula; 1,000 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, when the trained personnel who have received the required training from the manufacturer, consider it necessary if the system allows pulling down the threshold as much as possible to detect low amplifications, attention should be paid to keep the threshold line above the background.

The negative control is essential for accurate result analysis. Please check the negative control and ensure it shows no amplification outside the associated internal control filter. If the negative control has a signal outside the filter associated with the internal control, please do not report the results. Repeat the experiment after taking the necessary precautions against contamination.

If the same result is encountered again, please contact the manufacturer.

Internal control, standards, and positive control of Bosphore HDV Quantification-Detection Kit v1 are essential for accurate result analysis. The cycle threshold acceptance criteria for the internal control, standards, and positive control are listed below:

Component / Parameter	Threshold Value (C_T)
Standard 1	19.5±2
Standard 2	23±2
Standard 3	26±2
Standard 4	28.5±2
Positive Control	25±4
Internal Control	≤32
Correlation Coefficient	>0.970
PCR Efficiency*	>70%

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

In quantitative results; samples that cross the threshold in the FAM filter; it is evaluated as "Positive", samples that do not cross the threshold are shown as "No C_T" or "Negative". These samples are considered to have a negative or viral load below the detection limit of the assay. The internal control data in the HEX filter of these "undetected" samples should also be checked to avoid false negative results.

The delayed amplification of the internal control may indicate a problem in nucleic acid extraction / PCR inhibition or application failure. In this case, extraction and PCR should be repeated. Please consider that in the samples that contain high viral concentration, internal control can be suppressed therefore delayed or no increase in internal control signal may be detected.

Please note that this product only provides testing pathogens shown on the next page. Experimental results from the tube must be considered when providing a result for the patients, also in consideration of the patient's clinical findings, and the guidelines of the relevant health authorities. The table on the next page shows the possible results and their interpretation.

PCR Master Mix	HDV (FAM)	Internal Control (HEX)	Result
	+	+/-	The sample is HDV positive
	-	+	Sample is negative
	-	-	The test should be repeated!

In rare cases of PCR inhibition due to medication or other PCR inhibitors in the sample, we recommend repeating 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.

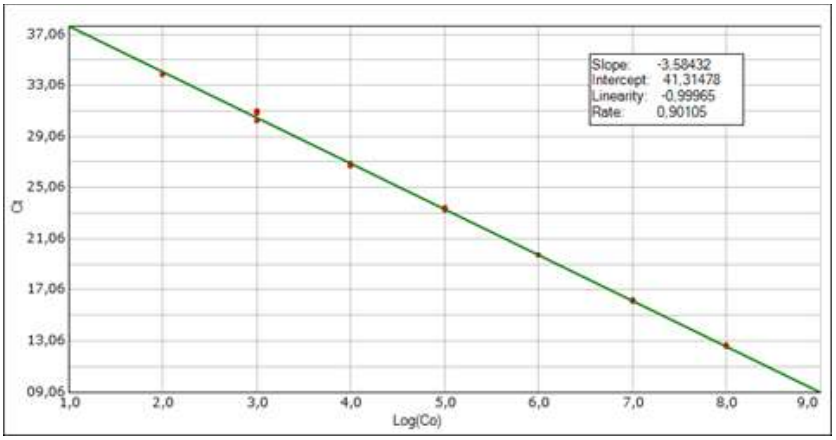
11. SPECIFICATIONS

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 of Bosphore HDV Quantification-Detection Kit 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 the probit 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 sequence comparison analysis using database alignment. To eliminate the risk of cross-reactivity; HBV, HCV, HIV, CMV, EBV, BKV, HSV-1, JCV, Adenovirus, Enterovirus, and RSV samples with known high positivity were tested and found negative. The experimental results indicated that the kit detects specifically and only pathogens that it only intends to detect, but not the others.

11.4. Reproducibility

Reproducibility data (on a C_T value basis) were obtained by the analysis of the previously quantitated positive samples. The test was performed in at least 4 replicates by 3 different operators, on multiple days, using 3 different lots. The resulting data is given below for 100 copies/mL.

Variability (HDV)	**Standard Deviation**	**Variance**	**Coefficient of Variation [%]**
Intra-assay (n=4)	0.12	0.0150	0.38
Inter-lot (n=3)	0.06	0.0032	0.17
Inter-operator (n=3)	0.05	0.0026	0.16
Total Inter-assay (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, by 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 Obtained	Negative Results Obtained
0/100	100/100

The clinical data has also been successfully obtained via QCMD 2015 Hepatitis D Virus EQA Pilot Study, QCMD 2016 Hepatitis D Virus EQA Pilot Study, QCMD 2020 Hepatitis D Virus EQA Programme, QCMD 2022 Hepatitis D Virus EQA Programme and QCMD 2023 Hepatitis D Virus EQA Programme 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

https://www.who.int/news-room/fact-sheets/detail/hepatitis-d (Access date: 14th April 2024)

13. SYMBOLS

	Use-by Date		Caution, consult accompanying documents.
	Batch Code		Manufacturer
	Catalog Number		*In vitro* Diagnostic Device

14. ORDERING INFORMATION

Catalog Number:

ABHDV3 (100 rxn/box)
ABHDV2 (50 rxn/box)
ABHDV1 (25 rxn/box)

15. CONTACT INFORMATION

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Document Revision History

Document Version No	Revision No	Date	Description
V1	01	16th December 2014	First Publishing
V2	02	14th January 2016	Partial content correction
V3	03	02nd May 2016	The general content and type check
V4	04	18th January 2017	The content has been updated and checked
V5	05	22nd May 2017	The general content and type check
V6	06	03rd June 2017	The content has been updated and checked
V7	07	29th December 2017	Partial content correction
V8	08	04th January 2018	The general content and type check
V9	09	06th May 2018	The content has been updated and checked
V10	10	12th September 2018	The general content and type check
V11	11	16th May 2019	The content has been updated and checked
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V13	13	01st January 2020	The general content and type check
V14	14	01st March 2022	The content has been updated and checked
V15	15	14th April 2024	Partial content correction

IVD CE

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INSTRUCTIONS FOR USE

HPV Genotyping
High Risk Kit v1

For *In Vitro* Diagnostic Use

MB09v14f
25th March 2025

Anatolia geneworks

CONTENTS

1. PRODUCT DESCRIPTION

Bosphore HPV Genotyping High Risk Kit v1 is a Real-Time PCR kit for *in vitro* diagnostics that detects and characterizes the high risk group of HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). It is compatible with cervical swabs (liquid-based cytology samples including those collected using *ThinPrep* (Hologic) and *SurePath* (Becton Dickinson)), semen samples, genital swabs from both females and males, as well as tissue biopsies and samples from warts. Fluorescence detection is performed using FAM, HEX, Texas RED, and Cy5 filters.

Component	**FAM** (Gene)	**HEX** (Gene)	**Texas RED** (Gene)	**Cy5**
PCR Master Mix 1	HPV 16 (L1 gene)	HPV 18 (L1 gene)	HPV 31 (L1 gene)	Internal Control (EXT or PCR)
PCR Master Mix 2	HPV 56 (L1 gene)	HPV 58 (L1 gene)	HPV 51 (L1 gene)	Internal Control (EXT or PCR)
PCR Master Mix 3	HPV 45 (L1 gene)	HPV 35 (L1 gene)	HPV 59 (L1 gene)	Internal Control (EXT or PCR)
PCR Master Mix 4	HPV 68 (L1 gene)	HPV 39 (L1 gene)	HPV 66 (L1 gene)	Internal Control (EXT or PCR)
PCR Master Mix 5	HPV 33 (L1 gene)	HPV 52 (L1 gene)	-	Internal Control (EXT or PCR)

Two internal controls have been integrated into the kit in order to check PCR inhibition, DNA extraction and application errors. Internal Control EXT is used in DNA extraction and Internal Control PCR is used in PCR step. Both internal controls can be used in all PCR Master Mixes; however, only one internal control should be used for each PCR Master Mix. Amplification data of the internal control is detected with the Cy5 filter.

2. CONTENT

Bosphore HPV Genotyping High Risk Kit v1 consists of the following dH_2O, Real-Time PCR Master Mixes, positive controls, and internal controls.

Component	**Reagent**	**100 Reactions**	**50 Reactions**	**25 Reactions**
1	dH_2O	(1,000 µL)	(1,000 µL)	(1,000 µL)
2	PCR Master Mix 1	(2,200 µL)	(1,100 µL)	(550 µL)
3	PCR Master Mix 2	(2,200 µL)	(1,100 µL)	(550 µL)
4	PCR Master Mix 3	(2,200 µL)	(1,100 µL)	(550 µL)

5	PCR Master Mix 4	(2,200 µL)	(1,100 µL)	(550 µL)
6	PCR Master Mix 5	(2,200 µL)	(1,100 µL)	(550 µL)
7	Internal Control PCR	(112 µL)	(56 µL)	(28 µL)
8	Internal Control EXT	(550 µL)	(275 µL)	(275 µL)
9	Positive Control 1	(88 µL)	(44 µL)	(44 µL)
10	Positive Control 2	(88 µL)	(44 µL)	(44 µL)
11	Positive Control 3	(88 µL)	(44 µL)	(44 µL)
12	Positive Control 4	(88 µL)	(44 µL)	(44 µL)
13	Positive Control 5	(88 µL)	(44 µL)	(44 µL)

3. STORAGE

PCR reagents for Bosphore HPV Genotyping High Risk Kit v1 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 minutes, and the PCR master 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 PCR master mix in a closed container.

If the components are stored according to the recommended conditions, they will remain stable until the expiry dates on the labels.

4. REQUIRED MATERIALS AND DEVICES

- Montania 4896 Real-Time PCR Instrument – Anatolia Geneworks, CFX96 Real-Time PCR Detection System - Bio-Rad, QuantStudio 5 Real-Time PCR System – ThermoFisher, LightCycler 480 Instrument II – Roche, Q qPCR Cycler - Quantabio, Rotor-Gene Q – QIAGEN or another Real-Time PCR system with FAM, HEX, Texas RED, Cy5 filters*
- 0.1 mL or 0.2 mL thin-wall PCR tubes, PCR plates or strips
- Bosphore STD EX-Tract Kit, Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Viral DNA/RNA Extraction Kit 600 µl, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit, Unio M32 Extraction System and Unio M32 Viral DNA/RNA Extraction Kit, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Viral DNA/RNA Extraction Kit, Bosphore Viral DNA Extraction Spin Kit, Bosphore Viral DNA/RNA Extraction Spin Kit or Bosphore Nucleic Acid Extraction Versatile Spin Kit,

Magnesia 16 Nucleic Acid Extraction Instrument and Magnesia Viral DNA/RNA Extraction Kit EP (Anatolia Geneworks) or other high-quality DNA extraction kits and systems

- Deep freezer (-20 °C)
- Desktop centrifuge with rotor for 2 mL or 1.5 mL microcentrifuge tubes
- DNase, RNase, pyrogen-free 1.5 mL or 2 mL microcentrifuge tubes
- Calibrated, adjustable micropipettes
- DNase, RNase, pyrogen-free micropipette tips with filters
- Disposable laboratory gloves, coats and caps

*For other Real-Time PCR devices that can be used with Bosphore HPV Genotyping High Risk Kit v1, please contact Anatolia Geneworks from the information in Section 15.

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

- The product should be delivered on dry ice. Check for the presence of dry ice upon arrival.
- Check for the expiration dates on the box and tube labels upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNase, RNase, and pyrogen-free micropipette tips with filters, and DNase, RNase, and 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 before use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared and quickly returned to -20 ºC.
- PCR and nucleic acid extraction 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 and in a microbiological safety cabinet of the appropriate class. Physical contact with pathogens should be avoided by wearing lab coats and gloves, making no allowance for eating or drinking within the workspace, and preventing unauthorized individuals' access to the working area.
- After working biological samples, all pathogenic wastes produced during the nucleic acid extraction step, including materials contacted with them, should be discarded into medical waste, and disposed of safely.

6. PRODUCT USE LIMITATIONS

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

7. INFECTION

Papillomaviruses are small DNA viruses that belong to the *Papovaviridae* family. The human pathogen papillomavirus type is called Human papillomavirus (HPV). The sexually transmitted HPV causes skin and mucosal disorders and genital infections. HPV infections are usually asymptomatic or cause mild clinical symptoms and do not require treatment. However, in some cases, both in women and men, HPV infections may cause genital papillomas and, rarely, anogenital or head-neck cancers.

HPV has more than 150 genotypes. Genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 are classified as high-risk HPV. Cervical cancer can develop in cases of persistent infections with these high-risk HPVs. It is estimated that HPV causes 500,000 cervical cancer cases per year, making it one of the leading causes of cancer-related death in women in developing countries.

HPV 16 and HPV 18 are responsible for 70% of cervical cancers worldwide. Furthermore, 15% of cervical cancer cases are related to types 31, 33, 35, 45, 52, and 58, while another 15% of cases are associated with rare types. HPV is a non-enveloped virus containing a circular double-stranded DNA genome that replicates in the nucleus of infected cells. The viral genome is 7.3–8.0 kb long and includes 8–10 open reading frames (ORFs) that encode early (E) and late (L) viral proteins (Gravitt et al., 2000; Juckett & Hartman-Adams, 2010).

8. METHOD

Bosphore HPV Genotyping High Risk Kit v1 is based on the Real-Time PCR method. The polymerase chain reaction is a technique that is used for the amplification of a DNA region. The reaction occurs through repeated cycles of heating and cooling. The main components of PCR are primers, dNTPs, *Taq* DNA Polymerase (with hot-start property), buffer solutions, and templates. As a brief explanation, primers are small synthetic DNA that anneals to the specific regions of the template to start the synthesis, dNTPs are the building blocks of the amplified products, and *Taq* DNA Polymerase amplifies the DNA template. Finally, buffer solutions provide the pH adjustment required for the reaction, and the template, as referred to, is the target region for synthesis. In the Real-Time PCR technique, in contrast to conventional PCR, PCR products can be monitored during the reaction.

Therefore, Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, thereby minimizing the risk of contamination. Dual-labeled probes employed in the reaction, in addition to the conventional PCR reagents, enable the detection of the amplified target with increased sensitivity.

The assay utilizes the 5' exonuclease activity of *Taq* DNA Polymerase to cleave a dual-labeled fluorescent hydrolysis 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 nearby, and the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step of PCR, *Taq* DNA Polymerase encounters and cleaves the probe bound to the template. As the reporter is freed from the suppressing effect of the quencher, a 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 the 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 DNA and its threshold cycle.

Bosphore HPV Genotyping High Risk Kit v1 uses multiplex qPCR and internal controls are included in the system to control the extraction procedure, PCR inhibition and application issues. The reaction is performed in five PCR tubes containing PCR Master Mix 1, 2, 3, 4, 5.

9. PROCEDURE

9.1. DNA Extraction

We recommend that Bosphore STD EX-Tract Kit*, Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Viral DNA/RNA Extraction Kit 600 µl, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit, Unio M32 Extraction System and Unio M32 Viral DNA/RNA Extraction Kit, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Viral DNA/RNA Extraction Kit, Bosphore Viral DNA Extraction Spin Kit, Bosphore Viral DNA/RNA Extraction Spin Kit or Bosphore Nucleic Acid Extraction Versatile Spin Kit, Magnesia 16 Nucleic Acid Extraction Instrument and Magnesia Viral DNA/RNA Extraction Kit EP (Anatolia Geneworks) or other high-quality DNA extraction kits and systems are used with Bosphore HPV Genotyping High Risk Kit v1. The DNA extraction should be performed according to the manufacturer's instructions.

*Bosphore STD EX-Tract Kit is a rapid sample preparation kit designed to make genital swab and urine specimens ready for use with the Bosphore Sexually Transmitted Diseases panel and the Bosphore Human Papilloma Virus (HPV) panel Real-Time PCR kits.

9.2. Kit Components

9.2.1. Negative Control

The negative control is an essential component of Bosphore HPV Genotyping High Risk Kit v1 for Real-Time PCR. It consists of distilled water (dH_2O) and serves as a reference sample to ensure accurate results by detecting any contamination or errors that could lead to false-positive outcomes. Including the negative control and carefully monitoring it will enable you to confidently interpret your data.

9.2.2. PCR Master Mix 1

PCR Master Mix 1 contains a highly specific and accurate *Taq* DNA Polymerase (with hot-start property), PCR buffers, and dNTPs mix. PCR Master Mix 1 also contains forward and reverse primers and dual-labeled probes specific for HPV 16, HPV 18, HPV 31, and internal control.

9.2.3. PCR Master Mix 2

PCR Master Mix 2 contains a highly specific and accurate *Taq* DNA Polymerase (with hot-start property), PCR buffers, and dNTPs mix. PCR Master Mix 2 also contains forward and reverse primers and dual-labeled probes specific for HPV 56, HPV 58, HPV 51, and internal control.

9.2.4. PCR Master Mix 3

PCR Master Mix 3 contains a highly specific and accurate *Taq* DNA Polymerase (with hot-start property), PCR buffers, and dNTPs mix. PCR Master Mix 3 also contains forward and reverse primers and dual-labeled probes specific for HPV 45, HPV 35, HPV 59, and internal control.

9.2.5. PCR Master Mix 4

PCR Master Mix 4 contains a highly specific and accurate *Taq* DNA Polymerase (with hot-start property), PCR buffers, and dNTPs mix. PCR Master Mix 4 also contains forward and reverse primers and dual-labeled probes specific for HPV 68, HPV 39, HPV 66, and internal control.

9.2.6. PCR Master Mix 5

PCR Master Mix 5 contains a highly specific and accurate *Taq* DNA Polymerase (with hot-start property), PCR buffers, and dNTPs mix. PCR Master Mix 5 also contains forward and reverse primers and dual-labeled probes specific for HPV 33, HPV 52, and internal control.

9.2.7. Internal Controls

Internal controls included in the kit to control DNA extraction, PCR inhibition and application errors are synthetic DNA molecules. Internal Control EXT is added to the sample, proteinase K, and Carrier RNA mixture at the beginning of DNA extraction to control extraction efficiency and application errors. If an internal control is to be added during nucleic acid extraction, add 5 µL per sample. Internal Control PCR added to control PCR inhibition adds 0.2 µL when added directly to PCR Master Mix. We recommend adding Internal Control EXT to the negative control to evaluate the efficiency of the extraction system.

Caution! If Internal Control EXT has already been added during the extraction step, it is not necessary to include Internal Control PCR in the PCR Master Mix. It should be considered that both internal controls can be used with any PCR Master Mix; however, when one internal control is used, the other should not be used. (For example, if Internal Control EXT is added during the extraction step, Internal Control PCR should not be added to the PCR Master Mix.)

The absence of internal control amplification in the Cy5 filters in negative samples may indicate a problem in extraction or application, or that the PCR reaction is inhibited. In this case, extraction and PCR should be repeated. In samples with high viral load, including the positive control, the internal control may be suppressed and an increase in fluorescent signal may not be detected. Therefore, internal control amplification should be evaluated according to the table in Section 10.

9.2.8. Positive Control

The kit contains 5 positive controls containing synthetic HPV DNAs. It must be included in the PCR reaction to test the reaction efficiency.

Positive Control 1: Synthetic HPV 16 DNA

Positive Control 2: Synthetic HPV 56 DNA

Positive Control 3: Synthetic HPV 45 DNA

Positive Control 4: Synthetic HPV 66, and HPV 68 DNAs

Positive Control 5: Synthetic HPV 33, and HPV 52 DNAs

The threshold C_T value of the positive control is given in the acceptance criteria table (Section 10). A threshold C_T value of the positive control greater than the upper limit of the range in the table may indicate a loss of yield in the reaction.

9.3. Preparing the PCR

The positive and negative controls should be added into the PCR reaction together with the samples. Ensure that all kit components are dissolved before use and refer to the table below to prepare the PCR reaction mix. The volumes given are for one reaction only, multiply these volumes by the number of samples to find the volumes required for the master mix. When preparing the mix for more than 5 samples, 10% should be added to the total number of samples.

Components	Volume
PCR Master Mix 1/2/3/4/5	20 µL
Internal Control PCR**	0.2 µL
Sample DNA (Negative / Positive Control)	5 µL
Total Volume	25 µL

**If Internal Control EXT has already been added during the extraction step, Internal Control PCR should not be added to the PCR reaction.

Pipette 20 µL of the PCR Master Mix into the PCR tubes or strips and add 5 µL of template (sample/ positive or negative control). Close the tube cap. Make sure that the mix 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 HPV Genotyping High Risk Kit v1 consists of initial denaturation for activation of the *Taq* DNA Polymerase (with hot-start property), a two-step amplification cycle, and a terminal hold. The Real-Time data is collected at the second step of the amplification cycle. The thermal protocol to be applied for the reaction is indicated below.

Steps	Temperature	Time	
Initial Denaturation	95 °C	14:30 min	
Denaturation	95 °C	00:30 min	40 Cycles
Annealing (Data Collection)	56 °C	00:45 min	
Hold	32 °C	02:00 min	

Before starting to work with Bosphore HPV Genotyping High Risk Kit v1, the following steps must be completed and checked:

- Choose all the filters to be used (FAM, HEX, Texas RED, and Cy5),
- Identify unknown samples, positive and negative controls,
- Select the correct thermal protocol,
- Start the experiment.

10. ANALYSIS

By the end of the thermal protocol, the Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold. 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 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 the manufacturer, consider it necessary if the system allows pulling down the threshold as much as possible to detect low amplifications, attention should be paid to keep the threshold line above the background.

The negative control is essential for accurate result analysis. Please check the negative control and ensure it shows no amplification outside the associated internal control filter. If the negative control has a signal outside the filter associated with the internal control, please do not report the results. Repeat the experiment after taking the necessary precautions against contamination. If the same result is encountered again, please contact the manufacturer.

Internal control, and positive control of Bosphore HPV Genotyping High Risk Kit v1 are essential for accurate result analysis. The cycle threshold acceptance criteria for the internal control, and positive control are listed below.

Component / Parameter	Threshold Value (C_T)
Positive Control	≤28
Internal Control	≤32

In qualitative test results; samples that cross the threshold in the FAM, HEX, and Texas RED filters for PCR Master Mix 1, PCR Master Mix 2, PCR Master Mix 3, and PCR Master Mix 4, and FAM and HEX filters for PCR Master Mix 5; it is evaluated as "Positive", samples that do not cross the threshold are shown as "No C_T" or "Negative".

These samples are considered to have a negative or viral load below the detection limit of the assay. The internal control data in the Cy5 filters of these "undetected" samples should also be checked to avoid false negative results.

The delayed amplification of the internal control may indicate a problem in nucleic acid extraction / PCR inhibition or application failure. In this case, extraction and PCR should be repeated. Please consider that in the samples that contain high viral concentration, internal control can be suppressed and therefore delayed or no increase in internal control signal may be detected.

Please note that this product only provides testing pathogens shown below. Experimental results from the tube must be considered when providing a result for the patients, also in consideration of the patient's clinical findings, and the guidelines of the relevant health authorities. The table below shows the possible results and their interpretation:

	HPV 16 (FAM)	HPV 18 (HEX)	HPV 31 (Texas RED)	Internal Control (Cy5)	Result
PCR Master Mix 1	+	-	-	+/-	The sample is HPV 16 positive
	-	+	-	+/-	The sample is HPV 18 positive
	-	-	+	+/-	The sample is HPV 31 positive
	-	-	-	+	Sample is negative
	+	+	-	+/-	The sample is HPV 16, and HPV 18 positive
	-	+	+	+/-	The sample is HPV 18, and HPV 31 positive
	+	-	+	+/-	The sample is HPV 16, and HPV 31 positive
	+	+	+	+/-	The sample is HPV 16, HPV 18, and HPV 31 positive
	-	-	-	-	The test should be repeated!
	HPV 56 (FAM)	**HPV 58 (HEX)**	**HPV 51 (Texas RED)**	**Internal Control (Cy5)**	**Result**
PCR Master Mix 2	+	-	-	+/-	The sample is HPV 56 positive
	-	+	-	+/-	The sample is HPV 58 positive
	-	-	+	+/-	The sample is HPV 51 positive
	-	-	-	+	Sample is negative
	+	+	-	+/-	The sample is HPV 56, and HPV 58 positive
	-	+	+	+/-	The sample is HPV 58, and HPV 51 positive

	+	-	+	+/-	The sample is HPV 56, and HPV 51 positive
	+	+	+	+/-	The sample is HPV 56, HPV 58, and HPV 51 positive
	-	-	-	-	The test should be repeated!
PCR Master Mix 3	**HPV 45 (FAM)**	**HPV 35 (HEX)**	**HPV 59 (Texas RED)**	**Internal Control (Cy5)**	**Result**
	+	-	-	+/-	The sample is HPV 45 positive
	-	+	-	+/-	The sample is HPV 35 positive
	-	-	+	+/-	The sample is HPV 59 positive
	-	-	-	+	Sample is negative
	+	+	-	+/-	The sample is HPV 45, and HPV 35 positive
	-	+	+	+/-	The sample is HPV 35, and HPV 59 positive
	+	-	+	+/-	The sample is HPV 45, and HPV 59 positive
	+	+	+	+/-	The sample is HPV 45, HPV 35, and HPV 59 positive
	-	-	-	-	The test should be repeated!
PCR Master Mix 4	**HPV 68 (FAM)**	**HPV 39 (HEX)**	**HPV 66 (Texas RED)**	**Internal Control (Cy5)**	**Result**
	+	-	-	+/-	The sample is HPV 68 positive
	-	+	-	+/-	The sample is HPV 39 positive
	-	-	+	+/-	The sample is HPV 66 positive
	-	-	-	+	Sample is negative
	+	+	-	+/-	The sample is HPV 68, and HPV 39 positive
	-	+	+	+/-	The sample is HPV 39, and HPV 66 positive
	+	-	+	+/-	The sample is HPV 68, and HPV 66 positive
	+	+	+	+/-	The sample is HPV 68, HPV 39, and HPV 66 positive
	-	-	-	-	The test should be repeated!

PCR Master Mix 5	HPV 33 (FAM)	HPV 52 (HEX)	X (Texas RED)	Internal Control (Cy5)	Result
	+	-	X	+/-	The sample is HPV 33 positive
	-	+	X	+/-	The sample is HPV 52 positive
	-	-	X	+	Sample is negative
	+	+	X	+/-	The sample is HPV 33, and HPV 52 positive
	-	-	X	-	The test should be repeated!

LightCycler 480 Instrument II - Roche: Please use **white plate** for Roche Light Cycler 480. "**Abs Quant/Fit Points**" should be chosen as the analysis type for the appropriate threshold level selection. Analysis should be performed with 'Cycle Range' option.

CFX96 Real-Time PCR Detection System - Bio-Rad: Use of **white plate** for the analysis is recommended.

Rotor-Gene Q – QIAGEN: Use of "Slope Correct" and "Dynamic Tube" options for the analysis is recommended. If the sigmoidal curve does not observe, try to analyze the sample without the "Slope Correct" option. It is recommended to use gain options as Green (FAM): 10, Yellow (HEX): 10, Red (Cy5): 10, Orange (T. RED):10.

Quant Studio 5-ThermoFisher: Use of **Passive Referrence** as **"None"** for the analysis is recommended.

Q qPCR MIC qPCR Cycler: "Standart TAQ" option should be selected while PCR setup. It is recommended to use gain options as Green (FAM): 10, Yellow (HEX): 10, Red (Cy5): 10, Orange (T.RED):10.

In rare cases of PCR inhibition due to medication or other PCR inhibitors in the sample, we recommend repeating the test of inhibited samples, by freezing and thawing the DNA samples and using them in the PCR after diluting them 1:2 with dH_2O.

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.

Sensitivities of the HPV genotypes (16 and 18) were determined using serial dilutions of 1st WHO International Standard for Human Papillomavirus (HPV) Type 16 DNA NIBSC code: 06/202 and 1st WHO International Standard for Human Papillomavirus (HPV) Type 18 DNA NIBSC code: 06/206. The dilutions were tested in different runs in replicates. The results were analyzed by probit method.

The analytical detection limit for Bosphore HPV Genotyping High Risk Kit v1 was found to be 1,469 IU/mL (1,469 x 10^3 GEq/ml) for HPV16, 1,400 IU/mL (1.4 x10^3 GEq/mL) for HPV18, 16 copies/ reaction for HPV31, 112 copies/reaction for HPV56, 15 copies/reaction for HPV58, 11 copies/reaction for HPV51, 14 copies/reaction for HPV45, 18 copies/reaction for HPV35, 20 copies/reaction for HPV59, 12 copies/reaction for HPV68, 3 copies/reaction for HPV39, 36 copies/reaction for HPV66, 100 copies/reaction for HPV33, and 20 copies/reaction for HPV52.

11.2. 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. To eliminate the risk of cross-reactivity; HSV, EBV, HBV, Parvovirus B19 samples with known high positivity were tested and found negative. The experimental results indicated that the kit detects specifically and only HPV genotypes that it intends to detect, but not the others.

12. REFERENCES

1. Garland, S. M., Steben, M., Sings, H. L., et al. (2009). Natural history of genital warts: Analysis of the placebo arm of two randomized phase III trials of a quadrivalent human papillomavirus (types 6, 11, 16, and 18) vaccine. Journal of Infectious Diseases, 199(6), 805–814. https://doi.org/10.1086/597071

2. Gravitt, P. E., Peyton, C. L., Alessi, T. Q., Wheeler, C. M., Coutle´E, F., Hildesheim, A., Schiffman, M. H., Scott, D. R., & Apple, R. J. (2000). Improved amplification of genital human papillomaviruses. Journal of Clinical Microbiology, 38(1), 357–361. https://doi.org/10.1128/JCM.38.1.357-361.2000

13. SYMBOLS

	Use-by Date		Caution, consult accompanying documents.
	Batch Code		Manufacturer
	Catalog Number		*In vitro* Diagnostic Device

14. ORDERING INFORMATION

Catalog Number:	**ABHPV2 (50 rxn/box)** **ABHPV1 (25 rxn/box)**

15. CONTACT INFORMATION

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V1	01	04th December 2012	First publishing
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V3	03	06th May 2014	The general content and type check
V4	04	05th January 2015	The content has been updated and checked
V5	05	04th May 2016	The general content and type check
V6	06	31st December 2016	The content has been updated and checked
V7	07	12th January 2018	Partial content correction
V8	08	06th May 2019	The general content and type check
V9	09	05th January 2020	The content has been updated and checked
V10	10	04th October 2020	The general content and type check
V11	11	15th January 2024	The content has been updated and checked
V12	12	10th February 2025	Partial content correction
V13	13	3rd March 2025	Additional sample type compatible with the kit have been included
V14	14	25th March 2025	Partial content correction

IVD CE

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INSTRUCTIONS FOR USE

Viral DNA/RNA
Extraction Spin Kit

For *In Vitro* Diagnostic Use

MB330v4f
25th October 2024

Anatolia geneworks

CONTENTS

1. PRODUCT DESCRIPTION

Bosphore Viral DNA/RNA Extraction Spin Kit has been designed for manual extraction of viral DNA/RNA from body fluids including blood, serum, plasma, sputum, cerebrospinal fluid, and as well as cell culture supernatants. The kit contains the required solutions for extraction as well as the plastic consumables for the spin column. Bosphore Viral DNA/RNA Extraction Spin Kit is highly compatible with Bosphore Real-Time PCR kits. The starting sample volume is 400 µL and DNA/RNA recovery (elution) volume is 60-100 µL.

2. CONTENT

Bosphore Viral DNA/RNA Extraction Spin Kit is composed of the following DNA/RNA extraction components.

Component Numbers	Content	Quantity (100 extractions)
1	Buffer LB1	40 mL
2	Buffer IR2	50 mL
3	Buffer W3	100 mL
4	Buffer EL4	10 mL
5	Proteinase K	11 mg x 4
6	PK Storage Buffer	1.25 mL x 4
7	Carrier RNA	1 mg x 1
8	DNase/RNase Free Water	1.25 mL x 1
9	Spin Column	100 pieces
10	Collection tubes	400 pieces

Table 1: Bosphore Viral DNA/RNA Extraction Spin Kit content

3. STORAGE

All of the Bosphore Viral DNA/RNA Extraction Spin Kit components are stored at +10 °C / +35 °C temperature. After resuspension, Proteinase K and Carrier RNA should be stored at +4 °C and -20 °C respectively. Kit components maintain their stability until the expiry dates on their labels if they are stored at recommended conditions.

4. REQUIRED MATERIALS AND DEVICES

- Refrigerator (+4 °C)
- Deep Freezer (-20 °C)

- Vortex
- Thermomixer or thermal block
- Disposable laboratory gloves, coats, and caps
- Calibrated adjustable micropipettes or multichannel pipettes
- DNase, RNase, pyrogen-free micropipette tips with filters
- DNase, RNase, pyrogen-free 1.5 mL or 2 mL microcentrifuge tubes
- Desktop centrifuge with rotor for 2 mL microcentrifuge tubes
- Ethanol* (**not provided with the kit**)
- Buffer LTX* (**not provided with the kit**)
- Dithiothreitol* (DTT) (**not provided with the kit**)
- Xylene* (**not provided with the kit**)

* To obtain these materials required to work with different sample types, please contact Anatolia Geneworks using the information in Section 11.

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

- 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 mixed well to ensure homogeneity prior to use.
- PCR and nucleic acid extraction must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- 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, and prevention of unauthorized individuals' access to the working area.
- Pathogen information should be reviewed to be aware of the health-related risks.
- All the pathogenic wastes produced during the nucleic acid extraction step; including the biological samples and material contact with them, should be discarded into the medical waste, and disposed of safely.
- This product is to be used by personnel specially trained to perform molecular genetic laboratory procedures, in accordance with this user manual.

6. PRODUCT USE LIMITATIONS

- This product may exclusively be used for *in vitro* diagnostics.
- This product should be used by personnel specially trained with this user manual.

7. METHOD

Bosphore Viral DNA/RNA Extraction Spin Kit is based on the silica membrane column separation method. The kit involves the extraction of nucleic acids from biological samples of patients by removing and purifying the viral nucleic acids from cellular components. In this manner, DNA/RNA extraction steps include disruption/lysis of the samples, inactivation of nuclease activity, binding of the DNA/RNA to the silica membrane, removal of the contaminants, and recovery of the nucleic acid respectively. So far, methods generally employed have been considered in two categories; the conventional 'liquid-phase extraction' and common 'solid-phase extraction' which is highly appreciated in the molecular biology field as a result of its practical use. Liquid-phase extraction methods include; Guanidinium Acid - Phenol- Extraction method, Alkaline Extraction method, Ethidium Bromide (EtBr)-Cesium Chloride (CsCl) Gradient centrifugation method, and Oligo(dT)-Cellulose Chromatography method. Recently, Solid-phase extraction methods are preferred as they avoid the phase separation problems usually faced in 'liquid-phase extraction' methods and are faster and more effective than conventional methods.

Extraction of the nucleic acid is carried out in 4 main steps that involve lysis, binding, washing, and elution. During the lysis/disruption step, the samples are lysed by using a lysis buffer solution with excessive denaturing conditions that inactivate the DNase and RNase activity. Using a buffer at a particular pH, the surface or functional groups on the solid/column are converted into a particular chemical form. Buffer conditions (pH and salt concentration) are optimized in order to achieve the effective binding of nucleic acid to the column surface. Other components are removed in further washing steps using the wash buffer. Mostly, alcohol solutions are applied during these wash steps. The components apart from nucleic acids are easily removed from nucleic acids as they tend to dissolve in alcohols. After this step, extraction is completed by eluting the bound DNA/RNA using an elution buffer. As Elution buffer, water or TE buffer is mainly used and nucleic acids are recovered during this last step.

The ion concentrations and pH degrees of the solutions used in nucleic acid extraction play an important role. The use of solid-surface material is very important. The main solid-surface (support) materials used in solid-phase extraction systems are usually; silica matrix, glass particles, diatomite, magnetic particles, or anion-change surfaces. Nowadays, most of the commercial manual extraction kits available in the market are based on silica-based column technology. In this technology, nucleic acids tend to bind to the silica column and separate from other components by applying a centrifuge.

8. PROCEDURE

8.1. Preparation of Different Types of Samples

Body Fluids (serum, plasma, urine, tears, cerebrospinal fluid, amniotic fluid, etc.): Homogenize the sample thoroughly before extraction. From the body fluid, take the appropriate volume and follow the procedure explained in Section 8.3 Viral DNA/RNA Extraction.

Liquid Swab Samples: Homogenize the samples thoroughly by shaking them before extraction. Then, take the appropriate volume from the homogenized sample and follow the procedure explained in Section 8.3 Viral DNA/RNA Extraction.

Dry Swab Samples: Place the tip of the swab in a microcentrifuge tube containing 500-1000 µL of DNase and RNase-free PBS or distilled water. The volume should be enough to completely submerge the swab. Incubate the swab by pressing it against the sides of the tube frequently for 5 minutes. This process transfers the sample to the liquid and then removes the swab. Take the appropriate volume from the liquid and follow the procedure explained in Section 8.3 Viral DNA/RNA Extraction.

Viscous Samples (BAL, sputum, or other mucus samples): It is important to liquefy the samples and make them liquified before DNA/RNA extraction. The liquefaction step can be performed using commercial liquefaction products or commercial liquefaction solutions containing 0.1% DTT (Dithiothreitol) (**not included in the kit - should be obtained separately**). The same amount as the sample volume can be added. Take the appropriate volume from the liquified sample and follow the procedure explained in Section 8.3 Viral DNA/RNA Extraction. Alternatively, the "Solid Samples" protocol below can be applied.

Saliva Samples: Add Buffer LTX (**not included in the kit - should be obtained separately**) equal to the sample volume and vortex thoroughly (start the extraction process within 2-3 hours after preparing this mixture. Long-term storage is not appropriate). Take the appropriate volume from this mixture and follow the procedure explained in Section 8.3 Viral DNA/RNA Extraction.

Solid Samples (Various tissue, wart, or stool samples): Cut 100-250 mg of fresh or frozen sample. Grind the sample and transfer it to a 1.5 mL microcentrifuge tube. For each sample, add 900 µL of Buffer LTX (**not included in the kit - should be obtained separately**) and 20 µL of Proteinase K and mix by vortexing. Incubate the mixture for at least 10 minutes at 56 °C (lysis).

Centrifuge the mixture for 3 minutes at 12,500 rpm. In a new microcentrifuge tube, take the appropriate volume from the clear supernatant and follow the procedure explained in Section 8.3 Viral DNA/RNA Extraction (do not add 20 µL of Proteinase K during the extraction procedure as it was added at this stage for each sample).

Liquid Stool Samples: Mix well and aspirate 750-1,000 µL of unprocessed sample and apply into a 1.5 mL microcentrifuge tube. Spin down at 12,500 rpm for 3 minutes. Discard the supernatant and add 20 µL Proteinase K and 900 µL of Buffer LTX (**not included in the kit - should be obtained separately**) on the pellet. Vortex and incubate at 56ºC for 10 minutes. Centrifuge the mixture for 3 minutes at 12,500 rpm. Aspirate the required amount from the supernatant into an adaptor tube and follow the procedure explained in Section 8.3 Viral DNA/RNA Extraction (do not add 20 µL of Proteinase K during the extraction procedure as it was added at this stage for each sample).

Stool Samples (Alternative Method): In addition to the protocol used for solid samples, nucleic acids can also be isolated using a rapid protocol. Add 900 µL of Buffer LTX (**not included in the kit-should be obtained separately**) and 20 µL of Proteinase K to a pea-sized stool sample and vortex thoroughly. Centrifuge the mixture at 3,000 rpm for 1 minute and transfer the appropriate volume of clear supernatant to a new microcentrifuge tube and follow the procedure explained in Section 8.3 Viral DNA/RNA Extraction (do not add 20 µL of Proteinase K during the extraction procedure as it was added at this stage for each sample). Homogenization efficiency may vary depending on the sample type and may affect sensitivity.

Paraffin-Embedded Tissue Samples: First, cut the sample into thin strips using a sterile scalpel. Transfer the cut pieces to a microcentrifuge tube and add 1 mL of xylene (**not included in the kit - should be obtained separately**). Vortex and wait for 5 minutes. Centrifuge at 8,000 rpm for 3 minutes and remove the liquid portion. Add 1 mL of ethanol (**not included in the kit - should be obtained separately**) to the pellet, vortex thoroughly, and centrifuge at 8,000 rpm for 3 minutes. Remove the liquid portion. Add 500 µL of dH_2O to the pellet, vortex thoroughly, and centrifuge at 8,000 rpm for 3 minutes. Remove the liquid portion. Add 650 µL of Buffer LTX (**not included in the kit - should be obtained separately**) and 20 µL of Proteinase K to each sample, vortex thoroughly. Incubate the mixture at 56 °C for at least 60 minutes (lysis). Centrifuge the mixture at 12,500 rpm for 5 minutes. Transfer the appropriate volume of clear supernatant to a new microcentrifuge tube and follow the procedure explained in Section 8.3 Viral DNA/RNA Extraction (do not add 20 µL of Proteinase K during the extraction procedure as it was added at this stage for each sample).

8.2. Extraction Preparation

Before use;

1.1 mL of PK Storage Buffer must be added to the Proteinase K tube and resuspended by vortexing. Proteinase K (10 mg/mL) solutions should be stored at +4 °C after resuspension.

1.0 mL RNase-free water must be added to the Carrier RNA tube and resuspended by vortexing. Carrier RNA (1 mg/mL) solutions should be stored at -20 °C after resuspension.

Caution! If you observe precipitation in Buffer LB1 and Buffer IR2, please heat the solution and dissolve the precipitate.

Caution! We strongly recommend to use serum samples for viral nucleic acid extraction in order to provide higher sensitivity and reproducibility.

Caution! Extraction protocol should be performed by carefully applying the solutions to the center of the membrane, and the tip of the micropipette should not touch the spin column membrane.

8.3. Viral DNA/RNA Extraction

Lysis Step

1. Prepare an extraction mix with 40 µL of Proteinase K, 10 µL of Carrier RNA (and the related internal control) for each sample and divide the extraction mix into one set of labeled microcentrifuge tubes.
2. Add 400 µL sample onto the Extraction Mix and mix by pipetting.
3. Add 400 µL of Buffer LB1 to each sample and mix by vortexing.
4. Incubate the mixture for 10 minutes at 56 °C.

Binding Step 1

5. Add 500 µL of Absolute Ethanol **(not included in the kit – obtained separately)** to the mixture, mix by vortexing.
6. Incubate for 5 minutes at room temperature.
7. Apply 650 µL of the mixture to the spin column.
8. Centrifuge at 8,000 rpm for 1 minute.
9. Discard the liquid flow-through together with the collection tube.
10. Place the spin column in a new collection tube.

Binding Step 2

11. Apply the remaining of the mixture to the spin column.
12. Centrifuge at 8,000 rpm for 1 minute
13. Discard the liquid flow-through together with the collection tube.
14. Place the spin column in a new collection tube.

Inhibitor Removal Step

15. Add 500 µL of Buffer IR2 to the spin column.
16. Centrifuge at 8,000 rpm for 1 minute.
17. Discard the liquid flow-through together with the collection tube.
18. Place the spin column in a new collection tube.

Wash Step 1

19. Add 500 µL of Buffer W3 to the spin column.
20. Centrifuge at 8,000 rpm for 1 minute.
21. Discard the liquid flow-through.
22. Place the spin column back into the same collection tube.

Wash Step 2

23. Add 500 µL of Buffer W3 is added to the spin column.
24. Centrifuge at 8,000 rpm for 1 minute.
25. Discard the liquid flow-through.
26. Place the spin column back into the same collection tube.
27. Centrifuge the empty spin column at 12,500 rpm for 2 minutes.
28. Discard the liquid flow-through together with the collection tube.
29. Place the spin column in a clean 1.5 mL microcentrifuge tube, open the cap of the spin column, and wait for 1 minute to evaporate any remaining alcohol.

Elution Step

30. Add 60–100 µL of Buffer EL4 to the spin column and wait for 5 minutes at room temperature.
31. Centrifuge at 12,500 rpm for 1 minute.
32. The liquid flow-through is the DNA/RNA sample. Store the sample at +4 °C in case of instant use or store it at -20 °C for later use.

9. SYMBOLS

	Use by		Caution, consult accompanying documents
	Lot/Batch		Manufacturer
	Catalog number		*In Vitro* Diagnostic Medical Device

10. ORDERING INFORMATION

Catalog Number: **ABXDR1 (100 extractions/box)**

11. CONTACT INFORMATION

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V1	01	05th December 2012	First publishing
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V3	03	11th May 2022	Partial content correction
V4	01	25th October 2024	Partial content correction

IVD CE 2962

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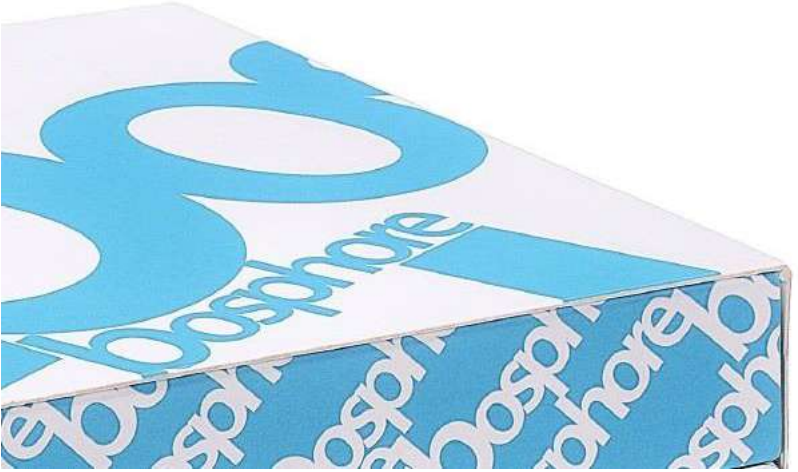
INSTRUCTIONS FOR USE

CMV
Quantification Kit

For *In Vitro* Diagnostic Use

MB195v13f
05th May 2025



Anatolia geneworks

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1. PRODUCT DESCRIPTION AND INTENDED PURPOSE

Bosphore CMV Quantification Kit is an in vitro diagnostic Real-Time Polymerase Chain Reaction (qPCR) kit intended for quantitative detection of Human Cytomegalovirus (HCMV), also known as Human Herpesvirus 5 (HHV-5), extracted from human Bronchoalveolar lavage (BAL), Whole Blood, Urine, Plasma, Cerebrospinal Fluid (CSF) and Amniotic Fluid (AF) samples from individuals suspected of being infected with related pathogen by health authorities.

The kit is designed for use in a multiplex format and aims to provide a quantitative measurement of HCMV viral load in a single tube.

The device is suitable for use on the general population, including EU population, all genders, Neonates, Infants, and Children (0 to 18 years), Adults (18 years and older), The elderly (65 years and older), as well as immunocompromised patients. The device is a CMV specific NAT test that can be used to determine the CMV infection status in patients with or without symptoms, and in immunocompromised patients including transplant patients, for detection and diagnosis of CMV infections, as well as monitoring their response to antiviral therapy. It can be used to ensure CMV diagnosis and management in transplant patients, yet has not been designed to assess the suitability of for transfusion, transplantation, or cell administration.

Positive results indicate the presence of HCMV viral DNA. However, a positive result does not exclude the possibility of infection with other pathogens. Negative results do not rule out HCMV infection and should not be used as the sole basis for patient management decisions. Results must be interpreted alongside clinical observations, patient history, and epidemiological information.

The kit is intended for use by qualified and trained clinical laboratory personnel who have received training in analyzing Real-Time PCR data and performing in vitro diagnostic procedures. Bosphore CMV Quantification Kit is a Real-Time PCR kit compatible with automated nucleic acid extraction systems and/or fully automated platforms that integrate both extraction and PCR setup. The results are automatically analyzed on the PCR device, and the viral load of the samples is quantitatively determined based on the standard curve. The standard curve, generated from known concentrations of the target, enables the conversion of Ct values into IU/ml, ensuring accurate viral load quantification.

The results are automatically calculated by the device software based on the standard curve. The kit is designed for automated analysis using compatible PCR instruments and software. The correlation between C_T values and IU/ml has been validated and is supported by internal controls and standard values.

Fluorescence detection is achieved using FAM and HEX filters. The conserved regions of the DNA polymerase gene for HCMV are used as target sites to specifically detect the pathogen.

Table 1: Filters used for the detection of the pathogen and internal control

FAM (Gene)	**HEX**
Cytomegalovirus (CMV) (DNA polymerase)	Internal Control

An internal control is included in the kit to control DNA extraction, PCR inhibition, and application mistakes. The internal control is a synthetic DNA molecule. The device is intended to be compatible and for use with the following Real-Time PCR systems and DNA extraction systems:

- Montania 4896 Real-Time PCR Instrument – Anatolia Geneworks, CFX96 Real-Time PCR Detection System - Bio-Rad, QuantStudio 5 Real-Time PCR System – ThermoFisher, LightCycler 480 Instrument II – Roche, Q qPCR Cycler - Quantabio, Rotor-Gene Q – QIAGEN
- Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Viral DNA/RNA Extraction Kit 600 µl or Unio Whole Blood Genomic DNA Extraction Large Volume Kit, Unio 96 Nucleic Acid Extraction Versatile Kit (Anatolia Geneworks)

2. CONTENT

Bosphore CMV Quantification Kit consists of the following dH_2O, Real-Time PCR Master Mix, positive control, internal control, and quantitation standards which have been calibrated against WHO First International Standard Human CMV DNA (NIBSC Code: 09/162).

Table 2: Bosphore CMV Quantification Kit content

Component	**Reagent**	**100 Reactions**	**50 Reactions**	**25 Reactions**
1	dH_2O	(1,000 µL x 2)	(1,000 µL)	(1,000 µL)
2	PCR Master Mix	(1,320 µL x 2)	(1,320 µL)	(660 µL)
3	Internal Control	(600 µL x 2)	(600 µL)	(600 µL)
4	Positive Control	(140 µL x 2)	(140 µL)	(140 µL)
5	Standard 1	(1,320 µL x 2)	(1,320 µL)	(1,320 µL)
6	Standard 2	(1,320 µL x 2)	(1,320 µL)	(1,320 µL)
7	Standard 3	(1,320 µL x 2)	(1,320 µL)	(1,320 µL)
8	Standard 4	(1,320 µL x 2)	(1,320 µL)	(1,320 µL)
⚠	For standards, must be remembered that they need to be extracted.			

	Prior to initial usage, please carefully inspect the product and its components to ensure they are complete in terms of quantity, type, and content. Do not utilize a defective or incomplete product, as it may compromise the performance of the kit. For standards to be added to PCR, it should be remembered that they need to be extracted.

3. STORAGE

PCR reagents for Bosphore CMV Quantification Kit 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 and the PCR master 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 PCR master mix in a closed container. If the components are stored according to the recommended conditions, they will remain stable until the expiry dates on the labels.

4. REQUIRED MATERIALS AND DEVICES

- Montania 4896 Real-Time PCR Instrument – Anatolia Geneworks, CFX96 Real-Time PCR Detection System - Bio-Rad, QuantStudio 5 Real-Time PCR System – ThermoFisher, LightCycler 480 Instrument II – Roche, Q qPCR Cycler - Quantabio, Rotor-Gene Q – QIAGEN
- 0.1 mL or 0.2 mL thin wall PCR tubes, PCR plates or strips
- Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Viral DNA/RNA Extraction Kit 600 µl or Unio Whole Blood Genomic DNA Extraction Large Volume Kit, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit (Anatolia Geneworks)
- Deep freezer (-20 °C)
- Desktop centrifuge with rotor for 1.5 mL or 2 mL microcentrifuge tubes
- Calibrated adjustable micropipettes
- DNase, RNase, pyrogen-free micropipette tips with filters
- DNase, RNase, pyrogen-free 1.5 mL or 2 mL microcentrifuge tubes
- Disposable laboratory gloves, coats, and caps

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

Delivery and Expiration Dates:

- The product should be delivered on dry ice. Check for its presence upon arrival.

- Check the expiration dates on the box and tube labels upon arrival. Do not use expired products or components.

Handling and Contamination Prevention:

- Handle the product components and samples properly to avoid contamination and maintain optimal performance.
- Perform nucleic acid extraction, PCR setup and PCR analysis in different compartments. Store samples separately to avoid contact with kit components.
- Avoid interchanging vials or bottle caps.
- Store samples and contaminated materials separately from the kit components.
- Refrain from opening the PCR plates and/or tubes after amplification.
- Use separate/assigned micropipette sets for PCR setup and nucleic acid extraction operations.
- Wear lab coats, caps, masks, and clean pairs of gloves specific/assigned for PCR setup, nucleic acid extraction and PCR analysis operations.
- Before starting work, ensure that the workspace will not cause contamination by cleaning work surfaces with 70% ethanol.

⚠	In cases where there is a serious risk of contamination, it is recommended to clean the relevant surfaces with 10% bleach. Bleach is a powerful and effective disinfectant. While it can disinfect non-porous surfaces in as little as 10 minutes and is available at a low cost, it can irritate mucous membranes, skin, and airways; degrades under heat and light; and easily reacts with other chemicals. Deviating from the recommended dilution (either stronger or weaker) can reduce its effectiveness for disinfection and pose a risk to healthcare workers. Adequate ventilation must be ensured during surface disinfection with bleach, and all relevant occupational health and safety guidelines must be strictly followed (NIH, 2014). After disinfection, the surface should be rinsed twice with the same volume of dH_2O as the bleach used (SIC, 2023). For more detailed information on contamination cleaning, please contact us.

Component Usage:

- Do not combine components from different kit lots, as it may compromise product performance.
- Only use the specified sample types provided with this kit. The use of other sample types can compromise product performance.
- Ensure proper centrifugation of product components after thawing to avoid contamination with reagent residues in the lids, which could compromise product performance.

PCR Setup and Analysis:

- The presence of PCR inhibitors may result in false negative or invalid results.
- Follow the specified volumes for component setup as outlined in the instructions for use. Using different volumes affects product performance.

	Exercise caution to avoid mixing up samples or sample IDs during PCR setup or transfer to the PCR instrument, as the incorrect assignment of samples can lead to false positive or false negative results.

- Adhere to the specified cycling conditions provided in the instructions for use. Using other cycling conditions may compromise product performance.
- Use only the control settings specified in the instructions for use for data analysis. Using different control settings can yield incorrect results.

Equipment and Thawing:

- Use calibrated or verified micropipettes, DNase, RNase and pyrogen-free micropipette tips with filters and DNase, RNase, and pyrogen-free microcentrifuge tubes.
- Thoroughly thaw all components before starting the test procedure. After thawing, centrifuge briefly (spin-down for 3-5 seconds) and mix well to ensure homogeneity before use.
- Keep the kit components on ice or a cooling block until the reaction is prepared and quickly return them to -20 °C.

Safety Precautions and Waste Disposal:

- Review pathogen information to be aware of health-related risks.
- Review cleaning/disinfection procedures suitable for the relevant pathogens.

	Handle biological samples with extreme caution in a microbiological safety cabinet of the appropriate class. Avoid physical contact with pathogens by wearing lab coats, goggles, caps, masks, and gloves. Do not eat or drink within the workspace and prevent unauthorized individuals' access to the working area.

- Discard all pathogenic wastes generated during the nucleic acid extraction step into medical waste bins in compliance with local regulations to ensure safe disposal.
- Kit packaging materials and plastic disposables can have environmental effects. Follow appropriate recycling or disposal protocols to minimize environmental impact. Laboratories must comply with local regulations for the safe disposal of packaging materials and plastic disposables.

5.1. Residual Risks

While this Real-Time PCR kit has been designed to facilitate the accurate detection and quantitation of Cytomegalovirus, it is important to be aware of certain residual risks associated with its usage. In compliance with regulations, the following limitations, contraindications, precautions, or warnings have been provided to ensure user safety and reliable results:

5.1.1. Sample and Detection Limitations

This kit is specifically intended for use with the following sample types: human Bronchoalveolar lavage (BAL), Whole Blood, Urine, Plasma, Cerebrospinal Fluid (CSF) and Amniotic Fluid (AF). It may not yield accurate results when used with other sample types.

The kit is designed for the detection and quantitation of CMV only. It may not be suitable for detecting other pathogens or microorganisms.

5.1.2. Contraindications

There are no specific contraindications associated with the use of this PCR kit. It can be used in all age groups, provided that appropriate samples are collected.

5.1.3. Precautions

For detailed precautions to be taken when using the kit, please read the entire "REQUIRED MATERIALS AND DEVICES" and this section of the user manual. These precautions cover aspects such as sample collection, reagent handling and safety measures to ensure accurate and reliable results.

It is important to note that when the kit is used within its shelf life, under appropriate storage conditions, in suitable laboratory environments and by competent technical personnel, there are no anticipated problems. However, it is crucial to adhere to the precautions and instructions provided in the user manual to minimize any potential risks associated with the use of the kit.

Please carefully review and understand all the information provided, including the limitations, precautions and guidelines outlined in the user manual, to ensure safe and effective usage of this Real-Time PCR kit.

	This information serves as an addition to the user manual and should be considered an essential part of the instructions for use.
	For the Summary of Safety and Performance (SSP) document, please visit: https://anatoliageneworks.com/SSP/SSP_Bosphore_CMV_Quantification_Kit_v03.pdf

6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for *in vitro* diagnostic.
- This product should be used by this user manual.
- This product is to be used by personnel specially trained to perform molecular genetics laboratory techniques.
- This device is intended for use in certain member states within the European Economic Area, Türkiye and Northern Ireland. Please refer to the manufacturer for the most up-to-date list of applicable countries.

7. INFECTION

7.1. CMV DNA as a Diagnostic Biomarker

The quantification of cytomegalovirus (CMV) DNA through nucleic acid amplification techniques (NAATs), particularly real-time PCR, has become the diagnostic mainstay for identifying active CMV replication. Unlike serologic methods, which reflect prior exposure or immune status, CMV DNA detection provides direct evidence of ongoing viral replication and allows quantitative tracking of viral kinetics over time (Al-Omari et al., 2016; Pavšič et al., 2015)

NAATs enable early diagnosis and clinical intervention across diverse populations. In transplant recipients—where CMV is a leading cause of morbidity and mortality—CMV DNA levels in plasma or whole blood serve as actionable thresholds for initiating preemptive antiviral therapy. This strategy has been shown to reduce the incidence of symptomatic disease and improve overall outcomes (Atkinson & Emery, 2011; Lawrence et al., 2024; Saldan et al., 2017). Several FDA-approved real-time PCR assays, such as Abbott RealTime CMV and Alinity M CMV, are routinely used for quantitative monitoring in transplant medicine (Albert et al., 2024; Lawrence et al., 2024). Quantitative monitoring also facilitates assessment of treatment efficacy and early identification of antiviral resistance (Atkinson & Emery, 2011).

In neonates, CMV DNA detection from urine or saliva within the first three weeks of life remains the diagnostic gold standard for congenital CMV (cCMV). Real-time PCR offers near-perfect sensitivity and specificity in these matrices (Liu et al., 2017; Pinninti et al., 2015; Razonable et al., 2020). Quantitative viral load levels are also under investigation as potential prognostic markers for neurodevelopmental outcomes such as sensorineural hearing loss (SNHL) and cognitive impairment (Lawrence et al., 2024; Liu et al., 2017; Saldan et al., 2017). Tanimura and Yamada emphasize that reliance on serologic tests alone can lead to significant underdiagnosis, particularly in symptomatic neonates who remain seronegative due to immature humoral responses (Tanimura & Yamada, 2018).

Beyond neonatology and transplantation, CMV DNA testing is increasingly applied in immunocompromised but non-transplant populations, including individuals with HIV, cancer, or inflammatory bowel disease (IBD). In such cases, CMV DNA can be detected in plasma, cerebrospinal fluid, or mucosal biopsies, helping differentiate true CMV disease from incidental or "bystander" viral shedding (Ciccocioppo et al., 2015; Römkens et al., 2016; Yokoyama et al., 2020) .In ulcerative colitis, CMV DNA quantification in intestinal biopsies supports diagnosis in steroid-refractory cases, often in combination with histologic or antigen-based assays (Yokoyama et al., 2020; Drjagunovs et al., 2022). ECCO guidelines also recommend tissue CMV PCR as a diagnostic adjunct in IBD-related CMV colitis when immunohistochemistry is inconclusive (Drjagunovs et al., 2022).

In the intensive care setting, CMV DNAemia has been increasingly documented in critically ill immunocompetent adults, including those with sepsis, ARDS, or multiorgan dysfunction. Several studies report associations between detectable CMV DNA and prolonged mechanical ventilation, secondary infections, and increased mortality (Al-Omari et al., 2016; Govender et al., 2017; Schattner, 2024). CMV DNA was also shown to predict pulmonary disease in infants with high accuracy when measured in bronchoalveolar lavage fluid, outperforming blood-based PCR (Govender et al., 2017). While causality remains debated, the consistent correlation supports the utility of CMV DNA as a dynamic biomarker of immune dysregulation and systemic stress.

The clinical performance of CMV DNA quantification has been enhanced by the implementation of the WHO International Standard for HCMV DNA (NIBSC 09/162), which enables harmonization of viral load results in international units per milliliter (IU/mL) across laboratories (Fryer et al., 2016; Hayden et al., 2015; Preiksaitis et al., 2016). However, despite this advancement, residual inter-laboratory variability persists due to differences in gene targets (e.g., UL123, UL54, UL55), extraction protocols, and amplification efficiencies between commercial and laboratory-developed assays (Atkinson & Emery, 2011; Novak et al., 2011; Pavšič et al., 2015)

In summary, CMV DNA quantification remains central to the clinical management of CMV infection. It enables early diagnosis, risk stratification, and therapeutic monitoring across a wide range of patient populations. Ongoing improvements in assay standardization, threshold validation, and prognostic modeling continue to enhance its role as both a diagnostic and monitoring tool (Albert et al., 2024; Atkinson & Emery, 2011; Razonable et al., 2020).

7.2. Clinical Conditions

Cytomegalovirus (CMV) is a widespread DNA virus that establishes lifelong latency and has the potential to reactivate in both immunocompromised and immunocompetent individuals. The clinical implications of CMV infection vary widely depending on the host's immune status, ranging from asymptomatic viral shedding to severe organ-specific disease and systemic complications.

Over the past two decades, the quantification of CMV DNA using molecular methods, particularly real-time polymerase chain reaction (qPCR)—has emerged as a cornerstone of CMV diagnostics. CMV DNA testing offers critical insights not only for diagnosis but also for risk stratification, therapeutic decision-making, and outcome prediction across a spectrum of clinical populations. This section reviews the clinical utility of CMV DNA quantification across key patient groups, including solid organ transplant recipients, neonates with congenital infection, individuals with gastrointestinal involvement, immunosuppressed patients with HIV or malignancies, and critically ill patients in intensive care settings.

7.2.1. Transplant

In solid organ transplant recipients, CMV DNA quantification has become a cornerstone of post-transplant surveillance, enabling early detection of CMV reactivation and guiding the initiation and monitoring of preemptive or therapeutic antiviral regimens. Quantitative nucleic acid amplification testing (QNAT) in plasma or whole blood provides a sensitive and reproducible method for detecting viral replication, which is particularly crucial during the high-risk period following transplantation (typically the first 12 weeks) (Razonable et al., 2020). The predictive value of CMV DNAemia extends beyond the identification of CMV syndrome to include organ-specific manifestations such as gastrointestinal disease and hepatitis. While there is no universal threshold for CMV viral load, many centers rely on assay-specific cutoffs to trigger intervention, often between 1,000 and 10,000 IU/mL depending on the clinical context (Atkinson & Emery, 2011; Fryer et al., 2016). Importantly, CMV DNA quantification also plays a role in assessing therapeutic response and detecting antiviral resistance, with persistent or rising viremia despite therapy prompting further investigation (Streck et al., 2023). Despite its widespread use and proven utility in transplant medicine, discussions of CMV DNA quantification in solid organ transplantation often focus on standardization challenges and inter-assay variability (Fryer et al., 2016). Assays such as COBAS AmpliPrep/COBAS TaqMan CMV and Abbott RealTime CMV, both calibrated to the WHO standard, have improved comparability between laboratories but still show up to 1000-fold variation in results due to differences in extraction methods, calibration materials, and qPCR platforms (Hayden et al., 2015; Preiksaitis et al., 2016).

7.2.2. Neonates and Congenital CMV

CMV DNA quantification plays a pivotal role in the detection, prognostication, and clinical management of congenital cytomegalovirus (cCMV) infection. Early postnatal diagnosis typically relies on detecting CMV DNA in saliva or urine specimens collected within the first 21 days of life using real-time PCR assays. Saliva-based screening has demonstrated high sensitivity and specificity and is feasible for large-scale newborn screening initiatives (Boppana et al., 2011).

In the prenatal setting, amniotic fluid CMV DNA quantification has emerged as a valuable biomarker for assessing fetal risk. Higher viral loads in amniotic fluid have been associated with a significantly increased risk of neurodevelopmental impairment and structural anomalies on imaging, such as ventriculomegaly or calcifications (Gilad et al., 2024). While fetal outcomes vary, data show that viral load thresholds above 10^5 copies/mL correlate with symptomatic disease at birth, particularly when imaging findings are also abnormal (Hui & Wood, 2015). Additionally, detection of CMV DNA in cerebrospinal fluid (CSF) has been linked to central nervous system involvement and sensorineural hearing loss, although its prognostic value beyond birth remains limited (Goycochea-Valdivia et al., 2017). Digital PCR has shown promise in predicting neurodevelopmental outcomes more accurately than conventional qPCR, with thresholds above 2,950 copies/mL correlating with adverse developmental outcomes (Lawrence et al., 2024). Emerging diagnostic platforms, such as the Alethia CMV isothermal assay and the Simplexa CMV Direct assay, offer rapid, non-invasive screening options with regulatory clearance for neonatal populations (Lawrence et al., 2024). Additionally, detection of CMV DNA in maternal cervical secretions has shown predictive value for fetal infection in CMV IgM-positive pregnancies (Tanimura et al., 2017).

7.2.3. Gastrointestinal CMV Disease

In the gastrointestinal (GI) tract, CMV DNA quantification has become increasingly important for diagnosing CMV colitis, particularly in patients with underlying inflammatory bowel disease (IBD) or in those receiving immunosuppressive therapies. Reactivation of latent CMV in the colon may mimic or exacerbate symptoms of IBD, especially in patients with steroid-refractory ulcerative colitis, leading to diagnostic uncertainty. Quantitative real-time PCR on intestinal biopsy specimens enables differentiation between CMV colitis and incidental viral shedding by establishing tissue viral load thresholds—typically ≥250 copies/mg (of tissue) or $\geq 10^3$ copies/10^5 cells—as indicators of clinically relevant infection (Ciccocioppo et al., 2015; Drjagunovs et al., 2022). These molecular assays offer superior sensitivity compared to traditional histopathology and can be used alongside immunohistochemistry to enhance diagnostic accuracy (Ciccocioppo et al., 2015). The European Crohn's and Colitis Organisation (ECCO) guidelines recognize CMV DNA testing as a valid alternative to immunohistochemistry for detecting active colonic CMV infection (Kucharzik et al., 2021). Moreover, CMV reactivation in the gastrointestinal tract is not limited to IBD patients—it has also been documented in immunocompromised individuals with AIDS or cancer, where it contributes significantly to morbidity (Drjagunovs et al., 2022). In elderly or immunocompetent patients, CMV gastrointestinal disease may present with severe ulceration, bleeding, or perforation, and the presence of CMV DNA in tissue correlates with worse prognosis (Schattner, 2024; Yokoyama et al., 2020).

7.2.4. HIV/AIDS and Other Immunosuppressed Conditions

In patients with HIV/AIDS and other forms of immunosuppression, CMV DNA serves as a key biomarker for identifying end-organ disease such as CMV retinitis, pneumonitis, or colitis. Among HIV-exposed or infected infants, CMV pneumonitis remains a significant contributor to morbidity and mortality, particularly in regions with high HIV prevalence. Quantitative PCR of bronchoalveolar lavage (BAL) fluid has shown improved diagnostic accuracy over plasma testing, with a proposed threshold of >4.03 log IU/mL suggesting active CMV pneumonitis (Govender et al., 2017). In adults with advanced HIV or hematologic malignancies, CMV reactivation in the gastrointestinal tract or lungs may be difficult to distinguish from other opportunistic infections; thus, CMV DNA quantification provides critical information to support diagnosis and guide antiviral therapy (Drjagunovs et al., 2022). The relevance of CMV DNA testing extends to patients undergoing chemotherapy, where latent viral reactivation is common and may contribute to secondary bacterial or fungal infections. Despite advances in antiviral prophylaxis, these patient groups remain at high risk for CMV complications, underscoring the ongoing need for sensitive and standardized molecular diagnostics (Razonable et al., 2020). In a large prospective study of hospitalized Kenyan children with HIV, CMV viremia ≥1000 IU/mL was independently associated with increased short-term mortality, longer hospitalization, and adverse clinical outcomes, especially in children under 2 years of age (Wamalwa et al., 2022). These findings demonstrate the prognostic utility of CMV DNA in resource-limited and high-burden settings.

7.2.5. Critically Ill Patients (Immunocompetent ICU Population)

CMV DNA quantification has gained increasing recognition as a biomarker of clinical relevance in immunocompetent but critically ill patients, particularly those admitted to intensive care units (ICUs) with sepsis, trauma, burns, or prolonged mechanical ventilation. While traditionally considered an opportunistic infection in immunocompromised hosts, CMV reactivation has been observed in 30–70% of seropositive ICU patients, often occurring within the first two weeks of admission (Al-Omari et al., 2016). This reactivation has been associated with adverse outcomes, including increased mortality, longer ICU stays, and heightened susceptibility to nosocomial infections. Although the clinical significance of CMV DNAemia in this population is still debated, accumulating evidence suggests that it reflects immune dysfunction and may exacerbate systemic inflammation through cytokine-mediated pathways (Griffiths et al., 2015). Quantitative PCR is used to monitor viral load dynamics, and studies have shown an inverse correlation between viral load and interferon-γ T cell responses in critically ill patients (Al-Omari et al., 2016). The detection of CMV DNA in blood or BAL fluid in this setting may thus serve as both a prognostic marker and a potential therapeutic trigger, although consensus on treatment thresholds remains to be established. In heart failure patients, even low-level CMV DNAemia (<100 copies/mL) has been independently associated with higher readmission and mortality rates, further supporting its prognostic relevance.

CMV reactivation has also been observed in patients with COVID-19, with studies suggesting a role for CMV in complicating recovery and worsening pulmonary inflammation (Schattner, 2024)

7.3. Existing Clinical Guidelines Supporting CMV DNA Testing

Several leading public health authorities have formally endorsed the use of CMV DNA testing for diagnostic and monitoring purposes in defined clinical settings. These endorsements underscore the analytical validity and clinical relevance of nucleic acid-based CMV diagnostics, particularly in transplant medicine, congenital infection, and assay standardization.

The World Health Organization (WHO) established the first International Standard (IS) for CMV DNA in 2010, developed by the National Institute for Biological Standards and Control (NIBSC) and adopted by the WHO Expert Committee on Biological Standardization. This standard (NIBSC code 09/162) provides a uniform reference for CMV DNA quantification in international units (IU/mL), supporting harmonization of assay performance across laboratories and platforms (Fryer et al., 2016; Hayden et al., 2015; Preiksaitis et al., 2016). Although the introduction of the WHO IS has significantly reduced inter-laboratory variation, studies have shown that clinically relevant variability still persists due to differences in extraction methods, amplification targets, and calibrator materials (Preiksaitis et al., 2016)

In the United States, the Food and Drug Administration (FDA) has cleared several CMV DNA tests for clinical use, including platforms for both quantitative and qualitative applications. Quantitative CMV PCR assays such as the Abbott RealTime CMV, Roche COBAS AmpliPrep/COBAS TaqMan CMV, and Aptima CMV Quant (Hologic) are FDA-approved and widely used for viral load monitoring in transplant recipients. In the context of congenital CMV, the FDA has also approved the Simplexa™ Congenital CMV Direct assay (DiaSorin), a qualitative real-time PCR test for saliva and urine specimens collected within 21 days of birth. While the assay provides qualitative results, its software displays cycle threshold (Ct) values, which can help clinicians approximate viral burden and guide retesting decisions (Lawrence et al., 2024).

The Centers for Disease Control and Prevention (CDC) supports CMV DNA PCR testing as the diagnostic gold standard for confirming congenital CMV infection. The CDC recommends that testing be performed using saliva or urine specimens collected within the first three weeks of life, after which it becomes difficult to distinguish congenital from postnatal CMV acquisition(Singh & Gaidhane, 2022). This recommendation aligns with national early hearing detection and intervention protocols, in which targeted CMV screening is advised for newborns who fail initial hearing tests or show clinical signs suggestive of infection.

In Europe, the European Crohn's and Colitis Organisation (ECCO) provides guideline-based support for CMV DNA testing in the context of inflammatory bowel disease (IBD).

The ECCO consensus recommends the use of quantitative CMV PCR on intestinal biopsy specimens, alongside immunohistochemistry, to diagnose CMV colitis in IBD patients—particularly those unresponsive to immunosuppressive therapies. While viral load thresholds have not been universally defined, the guidelines emphasize PCR as a reliable diagnostic adjunct in severe or refractory disease (Kucharzik et al., 2021)

These regulatory and public health endorsements reinforce the clinical validity and increasing standardization of CMV DNA testing, particularly in neonatal diagnostics, transplant surveillance, and assay calibration.

7.4. Overview of Current CMV Diagnostic Methods

Cytomegalovirus (CMV) diagnostics have evolved substantially over recent decades, with nucleic acid amplification techniques now representing the cornerstone of clinical detection. Among these, quantitative polymerase chain reaction (qPCR) has emerged as the preferred method due to its high sensitivity, specificity, and rapid turnaround time. It is routinely used to detect CMV DNA in a wide range of clinical specimens, including plasma, whole blood, urine, saliva, cerebrospinal fluid, bronchoalveolar lavage (BAL), and tissue biopsies (Albert et al., 2024; Al-Omari et al., 2016; Li et al., 2023). CMV qPCR is particularly valuable in managing transplant recipients, where viral load measurements guide preemptive therapy and monitor treatment response (Preiksaitis et al., 2016).

Historically, viral culture was considered the gold standard for CMV detection due to its specificity. However, its low sensitivity and slow turnaround time have rendered it largely obsolete in modern clinical settings (Al-Omari et al., 2016). CMV pp65 antigenemia assays remain in use, particularly in transplant settings, offering a relatively rapid and cost-effective alternative. Nevertheless, these assays are limited by their dependence on sufficient leukocyte counts and a lack of inter-laboratory standardization (Li et al., 2023; Yokoyama et al., 2020). Immunohistochemistry (IHC) and histopathology continue to serve as reference standards for diagnosing tissue-invasive CMV disease, although their invasive nature and requirement for specialized interpretation limit their routine use (Drjagunovs et al., 2022; Yokoyama et al., 2020).

Serologic testing, particularly the detection of CMV-specific IgG and IgM along with IgG avidity assays, is useful in determining primary CMV infection, especially in pregnant women. However, serology has limited utility in immunocompromised individuals, where CMV reactivation or reinfection rather than primary infection is often the clinical concern (Al-Omari et al., 2016; Razonable et al., 2020). In congenital CMV infections, serology plays only a minor role, with nucleic acid-based testing being the diagnostic method of choice. Studies have shown that IgM assays have suboptimal sensitivity and specificity, while IgG avidity may help differentiate recent from past infections, but only within specific gestational windows (Saldan et al., 2017).

Emerging molecular technologies have sought to overcome some of the limitations of qPCR. Droplet digital PCR (ddPCR), for instance, offers improved precision in quantification and is less affected by variability in standard curves, making it attractive for longitudinal monitoring and inter-laboratory comparison (Li et al., 2023; Pavšič et al., 2015). However, ddPCR is still undergoing clinical validation and is not yet widely adopted in routine diagnostics. Other innovations, including loop-mediated isothermal amplification (LAMP), CRISPR-based detection platforms, and metagenomic next-generation sequencing (mNGS), are under investigation and may offer point-of-care or multiplex testing capabilities in the future (Lawrence et al., 2024).

Despite efforts to harmonize testing, such as the introduction of the first WHO International Standard for CMV DNA, clinically meaningful inter-laboratory variability persists, especially when different platforms and specimen types (e.g., plasma vs. whole blood) are used (Hayden et al., 2015; Preiksaitis et al., 2016). This underscores the importance of using the same assay consistently for longitudinal monitoring within individual patients.

Ultimately, while several diagnostic tools are available, qPCR remains the standard of care for detecting and quantifying CMV across most clinical contexts. Its versatility across specimen types and its role in guiding treatment decisions underline its centrality in CMV diagnostics today (Al-Omari et al., 2016; Lawrence et al., 2024).

8. METHOD

Bosphore CMV Quantification Kit is a quantitative diagnostic tool designed to detect specific DNA regions using the Real-Time PCR method. Real-Time PCR, also known as quantitative PCR (qPCR), is a powerful technique that allows monitoring of the PCR reaction in Real-Time, eliminating the need for additional analysis methods such as gel electrophoresis.

The Real-Time PCR reaction begins with an initial denaturation step. During this step, the PCR tube containing the reaction mixture is heated to a temperature of 95 °C and this high temperature is maintained for a duration of 10 minutes. The purpose of the initial denaturation is to separate the double-stranded DNA template into single strands, providing the starting point for subsequent amplification. This step ensures that the DNA strands are available for binding with specific primers.

Following the initial denaturation, the reaction proceeds through a series of cycles, each consisting of denaturation and annealing steps. The denaturation step occurs during each cycle, where the reaction mixture is heated to a temperature of 97 °C for 15 seconds.

This high temperature ensures that the DNA strands separate completely, providing single-stranded templates for the subsequent steps. Denaturation involves the disruption of the hydrogen bonds between the DNA strands, leading to the separation of the double-stranded DNA into individual single-stranded DNA templates.

After denaturation, the reaction temperature is lowered to 62 °C for 50 seconds during the annealing step. At this lower temperature, the primers designed to be complementary to the target regions of the DNA template bind to their specific sequences. Primers are short DNA sequences that are designed to hybridize, or anneal, to the complementary sequences on the single-stranded DNA template. This step allows the primers to find their target regions and bind to them, indicating the presence of the target DNA sequences.

During the cycles, the denaturation and annealing steps repeat for a total of 45 repetitions. Each cycle consists of a denaturation step to separate the DNA strands and an annealing step to allow the primers to bind to their target sequences. These repeated cycles increase the chances of detecting the target DNA sequences, indicating a positive result.

Bosphore CMV Quantification Kit utilizes Real-Time PCR with an initial denaturation step followed by repeated cycles of denaturation and annealing and hold step. The denaturation step ensures complete separation of the DNA strands, while the annealing step facilitates the binding of primers to their target sequences, indicating the presence of the target DNA regions.

Threshold Cycle (C_T) and Result Interpretation:

During the Real-Time PCR reaction, the fluorescence generated by the reporter molecule is monitored. The point at which the fluorescence signal rises above the background level and becomes distinguishable is known as the threshold cycle (C_T). The samples that cross the threshold are regarded as positive. The samples that do not cut the threshold must be regarded as negative or having a viral load below the detection limit of the assay.

Bosphore CMV Quantification Kit is a quantitative assay, meaning it provides a positive or negative result for target DNA sequences and also allows for viral load measurement. A positive result indicates the presence of target DNA sequences, while a negative result suggests their absence or a viral load below the detection limit of the assay.

Multiplex qPCR and Internal Control:

Bosphore CMV Quantification Kit employs multiplex qPCR, allowing simultaneous detection and amplification of multiple target regions. Internal control is included in the system to monitor the extraction procedure, PCR inhibition and potential issues during the application. The internal control ensures the reliability of the PCR results by validating the overall PCR process and identifying any potential issues that may affect the interpretation of the results.

9. PROCEDURE

9.1. Specimen Collection and Handling

9.1.1. Amniotic Fluid

Amniocentesis is typically performed between the 15th and 20th weeks of pregnancy, but it can be done later if necessary. Initially, an ultrasound is used by a healthcare professional to determine the baby's position in the womb. Under ultrasound guidance, a thin, hollow needle is inserted into the uterus through the abdominal wall. A small amount of amniotic fluid is withdrawn using a syringe, after which the needle is removed (NHS 2022). Amniotic fluid should be collected via amniocentesis using aseptic techniques, with at least 10 mL transferred into a sterile, leak-proof tube. The first 2 to 5 mL of the amniotic fluid sample is discarded with the first syringe and the actual sample should be collected using a second syringe, thereby reducing the risk of maternal contamination (CLSI-MM13Ed2E Chapter 3).

9.1.2. Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) is a medical procedure used to collect fluid samples from the bronchoalveolar spaces in the lower respiratory tract. To ensure patient safety and the reliability of test results, strict aseptic techniques and protocols must be followed during sample collection. While BAL samples can be collected at any stage of the clinical process, it is often most beneficial to perform the procedure before initiating antibiotic or steroid treatments. To begin, position the patient in a semi-recumbent posture for comfort. Since the procedure can cause discomfort, administer local anesthesia to numb the throat and upper respiratory tract. Insert the bronchoscope carefully through the patient’s mouth or nose and guide it into the lower respiratory tract, ensuring it reaches the correct location. Using the bronchoscope channel, introduce sterile saline or sterile water into the bronchoalveolar spaces. Aspirate the fluid gently and collect it into a sterile container. Record the exact volume of fluid aspirated to ensure accurate documentation (CDC, 2024). Bronchoalveolar lavage (BAL) samples should be collected in sterile, nuclease-free containers with a minimum volume of 1–2 mL, avoiding excess mucosal contamination. While the sample may require pre-treatment for analysis, it must remain undiluted and unprocessed throughout storage and transportation to preserve its integrity (CLSI-MM13Ed2E Chapter 3).

9.1.3. Cerebrospinal Fluid

The purpose of this test is to measure cerebrospinal fluid (CSF) pressure and to collect a sample for diagnostic analysis. CSF studies are instrumental in diagnosing specific neurological conditions, such as brain or spinal cord injuries and infections like meningitis. In cases of suspected normal pressure hydrocephalus, a spinal tap may also be performed.

CSF samples are typically obtained via a lumbar puncture, the most commonly used technique. During the procedure, a spinal needle is carefully inserted into the lumbar region of the spine. After proper placement, 1 to 10 mL of CSF is collected, and CSF pressure is measured. This sample is then used for further diagnostic testing (Teunissen et al., 2009).

Cerebrospinal fluid (CSF) should be collected via lumbar puncture in a sterile, screw-cap tube, with at least 1 mL required for analysis, avoiding exposure to excessive heat or freezing to prevent nucleic acid degradation. While the sample may require pre-treatment for analysis, it must remain undiluted and unprocessed throughout storage and transportation to preserve its integrity (CLSI-MM13Ed2E Chapter 3).

9.1.4. Plasma

Whole blood should be collected into commercially available anticoagulant-treated tubes, EDTA tubes (commonly with lavender tops). To obtain plasma, samples are centrifuged—preferably using a chilled centrifuge to prevent cellular degradation. (Luque-Garcia & Neubert, 2007).

Plasma samples should be obtained from EDTA whole blood, centrifuged at 800×g –1600×g for 10–15 minutes, and carefully transferred into a fresh tube to avoid leukocyte contamination (CLSI-MM13Ed2E Chapter 3).

9.1.5. Urine

Urine samples should be midstream collections, with at least 10 mL in a sterile container (CLSI-MM13Ed2E Chapter 3). Sterile screw-cap container is labeled with the patient's name and date of birth. For sample collection, the patient should begin urination and then collect a "first catch" or "midstream" urine sample directly into the container. Once the sample is collected, the container should be tightly sealed, and the patient should wash their hands thoroughly (NHS 2022b).

9.1.6. Whole Blood

Whole blood consists of biological components such as red blood cells, white blood cells, and platelets, which are suspended in plasma—the liquid portion of the blood. Approximately 40% of blood volume is composed of cellular elements (red blood cells, white blood cells, and platelets), while the remaining 60% is the straw-colored fluid known as plasma. Hemolysis, the destruction of red blood cells, can alter laboratory test results and compromise sample quality. It may occur due to various factors, including temperature fluctuations, exposure to contaminants, or improper blood collection techniques (NHS 2023).

Whole blood should be collected in EDTA tubes, mixed gently to prevent clotting. All specimens must be clearly labelled (CLSI-MM13Ed2E Chapter 3). During blood collection, once the sample is obtained, the tourniquet is released, and the needle is withdrawn. Firm pressure is applied to the

puncture site with a sterile, dry cotton pad for about 10 minutes to stop bleeding and minimize bruising. Patients should be advised to continue applying gentle pressure to the site as needed (NHS 2023).

9.2. Specimen Stability

The specimen and viral CMV DNA stability studies and transportation conditions were conducted for the Bosphore CMV Quantification Kit and these studies were demonstrated that the kit meets the performance requirements. The study evaluated the specimen stability and transport conditions of CMV specimens in each matrix. It was also evaluated the stability of CMV DNA under various storage conditions.

Proper handling and transportation conditions are critical for maintaining the integrity of CMV DNA. Based on the study findings, the following storage and transport conditions are recommended.

Table 3: CMV Specimen and DNA Stability Conditions

Specimen	Transport/Storage conditions			Freeze and Thaw cycle
	15–30 °C (room temperature)	2–8 °C	≤-18 °C	
Whole blood	≤1 day	≤7 days	≤30 days	Up to 3
Plasma	≤1 day	≤7 days	≤30 days	Up to 3
CSF	≤1 day	≤7 days	≤30 days	Up to 3
Urine	≤1 day	≤1 days	≤30 days	Up to 3
Amniotic fluid	≤1 day	≤7 days	≤30 days	Up to 3
Bronchoalveolar lavage (BAL)	≤1 day	≤7 days	≤30 days	Up to 3
Viral CMV DNA	≤3 days	≤7 days	≤30 days	Up to 3

9.3. DNA Extraction

Viral DNA extraction should be performed following the instructions provided by the extraction kit manufacturer. Ensure that all steps of viral DNA extraction are conducted within a biological safety cabinet. For efficient DNA extraction, we recommend using the following kits and systems:

- Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Viral DNA/RNA Extraction Kit 600 µl and Unio Whole Blood Genomic DNA Extraction Large Volume Kit
- Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit (Anatolia Geneworks)

Please ensure that the viral DNA extraction is performed in accordance with the instructions provided by the manufacturers.

9.4. Kit Components

9.4.1. Negative Control

The negative control is an essential component of Bosphore CMV Quantification Kit for Real-Time PCR. It consists of distilled water (dH_2O) and serves as a reference sample to ensure accurate results by detecting any contamination or errors that could lead to false-positive outcomes.

To use the negative control:

- Label a separate tube or well as "Negative Control."
- Add the provided dH_2O to the labeled tube or well.
- Treat the negative control sample the same way as your experimental samples.
- Include the negative control in each PCR run.

Throughout the reaction, monitor the negative control closely. It should remain negative, showing no amplification signals (No C_T). Any amplification in the negative control indicates potential contamination. When the internal control is added during the extraction or PCR step, an amplification should be detected in the HEX filter of the negative control.

Negative control plays a vital role in Bosphore CMV Quantification Kit, ensuring the reliability of your results. Including negative control and carefully monitoring it will enable you to confidently interpret your data.

9.4.2. PCR Master Mix

The Real-Time PCR Master Mix is a comprehensive solution designed for efficient and accurate Real-Time PCR amplification. It combines a highly specific and accurate *Taq* DNA Polymerase with hot-start property, PCR buffers, a balanced mix of dNTPs, specific forward and reverse primers, dual-labeled probes, and essential additives. This master mix has been carefully formulated to enable simultaneous detection of CMV and internal control within a single reaction. To prepare the reaction, simply add 16 µl of template to 24 µl of the PCR Master Mix.

9.4.3. Internal Control

The kit includes an internal control that serves multiple purposes. The internal control is a synthetic DNA molecule specifically designed to assess the efficiency of DNA extraction, identify application errors, and detect any potential PCR inhibition.

This control is added to the sample, proteinase K, and carrier RNA mixture at the beginning of the DNA extraction process to evaluate the extraction efficiency and identify any application errors that may occur.

While adding the internal control during nucleic acid extraction, it is recommended to add 5 µL per sample. Conversely, when directly adding the internal control to the PCR Master Mix to monitor PCR inhibition, only 0.2 µL is required. For a comprehensive evaluation of the extraction system's efficiency, we suggest adding internal control to the negative control.

Please note that if the internal control has already been included during the extraction step, there is no need to incorporate it into the PCR Master Mix.

In negative samples, the absence of internal control amplification in the target filter may indicate issues with extraction or application, or potential PCR inhibition. In such cases, it is advisable to repeat both the extraction and PCR steps.

It is important to be aware that in samples with a high viral load, including the positive control, the internal control's fluorescent signal may be suppressed, making it difficult to detect an increase in the signal level.

9.4.4. Quantitation Standards

The quantitation standards for the Bosphore CMV Quantification Kit have been calibrated with the WHO First International Standard Human CMV DNA (NIBSC Code: 09/162). The standard concentrations for different extraction devices, kits, and matrices are shown in the table below. It should be noted that the standards must be extracted in order to be added to PCR.

Table 4: The standard concentrations used with different systems and kits

	Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Viral DNA RNA Extraction Kit 600 µl or Unio Whole Blood Genomic DNA Extraction Large Volume Kit					
	Plasma (IU/mL)	**Whole Blood (IU/ mL)**	**Urine (IU/ mL)**	**CSF (IU/ mL)**	**Amniotic Fluid (IU/ mL)**	**BAL (IU/mL)**
Standard 1	10^6	1.25×10^6	2×10^6	6.5×10^5	7×10^5	8×10^5
Standard 2	10^5	1.25×10^5	2×10^5	6.5×10^4	7×10^4	8×10^4
Standard 3	10^4	1.25×10^4	2×10^4	6.5×10^3	7×10^3	8×10^3
Standard 4	10^3	1.25×10^3	2×10^3	6.5×10^2	7×10^2	8×10^2
	Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit					
	Plasma (IU/mL)	**Whole Blood (IU/ mL)**	**Urine (IU/ mL)**	**CSF (IU/ mL)**	**Amniotic Fluid (IU/ mL)**	**BAL (IU/mL)**
Standard 1	7.5×10^5	10^6	2×10^6	7.5×10^5	7×10^5	8×10^5
Standard 2	7.5×10^4	10^5	2×10^5	7.5×10^4	7×10^4	8×10^4
Standard 3	7.5×10^3	10^4	2×10^4	7.5×10^3	7×10^3	8×10^3
Standard 4	7.5×10^2	10^3	2×10^3	7.5×10^2	7×10^2	8×10^2

9.4.5. Positive Control

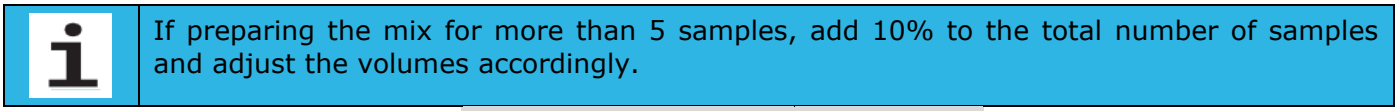
The provided kit encompasses a synthetic DNA positive control, which encompasses synthetic DNA from Cytomegalovirus. To assess the efficiency of the PCR reaction, it is imperative to incorporate this positive control into the testing process. To determine the acceptability of the PCR results, please refer to the acceptance criteria table outlined in Section 10 of this manual. The table specifies the threshold C_T value of the positive control.

If the threshold C_T value of the positive control exceeds the upper limit specified in the criteria table, it could indicate a potential decrease in the reaction's yield. Therefore, monitoring the C_T value of the positive control aids in assessing the overall efficiency of the reaction.

9.5. Preparing the PCR

Before starting the Real-Time PCR, it is important to prepare the PCR reaction mix accurately. Each PCR should include at least one negative control and positive control, and the samples with internal control. Please follow the detailed instructions below:

- Ensure that all kit components are properly dissolved before use. Thoroughly mix the reagents by gentle inversion or vortexing and perform spin down.
- Use the table provided below to calculate the volumes of each component required for the PCR reaction mix. The volumes mentioned in the table are for one reaction only.
- To determine the volumes needed for the master mix, multiply these volumes by the total number of samples.



Components	Volume
PCR Master Mix	24 µL
Internal Control*	0.2 µL
Sample DNA (Negative / Positive Control/Standards**)	16 µL
Total Volume	40 µL

*Internal control should not be added to the reaction if it has already been added during the extraction step.
**Quantitation standards should be added to the PCR after extraction.

Table 5: Bosphore CMV Quantification Kit components and volumes.

- Prepare PCR tubes or strips for each reaction. Pipette 24 µL of the PCR Master Mix into each PCR tube or well of the strip/plate.
- Add 16 µL of the appropriate template to each designated well or tube. Ensure that the DNA samples, positive control, standards, and negative control are added to separate wells or tubes.
- Close the tube caps/seal the PCR plate carefully to prevent contamination and evaporation.
- Ensure that the mix in each tube is at the bottom of the tube. If necessary, briefly spin down the tubes to collect the contents at the bottom.
- Once the PCR reaction mix is prepared, it is ready for the next steps in the PCR process. Follow the provided protocol to proceed with the Real-Time PCR analysis.

9.6. Programming the Real-Time PCR Instrument

The thermal protocol for Bosphore CMV Quantification Kit consists of initial denaturation for activation of the *Taq* DNA Polymerase (with hot-start property), a two-step amplification cycle and a terminal hold. The Real-Time data is collected at the second step of the amplification cycle. The thermal protocol to be applied for the reaction is indicated below:

Steps	Temperature	Time	
Initial Denaturation	95 °C	10:00 min	
Denaturation	97 °C	00:15 min	45 Cycles
Annealing (Data Collection)	62 °C	00:50 min	
Hold	32 °C	00:20 min	

Table 6: Bosphore CMV Quantification Kit thermal protocol.

Before starting to work with Bosphore CMV Quantification Kit, the following steps must be completed and checked:

- Choose all the filters to be used (FAM and HEX),
- Identify unknown samples, positive, standards, and negative controls,
- Select the correct thermal protocol,
- Start the experiment.

9.6.1. Experiment Setup for Montania 4896 Real-Time PCR Instrument

Preparation:

- Ensure that the Montania 4896 Real-Time PCR Instrument is set up correctly and connected to a computer installed by the manufacturer or authorized technical service.
- Verify that all the necessary reagents and components for the PCR reaction are available and stored under appropriate conditions for the experimental setup.

Sample and Reagent Setup:

- Prepare the PCR reaction mixture according to the specifications mentioned above.
- Distribute the PCR Master Mix homogeneously into PCR tubes.

Adding Samples:

- Add the extracted DNA templates, standards, positive control, and negative control to the PCR tubes.

- Gently mix the contents to ensure proper mixing of the template with the reaction mixture.

Loading the PCR Instrument:

- Open the software interface for the Montania 4896 Real-Time PCR Instrument on the connected computer.
- Create a new experiment or select a pre-existing experiment template suitable for your PCR reaction.
- Choose in which block the experiment will be performed.
- Define the necessary parameters such as reaction volume, cycling conditions and fluorescence detection filters.

Loading Samples:

- Transfer the prepared PCR tubes to the appropriate sample block of the Montania 4896 Real-Time PCR Instrument. Ensure that the lids are tightly closed, and the samples are properly aligned in the block to avoid potential issues due to sudden temperature changes during operation.

Initiating the PCR:

- Double-check that all the required settings and parameters have been accurately entered into the software interface.

	It is recommended to label the experiment at this stage to avoid mixing up the order of sample, standard, positive control, and negative control in the mixes.

- Start the PCR run using the software interface to initiate the cycling program and data collection process.
- Monitor the progress of the PCR run in the software interface by observing the amplification curves and fluorescence signals.

Data Analysis and Interpretation:

- After the completion of the PCR run, the Montania 4896 Real-Time PCR Instrument will automatically generate amplification curves for each sample.
- After ensuring that the standards are already selected, choose the analysis type Quantitative on the right side.

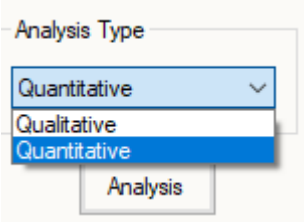


Figure 1: Selection of analysis type For Montania 4896 Real-Time PCR Instrument

- The instrument will automatically generate a standard curve and quantify the wells selected as sample. Interpret the generated data based on the information provided in this user manual.
- If the negative control, positive control and standards meet the acceptance criteria, analyze the standard and target amplifications accordingly.

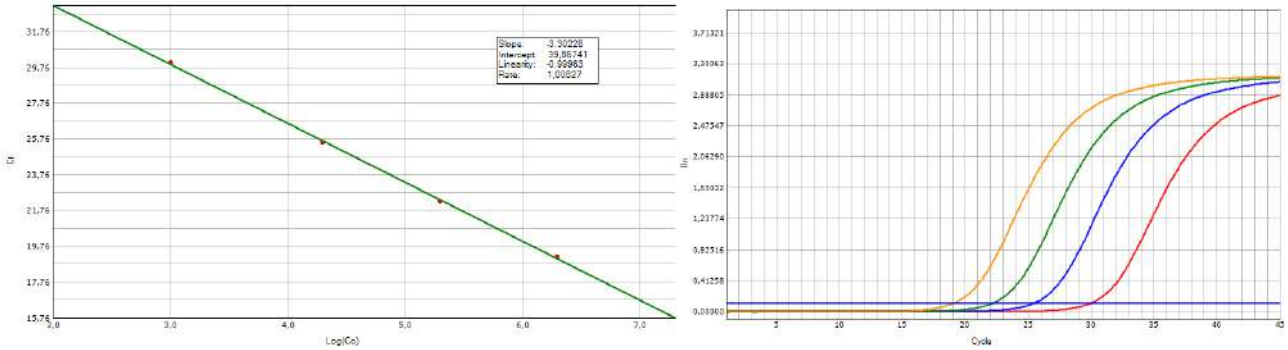


Figure 2: Standard And Amplification Curves For Montania 4896 Real-Time PCR Instrument

9.6.2. Experiment Setup for CFX96 Real-Time PCR Detection System

Instrument Preparation:

- Ensure that the CFX96 instrument is properly connected to a power source and turned on.
- Allow the instrument to warm up to the desired temperature (if applicable) following the manufacturer's instructions.
- Open the CFX Manager software on the connected computer.

Sample Preparation:

- Prepare your PCR reaction mix according to the instructions provided with Bosphore CMV Quantification Kit.
- Add your DNA sample, positive control, standards, and negative control to the PCR Master Mix.

- Mix the reaction thoroughly by pipetting gently and ensure that all components are well combined.

Plate Setup:

- Prepare a white PCR plate compatible with the CFX96 system. Ensure that the wells are labeled correctly to identify the samples.
- Transfer the PCR reaction mix into the appropriate wells of the PCR plate using a micropipette. Include standards, positive and negative controls.
- Cover the PCR plate with an optically clear adhesive film or seal to prevent contamination and evaporation.

Instrument Setup:

- Launch the CFX Manager software on the connected computer if not already open.
- Place the prepared PCR plate into the CFX96 instrument.
- Create a new experiment in the software and specify the experiment parameters (e.g., target, standards, positive control, negative control, reaction volume, plate properties etc.).
- Set up the thermal cycling conditions, including the initial denaturation temperature and time, amplification cycles and annealing temperatures.

Running the Experiment:

- Start the experiment by clicking the "Run" button in the CFX Manager software.
- The CFX96 instrument will perform the specified thermal cycling program while simultaneously monitoring fluorescence signals in real time.
- Once the run is complete, the CFX Manager software will generate a Real-Time PCR standard curve, amplification plot and provide data analysis options.

Data Analysis:

- Analyze the Real-Time PCR amplification plot using the CFX Manager software. Interpret the results according to the experimental objectives and acceptance criteria communicated in this manual.

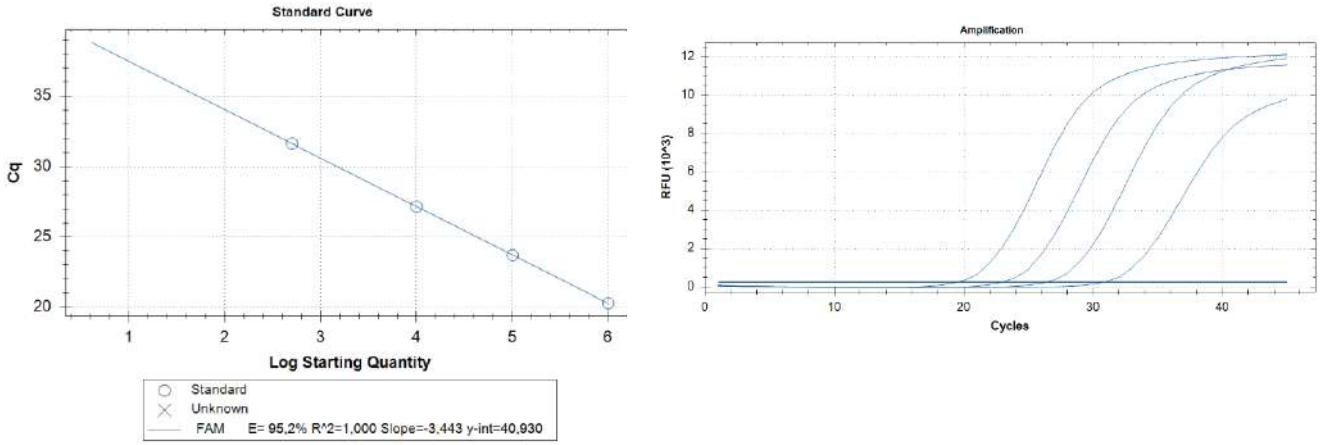


Figure 3: Standard And Amplification Curve For CFX96 Real-Time PCR Detection System

Applying Fluorescence Drift Correction and Baseline Setting:

- In the CFX Manager software, navigate to the experiment settings.
- Enable the Fluorescence Drift Correction feature to compensate for any potential variations in fluorescence intensity during the run.
- Set the Baseline Setting to the appropriate parameters to ensure accurate baseline calculations during data analysis.

9.6.3. Experiment Setup for LightCycler 480 Instrument II

Instrument Preparation:

- Ensure that the LightCycler 480 Instrument II system is properly connected to a power source and turned on.
- Make sure a white plate compatible with the Roche LightCycler 480 Instrument II is used.

⚠	Before conducting any experiments with the LightCycler 480 Instrument II, it is essential to perform the color compensation protocol once. This step is crucial for ensuring accurate results. Please use the Universal CC FAM (510) - VIC (580) specifically designed for this instrument. Follow the instructions provided with the kit carefully to execute the color compensation protocol accurately.

Perform Color Compensation Protocol:

- Perform a color compensation protocol using Universal CC FAM (510) - VIC (580) specifically designed for the LightCycler 480 Instrument II system.
- Follow the instructions provided with the kit to perform the color compensation protocol accurately.

Reagent Preparation:

- Prepare your PCR Master Mix according to the instructions provided with Bosphore CMV Quantification Kit.
- Ensure that your PCR Master Mix is properly aliquoted into individual wells of the white plate.

Sample Preparation:

- Prepare your samples and controls in appropriate volumes.
- Add your samples to the PCR Master Mix, making sure to include positive control, standards, and negative control.

Performing the Experiment

- Open the LightCycler 480 Instrument II software on the connected computer.

Create a New Experiment:

- Set up the experiment by specifying the type of experiment (quantification).
- Enter the necessary experimental details, such as the target filters, sample names and reaction conditions.
- Define quantification sample types as standards for the well with standard samples.
- Define the appropriate cycling protocol for your experiment, including denaturation, annealing and extension temperatures and times.

Set up the Plate Layout:

- Assign the sample names to the corresponding wells in the white plate.
- Include appropriate control wells (standards, positive and negative controls) for result interpretation.

Load the White Plate:

- Carefully load the white plate with the prepared PCR Master Mix and samples, following the plate layout defined in the software.
- Ensure that the plate is properly sealed to prevent contamination and evaporation.

Start the Experiment:

- Place the white plate into the LightCycler 480 Instrument II system multiwell plate loader.

- Retract the loader with the inserted PCR plate into the instrument.
- Start the run using the predefined cycling protocol.

Data Analysis and Interpretation

Monitor the amplification in Real-Time:

- During the experiment, the LightCycler 480 Instrument II system will continuously measure the fluorescence emitted by the samples.
- The instrument software will display real-time amplification curves for each well.

Analyze the data:

- Once the run is complete, open the LightCycler 480 Instrument II software for data analysis.
- Choose "Abs Quant/Fit Points" as the analysis type to ensure appropriate threshold level selection.
- Select “Abs Quant” from the create new analysis list.
- Click “Analysis” to display standard curve.
- If you included standards in the experiment, double-click the box next to the standard you want to add to the standard curve and select it.
- Select “Std curve (in run)”.
- After this, click “calculate” to calculate and display C_T value.
- Perform the analysis using the "Cycle Range" option.

Interpret the results:

- Refer to the acceptance criteria contained in this manual for result interpretation.

Remember to refer to the LightCycler 480 Instrument II system user manual for detailed instructions on instrument setup, operation, and data analysis. Additionally, follow the instructions provided with Universal CC FAM (510) - VIC (580) for accurate color compensation protocol execution.

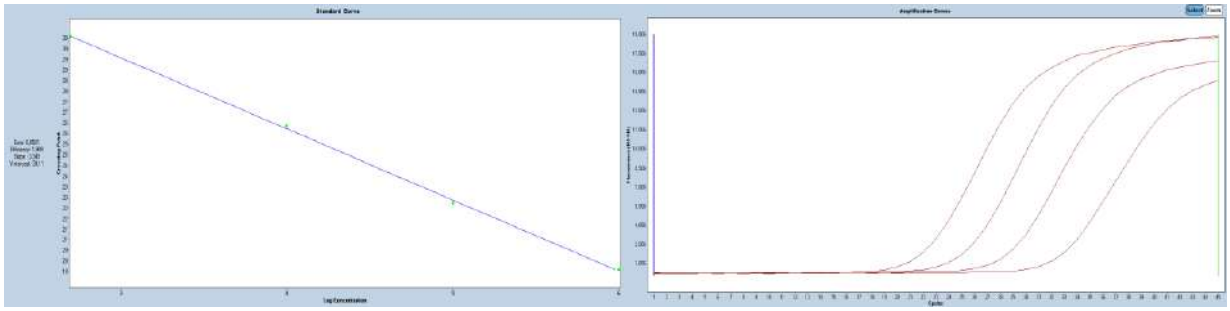


Figure 4: Standard and Amplification Curve for LightCycler480 Instrument II

9.6.4. Experiment Setup for RotorGene Q

Instrument Setup:

- Ensure that the RotorGene Q instrument is placed on a stable and level surface.
- Connect the instrument to a power source and switch it on.
- Allow the instrument to warm up according to the manufacturer's instructions.

Sample Preparation:

- Collect and prepare the samples according to Bosphore CMV Quantification Kit guidelines.

Reaction Setup:

- Dispense the PCR Master Mix into PCR tubes or strips, taking care to avoid any contamination.
- Add the DNA templates, standards and positive/negative controls to the appropriate wells or tubes, ensuring proper sample identification.

Load the Strips/Tubes:

- Carefully load the strips/tubes with the prepared PCR Master Mix and samples, following the proper sample identifications defined in the software.
- Ensure that the strip/tube caps are properly closed to prevent contamination and evaporation.
- When the device is initialized, the sample setup screen will open.
- Enter the sample names, click on "Type" next to the selected number to specify the well type.
- Then under the "Sample" tab, click on "Unknown" and select "Standard".
- In the "Given Conc." section, enter the concentration value.

Thermal Cycling Conditions:

- Set appropriate thermal cycling conditions on the RotorGene Q instrument for Bosphore CMV Quantification Kit.
- Disable automatic gain optimization and manually set the gain for each filter to 10.
- Optimize the cycling parameters, including denaturation temperature, annealing temperature, and the number of cycles, for your specific assay.

Data Analysis:

- After the Real-Time PCR run is complete, click "Analysis" to analyze the data.
- Select the channel to be analyzed and click "Show". In the window that opens, select "Linear Scale" under the graph.
- Then, if it is necessary to correct the graph, "Dynamic Tube" and "Slope correct" settings are activated.
- After the graphic image is proper, set the threshold to an appropriate value.

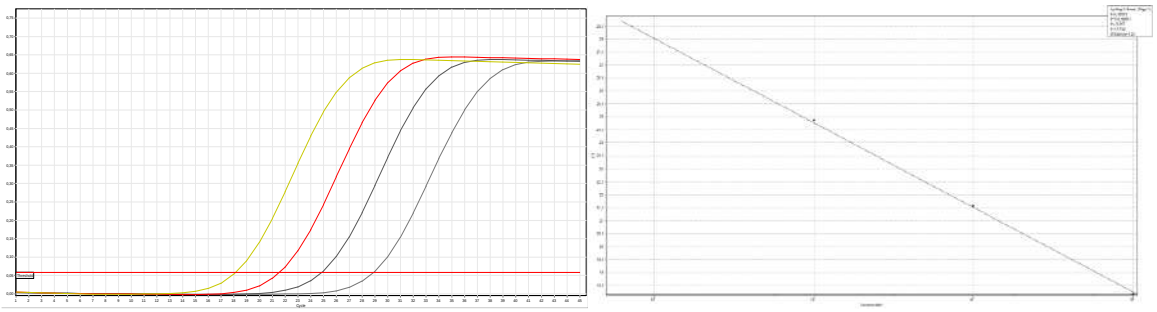


Figure 5: Amplification curve for RotorGene Q

9.6.5. Experiment Setup for Quant Studio 5 Real-Time PCR Instrument

Instrument Preparation:

- Ensure that the Quant Studio 5 instrument is powered on and connected to a computer with the appropriate software installed.
- Check that the instrument is clean and free from any contamination. Clean the instrument following the manufacturer's guidelines if necessary.

Plate Setup:

- Prepare a 96-well plate. Label the plate accordingly to track your samples and controls.
- Add the PCR Master Mix and the DNA templates and standards/positive/negative controls to the appropriate wells, ensuring accurate and precise pipetting.

Load the Plate:

- Carefully load the plate with the prepared PCR Master Mix and samples, following the plate layout defined in the software.
- Ensure that the plate is properly sealed to prevent contamination and evaporation.

Instrument Setup:

- Open the Quant Studio Design and Analysis Software on your computer and establish a connection with the Quant Studio 5 instrument.
- Select the Chemistry: *Taq*Man protocol for Bosphore CMV Quantification Kit based on the kit's specifications. Consult the software user manual for detailed instructions on protocol selection.
- Set the desired cycling parameters, including the number of cycles, denaturation, annealing and hold temperatures and times, as specified by Bosphore CMV Quantification Kit.
- Set the passive reference dye as "none".
- Choose the appropriate fluorescence filters for the probes used in Bosphore CMV Quantification Kit.

Run the Experiment:

- Load the prepared 96-well plate into the Quant Studio 5 instrument, ensuring it is properly aligned.
- Once the run is completed, the software will provide you with data, including amplification curves, C_T values and other relevant information.

Data Analysis:

- Interpret the results based on Bosphore CMV Quantification Kit guidelines provided in the kit's manual.
- The standard curve and related values can be controlled by clicking on the "Analysis Settings" button on the "Analysis" tab.

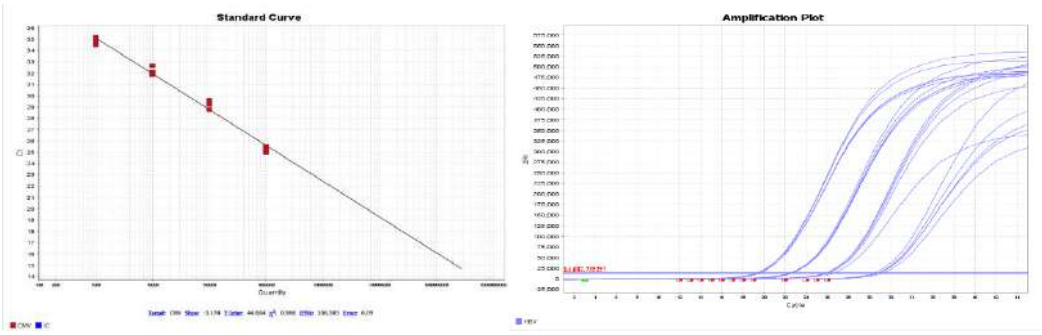


Figure 6: Standard and Amplification curve for Quant Studio 5

9.6.6. Experiment Setup for Q Real-Time Quantitative PCR Instrument

Instrument Setup:

- Ensure that the Q Real-Time qPCR Instrument is connected to a power source.
- Press the power button to turn on the instrument and allow it to warm up and stabilize according to the manufacturer's instructions.

Reaction Setup:

- Dispense the PCR Master Mix into PCR tubes or strips, taking care to avoid any contamination.
- Add the DNA templates, standards, and positive/negative controls to the appropriate wells, ensuring proper sample identification.

Load the Strips/Tubes:

- Carefully load the strips/tubes with the prepared PCR Master Mix and samples, following the proper sample identifications defined in the software.
- Ensure that the strip/tube caps are properly closed to prevent contamination and evaporation.

Thermal Cycling Conditions:

- Set the thermal cycling conditions on the Q Real-Time qPCR Instrument according to the instructions in Bosphore CMV Quantification Kit IFU.
- Manually set the gain for each filter to 10.
- Specify the denaturation and annealing temperatures and times for each cycle.

Data Analysis and Interpretation

Threshold Determination:

- Define a threshold fluorescence value that distinguishes the background signal from the amplification signal.
- If necessary, set the "Fluorescent Cutoff Level" up to 15% to optimize the threshold determination.

C_T Calculation:

- Determine the cycle threshold (C_T) value for each sample, representing the cycle number at which the fluorescence signal crosses the threshold.
- Use the instrument's software or appropriate data analysis tools to automatically calculate the C_T values.

Data Interpretation:

- Analyze the qPCR data to draw meaningful conclusions and interpretations based on the experimental objectives.
- Samples tab should be opened, and standards, positive control, negative control and samples should be selected.
- Compare and contrast the quantification levels between different samples or experimental conditions.

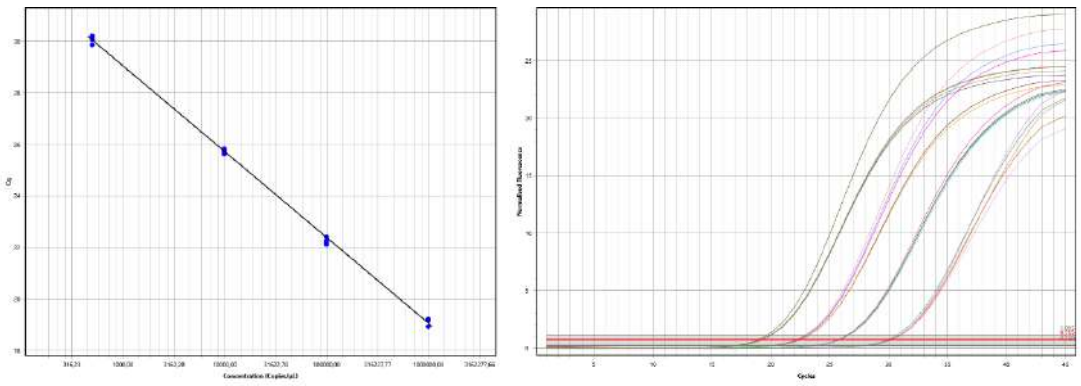


Figure 7: Standards and Amplification curve for Q Real-Time Quantitative PCR Instrument

10. ANALYSIS

By the end of the thermal protocol, the Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold. 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 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 the manufacturer, consider it necessary if the system allows pulling down the threshold as much as possible to detect low amplifications, attention should be paid to keep the threshold line above the background.

The negative control is essential for accurate result analysis. Please check the negative control and ensure it shows no amplification outside the associated internal control filter. If the negative control has a signal outside the filter associated with the internal control, please do not report the results. Repeat the experiment after taking the necessary precautions against contamination. If the same result is encountered again, please contact the manufacturer. Internal control, standards, and positive control of Bosphore CMV Quantification Kit are essential for accurate result analysis. The cycle threshold acceptance criteria for the internal control, standards, and positive control are listed below:

Table 7: Bosphore CMV Quantification Kit acceptance criteria.

		Montania 4896 Real-Time PCR Instrument (0.12 threshold)	**Rotor-Gene Q**	**LightCycler 480 Instrument II**	**CFX96 Real-Time PCR Detection System**	**QuantStudio 5 Real-Time PCR System**	**Q qPCR Cycler**
Extraction systems	**Component/ Parameter****	**Cycle Threshold Value (C_T)***					
Unio B24/B48/A24S Extraction Systems with Unio Viral DNA RNA Extraction Kit 600 µl (600 µl -60 µl)	**Standard 1**	20.7±2	19.5±2	23.6±2	23.2±2	23.2±2	21.5±2
	Standard 2	24±2	22.8±2	26.9±2	26.5±2	26.5±2	24.8±2
	Standard 3	27.3±2	26.1±2	30.2±2	29.8±2	29.8±2	28±2
	Standard 4	30.7±2	29.5±2	33.5±2	33.1±2	33.1±2	31.3±2
Unio B24/B48/A24S Extraction Systems with Whole Blood Genomic DNA Extraction Large Volume Kit (600 µl -60 µl)	**Standard 1**	20.5±2	19±2	23.2±2	22.5±2	22.5±2	20.8±2
	Standard 2	23.8±2	22.3±2	26.5±2	25.8±2	25.8±2	24.1±2
	Standard 3	27.1±2	25.6±2	29.8±2	29.1±2	29.1±2	27.4±2
	Standard 4	30.4±2	29±2	33.1±2	32.4±2	32.4±2	30.8±2
Unio 96 Extraction & PCR Setup System / Unio 96 Nucleic Acid Extraction Versatile Kit (200 µl -60 µl)	**Standard 1**	22.5±2	21±2	21.2±2	24±2	24±2	23.2±2
	Standard 2	25.8±2	24.3±2	24.4±2	27.4±2	27.4±2	26.5±2
	Standard 3	29.1±2	27.6±2	27.8±2	30.7±2	30.7±2	29.8±2
	Standard 4	32.4±2	31±2	31.1±2	34±2	34±2	33.1±2

Positive control	29±3	28.5±3	31±3	31±3	31.5±3	30±3
Correlation Coefficient	>0.970	>0.970	>0.970	>0.970	>0.970	>0.970
PCR Efficiency* **	>80%	>80%	>80%	>80%	>80%	>80%
Matrices	**Plasma**	**Whole Blood**	**CSF**	**Amniotic Fluid**	**BAL**	**Urine**
Internal control	≤30	≤30	≤30	≤30	≤30	≤35

⚠ * Cycle thresholds of the standards change depending on the starting volume and the efficiency of extraction system used.
**For standards to be added to PCR, it should be remembered that they need to be extracted.
*** PCR efficiency is calculated by the following formula: $10^{(-1/slope)}$-1x100

Bosphore CMV Quantification Kit is a real-time PCR test intended for the quantitative detection of Human Cytomegalovirus (HCMV) DNA in clinical samples. Quantitative measurement of CMV viral load is essential for monitoring disease progression, treatment efficacy, and patient follow-up. The kit analyzes viral DNA extracted from patient specimens and reports results as C_T values. These values are calculated automatically by the device software based on predefined acceptance criteria set by quantitation standards and internal controls.

C_T values are preferred over IU/ml as primary acceptance criteria to reduce the impact of variability introduced by different test platforms and sample matrices. The conversion of C_T values to IU/ml is performed through calibration with validated standard curves, allowing the device to report quantitative results in IU/ml.

Quantification is based on the number of PCR cycles (C_T) required for the fluorescence signal to exceed a defined threshold. C_T values are inversely proportional to the initial amount of target nucleic acid. The use of validated standard curves ensures accurate, reliable, and reproducible quantitative performance.

Test results should not be reported unless there is an amplification of the internal control in negative samples. 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 filter are displayed with their positive/negative results, samples that do not cut the threshold are displayed as "No C_T". These samples must be regarded as negative or having a viral load below the detection limit of the assay.

For these undetectable samples, HEX data of the internal control should also be checked to avoid false negative results. The delayed amplification of the internal control may indicate a problem in nucleic acid extraction, PCR inhibition, or application errors.

In this case, extraction and PCR should be repeated. The table below shows the possible results and their interpretation. Please note that this product only provides testing for Cytomegalovirus experimental results from the tube must be considered when providing a result for the patients, also in consideration of the patient's clinical findings and the guidelines of the relevant health authorities.

Table 8: Bosphore CMV Quantification Kit Results Interpretation

PCR Master Mix	CMV (FAM)	Internal Control (HEX)	Result
	+	+/-	The sample is CMV positive
	-	+	Sample is negative
	-	-	The test should be repeated!

In cases of high viral load, suppression of the internal control in the Bosphore CMV Quantification Kit may occur.

In rare cases of PCR inhibition due to medication or other PCR inhibitors in the sample, we recommend repeating the test of inhibited samples, by freezing and thawing the DNA samples and using them in the PCR after diluting them 1:2 with dH_2O.

The dilution factor must be taken into account while reporting the Real-Time PCR quantitative results. If the inhibition problem continues, we recommend that another sample from the patient is collected, and the test is repeated starting from the nucleic acid extraction.

- **Montania 4896 Real-Time PCR Instrument:**

  PCR Master Mix: Cross Talk Option should be adjusted as shown below:

Cross Talk Option

	Channel 1	Channel 2
Channel 1	0,00	0,50
Channel 2	0,00	0,00

Figure 8: Montania 4896 Cross Talk Option

Channel	Target	Begin Baseline	End Baseline	Auto Threshold	Manual Threshold	Optimization	Analysis Type	Digital Filter
1	CMV	6	12	☐	0,12	Auto	Quantitative	☐
2	IC	6	12	☐	0,12	Auto	Qualitative	☐

Figure 9: Montania 4896 Analysis Parameters.

- **LightCycler 480 Instrument II - Roche:** Please use a white plate for Roche LightCycler 480 Instrument II. "Abs Quant/Fit Points" should be chosen as the analysis type for the appropriate threshold level selection. Analysis should be performed with the 'Cycle Range' option. For LC480, a color compensation protocol must be performed Universal CC FAM (510) - VIC (580).
- **CFX96 Real-Time PCR Detection System - Bio-Rad:** Use of a white plate. If there are any irregularities in the baseline, it is recommended to select "Apply Fluorescence Drift Correction" in "Baseline Setting" for the analysis.
- **Q qPCR Cycler - QuantaBio:** Please use "Fluorescent Cutoff Level" up to 15% if necessary. Make sure that the gain for each filter has been set to 10 manually.
- **Rotor-Gene Q - QIAGEN:** Use of "Slope Correct" and "Dynamic Tube" options for the analysis is recommended. If the sigmoidal curve does not observe, try to analyze the sample without the "Slope Correct" option. Make sure that the gain for each filter has been set to 10 manually.
- **QuantStudio 5 Real-Time PCR System – ThermoFisher:** Make sure that the passive reference dye is set to none.

11. SPECIFICATIONS

11.1. Analytical Sensitivity

11.1.1. Limit of Blank (LoB)

The Limit of Blank (LoB) of the Bosphore CMV Quantification Kit was determined using confirmed CMV-negative samples. A total of 32 samples were tested using two different lots. No amplification was observed in any sample. These results demonstrate that the test does not generate false positive signals in blank samples, and no additional cut-off value is required.

11.1.2. Calibration to WHO International Standard

Bosphore CMV Quantification Kit's standards were calibrated with WHO First International Standard Human CMV DNA (NIBSC Code: 09/162) dilutions over three days using six different matrices and extraction systems (Unio B24 Extraction Systems and Unio 96 Extraction & PCR Setup System) at four different concentrations, with three replicates each.

Bosphore CMV Quantification Kit's standards have been calibrated according to WHO First International Standard Human CMV DNA (NIBSC Code: 09/162) for extraction systems and different matrices, and the relevant values are provided in the table below.

Table 9: Calibration according to WHO International Standards

	Unio B24/B48 Extraction Systems and Unio A24S Extraction &PCR Setup System / Unio Viral DNA RNA Extraction Kit 600 µL (600 µL starting and 60 µL elution volume)					Unio B24 Extraction System- Unio Whole Blood Genomic DNA Extraction Large Volume Kit (600 µL starting - 60 µL elution)
	Plasma (IU/ mL)	**Urine (IU/ mL)**	**Amniotic Fluid (IU/ mL)**	**CSF (IU/ mL)**	**BAL (IU/ mL)**	**Whole Blood (IU/ mL)**
Standard 1	10^6	$2x10^6$	$7x10^5$	$6.5x10^5$	$8x10^5$	$1.25x10^6$
Standard 2	10^5	$2x10^5$	$7x10^4$	$6.5x10^4$	$8x10^4$	$1.25x10^5$
Standard 3	10^4	$2x10^4$	$7x10^3$	$6.5x10^3$	$8x10^3$	$1.25x10^4$
Standard 4	10^3	$2x10^3$	$7x10^2$	$6.5x10^2$	$8x10^2$	$1.25x10^3$

	Unio 96 Extraction & PCR Setup System- Unio 96 Nucleic Acid Extraction Versatile Kit (200 µl starting - 60 µl elution)					
	Plasma (IU/ mL)	**Urine (IU/ mL)**	**Amniotic Fluid (IU/ mL)**	**CSF (IU/ mL)**	**BAL (IU/ mL)**	**Whole Blood (IU/ mL)**
Standard 1	$7.5x10^5$	$2x10^6$	$7x10^5$	$7.5x10^5$	$8x10^5$	10^6
Standard 2	$7.5x10^4$	$2x10^5$	$7x10^4$	$7.5x10^4$	$8x10^4$	10^5
Standard 3	$7.5x10^3$	$2x10^4$	$7x10^3$	$7.5x10^3$	$8x10^3$	10^4
Standard 4	$7.5x10^2$	$2x10^3$	$7x10^2$	$7.5x10^2$	$8x10^2$	10^3

11.1.3. Limit of Detection (LoD)

The LOD is the lowest analyte concentration that can be reliably detected in 95% of samples tested in a regular laboratory setting using the convenient sample matrix. Biostat Pro v 7.6.5.0 probit program was used to calculate the sensitivity levels and the LOD 95% was determined. Detection ratios of the LoD levels detected in other devices are also summarized in the table below.

Table 10: LOD Values for the Bosphore CMV Quantification Kit

Extraction System/Kit	Sample Type	LoD Level (Obtained with Montania 4896)
Unio B24/B48 Extraction Systems and Unio A24S Extraction & PCR Setup System / Unio Viral DNA RNA Extraction Kit 600 µl OR Unio Whole Blood Genomic DNA Extraction Large Volume Kit (600 µL starting and 60 µL elution volume)	Plasma	40.52 IU/mL
	Whole Blood	27.05 IU/mL
	Urine	67.50 IU/mL
	Amniotic Fluid	25.25 IU/mL
	Cerebrospinal Fluid (CSF)	34.35 IU/mL
	Bronchoalveolar Lavage (BAL)	38.41 IU/mL

Unio 96 Extraction & PCR Setup System / Unio 96 Nucleic Acid Extraction Versatile Kit (200 µl starting - 60 µL elution)	Plasma	50.97 IU/mL
	Whole Blood	94.71 IU/mL
	Urine	117.02 IU/mL
	Amniotic Fluid	39.52 IU/mL
	Cerebrospinal Fluid (CSF)	43.44 IU/mL
	Bronchoalveolar Lavage (BAL)	46.40 IU/mL

Confirmatory LoD studies demonstrated that the related levels determined on Montaina 4896 Real-Time PCR Instrument is confirmed with other qPCR devices.

Table 11: Confirmatory LoD studies

	Unio B24/B48 Extraction Systems and Unio A24S Extraction & PCR Setup System / Unio Viral DNA RNA Extraction Kit 600 µl OR Unio Whole Blood Genomic DNA Extraction Large Volume Kit					**Unio 96 Extraction & PCR Setup System / Unio 96 Nucleic Acid Extraction Versatile Kit**				
	Biorad	**QS5**	**LC**	**Rotor**	**Q**	**Biorad**	**QS5**	**LC**	**Rotor**	**Q**
Amniotic Fluid	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
BAL	100%	100%	100%	100%	100%	100%	100%	100%	100%	95%
CSF	100%	100%	100%	100%	100%	100%	100%	100%	95%	100%
Urine	100%	100%	100%	95%	100%	100%	100%	100%	95%	100%
Plasma	100%	100%	100%	100%	100%	100%	100%	100%	95%	100%
Whole Blood	100%	100%	100%	100%	100%	100%	100%	100%	100%	95%

11.1.4. Limit of Quantitation (LoQ)

The LoQ results indicate that the assay demonstrates consistent and reliable quantification capabilities at low concentration levels.

The established LoQ values are within acceptable ranges, confirming that the assay can accurately quantify the target analyte with minimal variability and high precision. The LoQ limit is 100 IU/mL for Unio 96 Extraction & PCR Setup and 120 IU/mL for Unio B24 Extraction Systems determined in plasma matrix.

11.1.5. Linear Measuring Range

The results were analyzed and showed that the standard deviation (SD), coefficient of variation (CV%), percent deviation and bias values were within ±10% and the non-linearity value was within ±1%. The linear measuring range of the Bosphore CMV Quantification Kit was determined to be 120 IU/mL - 10^9 IU/mL for Unio B24 Extraction System and 100 IU/mL - 10^9 IU/mL for Unio 96 Extraction & PCR Setup System.

11.2. Analytical Specifity

11.2.1. Inclusivity (in-silico analysis)

In-silico analysis is a critical step in the design and validation of molecular assays, especially to ensure specificity and minimize the risk of cross-reactivity with non-target organisms. This involves assessing primer and probe sequences through computational alignment tools and sequence databases to confirm they will detect only the intended target without amplifying other sequences.

In-silico analysis is conducted using extensive nucleotide databases, typically including GenBank, EMBL, DDBJ, PDB, and RefSeq. These databases are optimized for redundancy reduction by consolidating identical sequences into a single record while preserving essential metadata (e.g., accession numbers, taxonomy). Searches are filtered to exclude sequences that are incomplete or irrelevant, such as environmental samples, patents, and large sequences over 100 Mb.

For primer and probe specificity, highly similar sequence search algorithms (e.g., BLAST) are used. Parameters are optimized to maximize the sensitivity of the search for closely related sequences with minimal chance of cross-reactivity, usually with an expect threshold around 0.05 and a larger word size to balance sensitivity and processing efficiency. Sequences with high similarity to the assay's targets are evaluated in close proximity (typically within 500 base pairs), focusing on whether the primers and probes align in an appropriate orientation for PCR amplification.

Cross-reactivity assessments are crucial to prevent non-specific amplification in assays. During the in-silico analysis, a sequence comparison analysis is conducted on potential non-target organisms known to commonly present in relevant sample types. Cross-reactivity is generally flagged if primers and probes demonstrate over 80% homology with sequences on opposite strands within the 500 bp range, indicating a possible risk of non-specific product formation.

Further, any sequences that meet the initial cross-reactivity criteria undergo additional scrutiny for specific sequence identity, orientation, and base positioning, as high similarity or homology alone does not always indicate a functional PCR interaction.

Inclusivity is tested to ensure that the assay can detect all expected genetic variations of the target, while exclusivity testing is used to confirm the assay does not amplify non-target organisms with similar sequences. This dual approach helps in verifying the robustness and accuracy of the assay.

For inclusivity, sequences from various strains and types of the target organisms are aligned to ensure coverage. Exclusivity testing involves comparing the assay sequences against a panel of non-target organisms known to be present in the test environment or sample matrix.

In-silico analysis is an essential component of assay development, providing insights into potential cross-reactivity, inclusivity, and exclusivity before experimental validation. While computational predictions are indicative, they are often followed by empirical testing to further validate assay performance and specificity.

11.2.2. Cross Reactivity

HSV-1, HSV-2, VZV, EBV, HHV-6, HHV-7, HHV-8, HCV, HIV-1, HDV, HBV, ADV, HIV-2, Influenza A, Influenza B, RSV-A, RSV-B, HPV 16, HPV 18, and SARS-CoV-2 pathogens at high concentrations were tested for cross-reactivity with the Bosphore CMV Quantification Kit. The results consistently showed no cross-reactivity across all tested pathogens, as evidenced by negative results (No C_T) in all replicates. This demonstrates the kit's high CMV specificity, which reduces the possibility of false-positive results caused on by other viral infections.

11.2.3. Interference

In the interference study, various exogenous substances were added to or endogenous substances were tested in negative and low positive CMV samples for bronchoalveolar lavage (BAL), whole blood, urine, plasma, cerebrospinal fluid (CSF), and amniotic fluid (AF) matrices. The substances tested included common medications as well as numerous endogenous biological substances. It has been proven that the concentrations of the interference substances specified in the table below do not interfere with the Bosphore CMV Quantification Kit.

Table 12: Interferences Study for the Bosphore CMV Quantification Kit

Substance	Urine	Plasma	Bronchoalveolar lavage (BAL)	Cerebrospinal fluid (CSF)	Whole Blood	Amniotic Fluid	Results
No substance	-	-	-	-	-	-	-
Antibiotic (Amoxicillin)	5.4 mg/dL	5.4 mg/dL	5.4 mg/dL	5.4 mg/dL	5.4 mg/dL	5.4 mg/dL	No interference was detected
Anti-inflammatory (Prednisone)	9.9x10-3 mg/dL	9.9x10-3 mg/dL	9.9x10-3 mg/dL	9.9x10-3 mg/dL	9.9x10-3 mg/dL	9.9x10-3 mg/dL	No interference was detected
Antiviral (Oseltamivir)	3.99x10-2 mg/dL	3.99x10-2 mg/dL	3.99x10-2 mg/dL	3.99x10-2 mg/dL	3.99x10-2 mg/dL	3.99x10-2 mg/dL	No interference was detected
Painkiller (Paracetamol)	15.6 mg/dL	15.6 mg/dL	15.6 mg/dL	15.6 mg/dL	15.6 mg/dL	15.6 mg/dL	No interference was detected
Non-steroid anti-inflammatory (Ibuprofen)	21.9 mg/dL	21.9 mg/dL	21.9 mg/dL	21.9 mg/dL	21.9 mg/dL	21.9 mg/dL	No interference was detected
Antiviral (Tenofovir disoproxil fumarate)	9.78x10-2 mg/dL	9.78x10-2 mg/dL	9.78x10-2 mg/dL	9.78x10-2 mg/dL	9.78x10-2 mg/dL	9.78x10-2 mg/dL	No interference was detected
Antiviral (Lamivudine)	1.05 mg/dL	1.05 mg/dL	1.05 mg/dL	1.05 mg/dL	1.05 mg/dL	1.05 mg/dL	No interference was detected

Antiviral (Valasiklovir)	1 mg/dL	1 mg/dL	1 mg/dL	1 mg/dL	1 mg/dL	1 mg/dL	No interference was detected
Antifungal (Fluconazole)	2.55 mg/dl	2.55 mg/dl	2.55 mg/dl	2.55 mg/dl	2.55 mg/dl	2.55 mg/dl	No interference was detected
Caffeine	10.8 mg/dl	10.8 mg/dl	10.8 mg/dl	10.8 mg/dl	10.8 mg/dl	10.8 mg/dl	No interference was detected
Antiviral (Asiviral)	6.6 mg/dl	6.6 mg/dl	6.6 mg/dl	6.6 mg/dl	6.6 mg/dl	6.6 mg/dl	No interference was detected
Bilirubin	40 mg/dL	40 mg/dL	40 mg/dL	40 mg/dL	40 mg/dL	40 mg/dL	No interference was detected
Albumin	60 mg/dL	60 mg/dL	60 mg/dL	60 mg/dL	60 mg/dL	60 mg/dL	No interference was detected
Haemoglobin	1000 mg/dL	1000 mg/dL	1000 mg/dL	1000 mg/dL	1000 mg/dL	1000 mg/dL	No interference was detected
Urea	120 mg/dL	120 mg/dL	120 mg/dL	120 mg/dL	120 mg/dL	120 mg/dL	No interference was detected
Uric acid	23.5 mg/dL	23.5 mg/dL	23.5 mg/dL	23.5 mg/dL	23.5 mg/dL	23.5 mg/dL	No interference was detected
Glucose	1000 mg/dL	1000 mg/dL	1000 mg/dL	1000 mg/dL	1000 mg/dL	1000 mg/dL	No interference was detected

11.3. Accuracy

11.3.1. Trueness of measurement

Trueness study, which performed in 24 replicates of 3 different concentrations of WHO First International Standard Human CMV DNA (NIBSC Code: 09/162), all samples were detected as positive likely expected and CV value less than 20%.

11.3.2. Precision (Reproducibility)

In experiments, precision study performed by 2 different operators, on 3 different sites, on 5 different days, using 2 different lots, in 6 replicates, it was observed that there was no significant difference (≤10% differences) among those using the same concentration of samples. The results of the repeatability and reproducibility are presented below.

Table 13: Calculations and comparisons of log10 concentration values obtained in the CMV for repeatability, reproducibility and within laboratory.

Sample concentration	Mean value	N	Repeatability				Reproducibility				Within Laboratory			
			SD	CV%	95% CI Lower	95% CI Upper	SD	CV%	95% CI Lower	95% CI Upper	SD	CV%	95% CI Lower	95% CI Upper
Low positive sample	2.311	180	0.108	4.7	2.295	2.326	0.132	5.7	2.292	2.33	0.132	5.7	2.292	2.330
Moderate positive sample	3.770	180	0.040	1.1	3.764	3.775	0.059	1.6	3.761	3.779	0.056	1.5	3.762	3.778
High positive sample	5.412	180	0.023	0.3	5.408	5.415	0.027	0.5	5.408	5.416	0.027	0.5	5.408	5.416

Table 14: Calculations and comparisons of log10 concentration values obtained in the CMV for between run, laboratory.

Sample concentration	Mean value	N	Between Run				Between Laboratory			
			SD	CV%	95% CI Lower	95% CI Upper	SD	CV%	95% CI Lower	95% CI Upper
Low positive sample	2.311	180	0.070	3.3	2.301	2.321	0.003	0.1	2.310	2.311
Moderate positive sample	3.770	180	0.039	1.0	3.764	3.776	0.018	0.5	3.767	3.77
High positive sample	5.412	180	0.021	0.4	5.409	5.415	0.000	0.0	5.412	5.412

Table 15: Calculations and comparisons of log10 concentration values obtained in the CMV for between operator and lot.

Sample concentration	Mean value	N	Between Operator				Between Lot			
			SD	CV%	95% CI Lower	95% CI Upper	SD	CV%	95% CI Lower	95% CI Upper
Low positive sample	2.311	180	0	0	2.311	2.311	0.033	1.4	2.306	2.316
Moderate positive sample	3.770	180	0	0	3.770	3.770	0.000	0.0	3.770	3.770
High positive sample	5.412	180	0	0	5.412	5.412	0.000	0.0	5.412	5.412

! The "0.0" results given in the tables above are the results that the "*Analyse-it* (Method Validation Edition)" application used when performing the statistical calculations found "not significant".

11.4. Product Stability

11.4.1. Claimed Shelf Life

Based on the stability data, the shelf life was determined to be 18 months from the production date.

11.4.2. In-Use Stability

The in-use stability of the kit has been tested for thaw and freeze stability. The statistical data obtained shows that the kit is stable up to three cycles of freeze and thaw. For this reason, the maximum freeze and thaw number of the kit is determined as three.

The in-use stability of the kit has also been tested cold rack incubation period stability. The Bosphore CMV Quantification Kit Real-time PCR Kit is stable despite it was kept in the cold rack for 120 minutes according to statistical data.

11.5. Shipping / Transport Stability

This study leverages data from prior validated reports on Bosphore CT/NG/MG Panel Kit v1 for winter stability and Bosphore HBV Quantification Kit for summer stability, as both kits share similar components, packaging, and handling requirements.

These references provide a foundation for establishing reliable shipping protocols that ensure the integrity and performance of Bosphore CMV Quantification Kit throughout its transit.

According to the report from the study conducted for the CT/NG/MG Panel Kit, the kit maintained its stability under winter conditions during a transport period lasting 6 days, 1 hour, and 30 minutes. This study employed a styrofoam box with outer dimensions of 675x520x300 mm, inner dimensions of 575x420x200 mm, a wall thickness of 50 mm, and a density of 30 kg/m³. The package included 25 kg of dry ice, two cold chain gel packs weighing 500±50 grams each, and a thermal indicator. The box was sealed tightly with duct tape applied in at least three rounds, ensuring no gaps along the lid. Temperature monitoring confirmed that all components remained within the required temperature range, with no temperature excursions observed. These results validated the shipping stability of the CT/NG/MG Panel Kit under subzero conditions, providing essential data for similar winter transport scenarios for the CMV Quantification Kit.

Summer stability data for the CMV Quantification Kit is drawn from the report on Bosphore HBV Quantification Kit. This study simulated extreme summer conditions during a round-trip transport over six days, involving air freight and storage at multiple locations. The packaging specifications included a styrofoam box of similar dimensions to the CT/NG/MG study, but with 28 kg of dry ice supplemented by two cold chain gel packs. The statistical analysis revealed no significant differences (p-value > 0.05) in test results before and after shipment. These findings validate the kit's stability under summer conditions for up to 5 days and 18 hours.

The results from these studies confirm that the Bosphore CMV Quantification Kit can be transported under conditions similar to those validated for the Bosphore CT/NG/MG Panel Kit and HBV Quantification Kit. For winter shipments, a minimum of 25 kg of dry ice, two cold chain gel packs, and a thermal indicator are required.

For summer shipments, 28 kg of dry ice must be used to ensure extended stability. In both scenarios, the styrofoam box must meet the specified dimensions and density requirements, and the package must remain tightly sealed with duct tape applied in multiple layers.

In conclusion, the validated shipping stability conditions outlined in this section ensure the integrity and performance of the Bosphore CMV Quantification Kit under both winter and summer transportation scenarios. Any alterations to the packaging specifications or transport conditions will require a new stability study to be conducted.

11.6. Robustness

11.6.1. Carry Over

For carry over studies, at least 5 runs are required according to the relevant directive. Five high-positive clinical samples were tested in 5 runs, with 5 negative clinical samples included between them. Specifically, the expectation was that all 5 negative samples would yield negative results in every run, without any contamination from the preceding high positive specimen.

11.6.2. Whole System Failure

To determine the whole system failure rate of the device, 100 CMV positive clinical sample with 3xLoD concentration were tested. Results show that Bosphore CMV Quantification Kit have 100% detection rate.

11.6.3. Metrological Traceability

Metrological traceability of the positive control, internal control and standards of Bosphore CMV Quantification Kit were evaluated by experimental studies. The table in Section 10 shows the results of the Metrological Traceability study.

12. TECHNICAL ASSISTANCE

For any technical assistance or inquiries regarding your Real-Time PCR kit, we are here to help you. We understand that there might be situations where you require expert guidance and support. Whether you have questions about kit usage, troubleshooting, or data interpretation, our dedicated team of technical experts is available to assist you. To reach our technical support team, please use the contact information in Section 16.

When contacting us, please provide detailed information about your specific query or concern. This will enable us to provide you with the most accurate and efficient assistance. We kindly request that you have the following information ready.

Experimental Details: Describe your experimental setup, including the sample type, target gene, reaction conditions and any specific issues you are encountering. The more information you can provide, the better we can understand your needs and offer the correct support. Instrumentation and Software: If applicable, please mention the Real-Time PCR instrument and software you are using. This information will allow us to provide guidance specific to your instrument's capabilities and features.

13. REFERENCES

1. Albert, E., Giménez, E., Alberola, J., Torres, I., López, Y., Marcos, A., Reinhardt, B., & Navarro, D. (2024). Performance evaluation of the Alinity m system for quantifying cytomegalovirus DNA in samples of the respiratory, gastrointestinal, and urinary tract. Microbiology Spectrum, 12(7), e0420123. https://doi.org/10.1128/spectrum.04201-23

2. Al-Omari, A., Aljamaan, F., Alhazzani, W., Salih, S., & Arabi, Y. (2016). Cytomegalovirus infection in immunocompetent critically ill adults: Literature review. Annals of Intensive Care, 6(1), 110. https://doi.org/10.1186/s13613-016-0207-8

3. Atkinson, C., & Emery, V. C. (2011). Cytomegalovirus quantification: Where to next in optimising patient management? Journal of Clinical Virology, 51(4), 223–228. https://doi.org/10.1016/j.jcv.2011.04.007

4. Boppana, S. B., Ross, S. A., Shimamura, M., Palmer, A. L., Ahmed, A., Michaels, M. G., Sánchez, P. J., Bernstein, D. I., Tolan, R. W. J., Novak, Z., Chowdhury, N., Britt, W. J., & Fowler, K. B. (2011). Saliva polymerase-chain-reaction assay for cytomegalovirus screening in newborns. The New England Journal of Medicine, 364(22), 2111–2118. https://doi.org/10.1056/NEJMoa1006561

5. Ciccocioppo, R., Racca, F., Paolucci, S., Campanini, G., Pozzi, L., Betti, E., Riboni, R., Vanoli, A., Baldanti, F., & Corazza, G. R. (2015). Human cytomegalovirus and Epstein-Barr virus infection in inflammatory bowel disease: Need for mucosal viral load measurement. World Journal of Gastroenterology, 21(6), 1915–1926. https://doi.org/10.3748/wjg.v21.i6.1915

6. Drjagunovs, I., Laivacuma, S., Zeltiņa, I., & Derovs, A. (2022). A quick guide to diagnosis and treatment of cytomegalovirus infection in the gut: Current dilemmas. Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences., 76(5–6), 585–594. https://doi.org/10.2478/prolas-2022-0091

7. Fryer, J. F., Heath, A. B., Minor, P. D., Kessler, H., Rawlinson, W., Boivin, G., Preiksaitis, J., Pang, X.-L., Barranger, C., Alain, S., Bressollette-Bodin, C., Hamprecht, K., Grewing, T., Constantoulakis, P., Ghisetti, V., Capobianchi, M. R., Abbate, I., Olivo, C., Lazzarotto, T., … Guiver, M. (2016). A collaborative study to establish the 1st WHO International Standard for human cytomegalovirus for nucleic acid amplification technology. Biologicals, 44(4), 242–251. https://doi.org/10.1016/j.biologicals.2016.04.005

8. Gilad, N., Agrawal, S., Philippopoulos, E., Murphy, K. E., & Shinar, S. (2024). Is a Higher Amniotic Fluid Viral Load Associated with a Greater Risk of Fetal Injury in Congenital Cytomegalovirus Infection—A Systematic Review and Meta-Analysis. Journal of Clinical Medicine, 13(7), Article 7. https://doi.org/10.3390/jcm13072136

9. Govender, K., Jeena, P., & Parboosing, R. (2017). Clinical utility of bronchoalveolar lavage cytomegalovirus viral loads in the diagnosis of cytomegalovirus pneumonitis in infants. Journal of Medical Virology, 89(6), 1080–1087. https://doi.org/10.1002/jmv.24730

10. Goycochea-Valdivia, W.-A., Baquero-Artigao, F., Del Rosal, T., Frick, M.-A., Rojo, P., Echeverría, M.-J., Noguera-Julian, A., Bringué, X., Saavedra-Lozano, J., Vives-Oñós, I., Moliner, E., Cilleruelo, M.-J., Cuadrado, I., Colino, E., Castells, L., Tagarro, A., Vilas, J., Soler-Palacin, P., & Blázquez-Gamero, D. (2017). Cytomegalovirus DNA Detection by Polymerase Chain Reaction in Cerebrospinal Fluid of Infants With Congenital Infection:

Associations With Clinical Evaluation at Birth and Implications for Follow-up. Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America, 64(10), 1335–1342. https://doi.org/10.1093/cid/cix105

11. Griffiths, P., Baraniak, I., & Reeves, M. (2015). The pathogenesis of human cytomegalovirus. The Journal of Pathology, 235(2), 288–297. https://doi.org/10.1002/path.4437

12. Griffiths, P., & Reeves, M. (2021). Pathogenesis of human cytomegalovirus in the immunocompromised host. Nature Reviews Microbiology, 19(12), 759–773. https://doi.org/10.1038/s41579-021-00582-z

13. Hayden, R. T., Preiksaitis, J., Tong, Y., Pang, X., Sun, Y., Tang, L., Cook, L., Pounds, S., Fryer, J., & Caliendo, A. M. (2015). Commutability of the First World Health Organization International Standard for Human Cytomegalovirus. Journal of Clinical Microbiology, 53(10), 3325–3333. https://doi.org/10.1128/JCM.01495-15

14. Hui, L., & Wood, G. (2015). Perinatal outcome after maternal primary cytomegalovirus infection in the first trimester: A practical update and counseling aid. Prenatal Diagnosis, 35(1), 1–7. https://doi.org/10.1002/pd.4497

15. Kucharzik, T., Ellul, P., Greuter, T., Rahier, J. F., Verstockt, B., Abreu, C., Albuquerque, A., Allocca, M., Esteve, M., Farraye, F. A., Gordon, H., Karmiris, K., Kopylov, U., Kirchgesner, J., MacMahon, E., Magro, F., Maaser, C., De Ridder, L., Taxonera, C., … Vavricka, S. (2021). ECCO Guidelines on the Prevention, Diagnosis, and Management of Infections in Inflammatory Bowel Disease. Journal of Crohn's and Colitis, 15(6), 879–913. https://doi.org/10.1093/ecco-jcc/jjab052

16. Lawrence, S. M., Goshia, T., Sinha, M., Fraley, S. I., & Williams, M. (2024). Decoding human cytomegalovirus for the development of innovative diagnostics to detect congenital infection. Pediatric Research, 95(2), 532–542. https://doi.org/10.1038/s41390-023-02957-9

17. Li, X., Zhong, Y., Qiao, Y., Li, H., Hu, X., Imani, S., Zheng, S., & Li, J. (2023). Advances and Challenges in Cytomegalovirus Detection Methods for Liver Transplant Donors. Diagnostics, 13(21), Article 21. https://doi.org/10.3390/diagnostics13213310

18. Liu, G., Hai, R., & Liu, F. (2017). Detection of congenital cytomegalovirus in newborns using nucleic acid amplification techniques and its public health implications. Virologica Sinica, 32(5), 376–386. https://doi.org/10.1007/s12250-017-4055-y

19. Novak, Z., Chowdhury, N., Ross, S. A., Pati, S. K., Fowler, K., & Boppana, S. B. (2011). Diagnostic consequences of cytomegalovirus glycoprotein B polymorphisms. Journal of Clinical Microbiology, 49(8), 3033–3035. https://doi.org/10.1128/JCM.01039-11

20. Pavšič, J., Devonshire, A. S., Parkes, H., Schimmel, H., Foy, C. A., Karczmarczyk, M., Gutiérrez-Aguirre, I., Honeyborne, I., Huggett, J. F., McHugh, T. D., Milavec, M., Zeichhardt, H., & Žel, J. (2015). Standardization of Nucleic Acid Tests for Clinical Measurements of Bacteria and Viruses. Journal of Clinical Microbiology, 53(7), 2008–2014. https://doi.org/10.1128/JCM.02136-14

21. Pinninti, S. G., Ross, S. A., Shimamura, M., Novak, Z., Palmer, A. L., Ahmed, A., Tolan, R. W. J., Bernstein, D. I., Michaels, M. G., Sánchez, P. J., Fowler, K. B., & Boppana, S. B. (2015). Comparison of saliva PCR assay versus rapid culture for detection of congenital cytomegalovirus infection. The Pediatric Infectious Disease Journal, 34(5), 536–537. https://doi.org/10.1097/INF.0000000000000609

22. Preiksaitis, J. K., Hayden, R. T., Tong, Y., Pang, X. L., Fryer, J. F., Heath, A. B., Cook, L., Petrich, A. K., Yu, B., & Caliendo, A. M. (2016). Are We There Yet? Impact of the First International Standard for Cytomegalovirus DNA on the Harmonization of Results Reported on Plasma Samples. Clinical Infectious Diseases, 63(5), 583–589. https://doi.org/10.1093/cid/ciw370

23. Razonable, R. R., Inoue, N., Pinninti, S. G., Boppana, S. B., Lazzarotto, T., Gabrielli, L., Simonazzi, G., Pellett, P. E., & Schmid, D. S. (2020). Clinical Diagnostic Testing for Human Cytomegalovirus Infections. The Journal of Infectious Diseases, 221(Suppl 1), S74–S85. https://doi.org/10.1093/infdis/jiz601

24. Römkens, T. E. H., Bulte, G. J., Nissen, L. H. C., & Drenth, J. P. H. (2016). Cytomegalovirus in inflammatory bowel disease: A systematic review. World Journal of Gastroenterology, 22(3), 1321–1330. https://doi.org/10.3748/wjg.v22.i3.1321

25. Saldan, A., Forner, G., Mengoli, C., Gussetti, N., Palù, G., & Abate, D. (2017). Testing for Cytomegalovirus in Pregnancy. Journal of Clinical Microbiology, 55(3), 693–702. https://doi.org/10.1128/jcm.01868-16

26. Schattner, A. (2024). The Wide Spectrum of Presentations of Cytomegalovirus Infection in Immunocompetent Hosts: An Exhaustive Narrative Review. Pathogens (Basel, Switzerland), 13(8). https://doi.org/10.3390/pathogens13080667

27. Singh, G., & Gaidhane, A. (2022). A Review of Sensorineural Hearing Loss in Congenital Cytomegalovirus Infection. Cureus. https://doi.org/10.7759/cureus.30703

28. Streck, N. T., Espy, M. J., Ferber, M. J., Klee, E. W., Razonable, R. R., Gonzalez, D., Sayada, C., Heaton, P. R., Chou, S., & Binnicker, M. J. (2023). Use of next-generation sequencing to detect mutations associated with antiviral drug resistance in cytomegalovirus. Journal of Clinical Microbiology, 61(10), e00429-23. https://doi.org/10.1128/jcm.00429-23

29. Tanimura, K., Ebina, Y., & Yamada, H. (2017). PCR tests for uterine cervical secretion are promising noninvasive methods for predicting congenital cytomegalovirus infection. Expert Review of Molecular Diagnostics, 17(6), 535–537. https://doi.org/10.1080/14737159.2017.1318068

30. Tanimura, K., & Yamada, H. (2018). Potential Biomarkers for Predicting Congenital Cytomegalovirus Infection. International Journal of Molecular Sciences, 19(12). https://doi.org/10.3390/ijms19123760

31. Wamalwa, D., Njuguna, I., Maleche-Obimbo, E., Begnel, E., Chebet, D. J., Onyango, J. A., Cranmer, L. M., Huang, M.-L., Richardson, B. A., Boeckh, M., John-Stewart, G., & Slyker, J. (2022). Cytomegalovirus Viremia and Clinical Outcomes in Kenyan Children Diagnosed With Human Immunodeficiency Virus (HIV) in Hospital. Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America, 74(7), 1237–1246. https://doi.org/10.1093/cid/ciab604

32. Yokoyama, Y., Yamakawa, T., Hirano, T., Kazama, T., Hirayama, D., Wagatsuma, K., & Nakase, H. (2020). Current Diagnostic and Therapeutic Approaches to Cytomegalovirus Infections in Ulcerative Colitis Patients Based on Clinical and Basic Research Data. International Journal of Molecular Sciences, 21(7), Article 7. https://doi.org/10.3390/ijms21072438

14. SYMBOLS

	Manufacturer		This product is in compliance with relevant EU requirements
	*In vitro* Diagnostic Device		Unique Device Identifier
	Catalog Number		Batch Number
	Note		Consult (Electronic) Instructions for Use http://www.anatoliageneworks.com/support
	Expiry Date		Temperature Limit
	Caution		

15. ORDERING INFORMATION

Catalog Number:

ABCMQ3 (100 rxn/box)
ABCMQ2 (50 rxn/box)
ABCMQ1 (25 rxn/box)

16. CONTACT INFORMATION

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Document Revision History

Revised on	Version	Description	Approved by
04th January 2011	V1	First Publishing	Ayşe Kanneci
12th April 2011	V2	Partial content correction	Merve Ölçen Erdem
06th May 2014	V3	The general content and type check	Ayşe Kanneci
05th December 2016	V4	The content has been updated and checked	Merve Ölçen Erdem
04th May 2017	V5	The general content and type check	Ayşe Kanneci
13th November 2019	V6	The content has been updated and checked	Merve Ölçen Erdem
14th February 2023	V7	The general content and type check	Merve Ölçen Erdem
13th March 2024	V8	Partial content correction	Ömer Alperen Arslantaş
17th April 2024	V9	The content has been updated and checked	Ömer Alperen Arslantaş
27th September 2024	V10	Partial content correction	Ömer Alperen Arslantaş
29th November 2024	V11	Added analytical performance data and aligned IFU to IVDR	Ömer Alperen Arslantaş
02nd April 2025	V12	The intended purpose has been aligned with Annex II, 1.1(c), and the definition of "General Population" has been revised to include details such as sex, age, race, and medical condition. It has been clarified that the device is not intended for transplantation recipient matching. The device is defined as a Real-Time PCR-based quantitative assay compatible with automated systems. The relationship between C_T values and IU/mL has been explained, and the description of the standard curve and data analysis process has been improved.	Ömer Alperen Arslantaş
05th May 2025	V13	Added details to the intended use regarding use in immunocompromised and transplant patients, and clarified that the device is not intended to assess suitability for transfusion, transplantation, or cell administration.	Ömer Alperen Arslantaş