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bosphore

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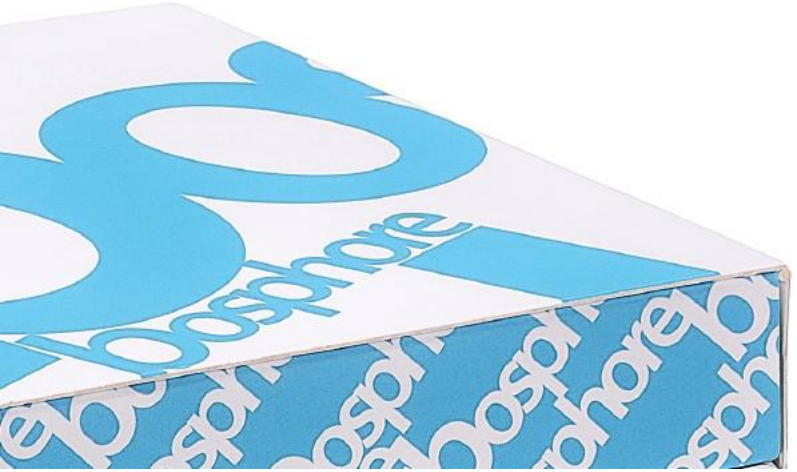
INSTRUCTIONS FOR USE

Bacterial Meningitis
Panel Kit v3

For *In Vitro* Diagnostic Use

MB526v3f
14th July 2023



Anatolia geneworks

CONTENTS

1. PRODUCT DESCRIPTION

Bosphore Bacterial Meningitis Panel Kit v3 is a Real-Time PCR kit for *in vitro* diagnostics that detects and characterizes the *omp6* gene of *Haemophilus influenzae*, *ctrA* gene of *Neisseria meningitidis* and *lytA* gene of *Streptococcus pneumoniae* from whole blood, serum, plasma, tissue/biopsy samples, and CSF* samples. Fluorescence detection is performed using FAM, HEX, Texas RED, and Cy5 filters. *Haemophilus influenzae* DNA is amplified and fluorescence detection is performed using the FAM filter, *Neisseria meningitidis* DNA is amplified and fluorescence detection is performed using the HEX filter, and *Streptococcus pneumoniae* DNA is amplified and fluorescence detection is performed using the Texas RED filter.

* CSF samples are the "gold standard" for the diagnosis of meningitis.

Component	**FAM** (Gene)	**HEX** (Gene)	**Texas RED** (Gene)	**Cy5**
PCR Master Mix	*Haemophilus influenzae (omp6)*	*Neisseria meningitidis (ctrA)*	*Streptococcus pneumoniae (lytA)*	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 Cy5 filter. The internal control can be added either during DNA extraction or the PCR step.

2. CONTENT

Bosphore Bacterial Meningitis Panel Kit v3 consists of the following dH_2O, Real-Time PCR Master Mix, positive control, 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)	(830 µL)	(415 µL)
3	Internal Control	(550 µL)	(275 µL)	(275 µL)
4	Positive Control	(176 µL)	(88 µL)	(88 µL)

3. STORAGE

PCR reagents for Bosphore Bacterial Meningitis Panel Kit v3 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
- Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Bacterial DNA Extraction Kit and Unio Whole Blood Genomic DNA Extraction Kit or Unio Whole Blood Genomic DNA Extraction Large Volume Kit, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit or Unio 96 Large Volume Whole Blood Nucleic Acid Extraction Kit, Unio M32 Extraction System and Unio M32 Whole Blood Genomic DNA Extraction Kit, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Bacterial DNA Extraction Kit or Magrev Whole Blood Genomic DNA Extraction Kit, Bosphore Bacterial DNA Extraction Spin Kit v2 or Bosphore Nucleic Acid Extraction Versatile Spin Kit or Magnesia 16 Nucleic Acid Extraction Instrument and Magnesia Genomic DNA Bacterial Kit or Magnesia Whole Blood Genomic DNA 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, coats and caps

*For other Real-Time PCR devices that can be used with Bosphore Bacterial Meningitis Panel Kit v3, 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

Haemophilus influenzae, *Neisseria meningitidis*, and *Streptococcus pneumoniae* are three different bacterial species that can cause significant harm to human health. These bacteria can lead to severe infections and illnesses, and therefore, understanding their behavior and how to prevent and treat them is crucial.

Haemophilus influenzae is a bacterium that normally lives in the nasal and throat mucous membranes, but can sometimes cause infections. These infections can be serious, especially in individuals with weakened immune systems. *Haemophilus influenzae* infections can manifest as ear infections, pneumonia, and meningitis (Todar, 2012).

Neisseria meningitidis is a bacterium that can cause meningitis, sepsis, or infections of the sensory organs. This bacterium is easily spread through coughing, sneezing, or close personal contact (CDC, 2019). *N. meningitidis* infections can progress rapidly and cause severe harm to an infected individual (CDC, 2019).

Streptococcus pneumoniae is another bacterium that can cause severe respiratory tract infections, meningitis, and sepsis. Like *N. meningitidis*, *Streptococcus pneumoniae* can also be easily spread through coughing, sneezing, or close personal contact (WHO, 2019). This bacterium poses a serious threat, especially to the elderly, children, and individuals with weakened immune systems (CDC, 2019). Preventing and treating these bacterial species can be done through a variety of methods.

The most effective prevention method is vaccination, which can provide protective immunity against these bacteria (CDC, 2019). Additionally, practicing good hygiene, such as frequently washing hands, and avoiding close contact with infected individuals can also help prevent the spread of infections (WHO, 2019).

8. METHOD

Bosphore Bacterial Meningitis Panel Kit v3 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 Bacterial Meningitis Panel Kit v3 uses multiplex qPCR and internal control is included in the system to control the extraction procedure, PCR inhibition, and application issues.

In PCR Master Mix, *Haemophilus influenzae* genome amplification is screened using FAM filter, *Neisseria meningitidis* genome amplification is screened using HEX filter, *Streptococcus pneumoniae* genome amplification is screened using Texas RED filter, and internal control genome amplification is screened using Cy5 filter.

9. PROCEDURE

9.1. DNA Extraction

We recommend that Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Bacterial DNA Extraction Kit and Unio Whole Blood Genomic DNA Extraction Kit or Unio Whole Blood Genomic DNA Extraction Large Volume Kit, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit or Unio 96 Large Volume Whole Blood Nucleic Acid Extraction Kit, Unio M32 Extraction System and Unio M32 Whole Blood Genomic DNA Extraction Kit, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Bacterial DNA Extraction Kit or Magrev Whole Blood Genomic DNA Extraction Kit, Bosphore Bacterial DNA Extraction Spin Kit v2 or Bosphore Nucleic Acid Extraction Versatile Spin Kit or Magnesia 16 Nucleic Acid Extraction Instrument and Magnesia Genomic DNA Bacterial Kit or Magnesia Whole Blood Genomic DNA Extraction Kit (Anatolia Geneworks) or other high-quality DNA extraction kits and systems, are used with Bosphore Bacterial Meningitis Panel Kit v3. The DNA extraction should be performed according to the manufacturer's instructions.

9.2. Kit Components

9.2.1. Negative Control

The negative control is an essential component of Bosphore Bacterial Meningitis Panel Kit v3 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 *Haemophilus influenzae, Neisseria meningitidis, Streptococcus pneumoniae,* and internal control.

9.2.3. Internal Control

The internal control included in the kit to check for DNA extraction, PCR inhibition, and application errors, the internal control 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, 0.2 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 Cy5 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 bacterial 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.

9.2.4. Positive Control

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

Positive Control: Synthetic *Haemophilus influenzae, Neisseria meningitidis,* and *Streptococcus pneumoniae* 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 to the PCR reaction together with the samples. Ensure that all kit components are dissolved before use and refer to the table on the next page 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)	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). 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 Bacterial Meningitis Panel Kit v3 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	06:00 min	
Denaturation	97 °C	00:15 min	38 Cycles
Annealing (Data Collection)	58 °C	01:10 min	
Hold	32 °C	00:20 min	

Before starting to work with Bosphore Bacterial Meningitis Panel Kit v3, 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 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 and positive control of Bosphore Bacterial Meningitis Panel Kit v3 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	≤32
Internal Control	≤33

In qualitative test results; samples that cross the threshold in the FAM, HEX, and Texas RED filters; 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 bacterial load below the detection limit of the assay. The internal control data in the Cy5 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 bacterial 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:

	***Haemophilus influenzae* (FAM)**	***Neisseria meningitidis* (HEX)**	***Streptococcus pneumoniae* (Texas RED)**	**Internal Control (Cy5)**	**Result**
PCR Master Mix	+	-	-	+/-	The sample is *Haemophilus influenzae* positive
	-	+	-	+/-	The sample is *Neisseria meningitidis* positive
	-	-	+	+/-	The sample is *Streptococcus pneumoniae* positive
	-	-	-	+	Sample is negative
	+	+	-	+/-	The sample is *Haemophilus influenzae* and *Neisseria meningitidis* positive
	-	+	+	+/-	The sample is *Neisseria meningitidis* and *Streptococcus pneumoniae* positive
	+	-	+	+/-	The sample is *Haemophilus influenzae* and *Streptococcus pneumoniae* positive
	+	+	+	+/-	The sample is *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* positive
	-	-	-	-	The test should be repeated!

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 'Cycle Range' option. For LC480, color compensation protocol must be performed (Bosphore Color Compensation Plate Set F could be used the for analysis of PCR Master Mix.)

Rotor-Gene Q – QIAGEN: It is recommended to use the "**Gain**" settings as 10 for all filters, to analyze with "Slope Correct" and "Dynamic tube" selected. Please use outlier removal options **%5** for the Yellow filter and **%10** for the Red filter if it is necessary.

CFX96 Real-Time PCR Detection System - Bio-Rad: Use of a **white plate** and turning "**Apply Fluorescence Drift Correction**" in "**Baseline Setting**" for the analysis is recommended.

Quant Studio 5-ThermoFisher: Use of **Passive Referrence** as **"None"** for the analysis 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 (ROX):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.

AMPLIRUN HAEMOPHILUS DNA CONTROL, AMPLIRUN NEISSERIA MENINGITIDIS DNA CONTROL, and AMPLIRUN STREPTOCOCCUS PNEUMONIAE DNA CONTROL samples are utilized for analytical sensitivity studies.

The analytical detection limit for Bosphore Bacterial Meningitis Panel Kit v3 was found to*, be* 10.5 copies/reaction for *Haemophilus infuenzae*, 9.3 copies/reaction for *Neisseria meningitidis* and 23.2 copies/reaction for *Streptococcus pneumoniae*. The sensitivity was determined using serial dilutions of the previously quantitated DNA Controls. The dilutions were tested in different runs in replicates. The results were analyzed by the probit method.

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; To eliminate the risk of cross-reactivity; *M. pneumoniae*, Brucella, *U. urealyticum*, *N. gonorrhea, M. genitalium, M. hominis*, *T. vaginalis, K. pneumoniae*, *S. aureus, S. enteritidis, A. fumigatus, C. albicans, E. coli,* and *L. monocytogenes* samples with known high

positivity were tested, and found negative. The experimental results indicated that the kit detects specifically only *Haemophilus influenzae, Neisseria meningitidis,* and *Streptococcus pneumoniae* DNA pathogens that are only intended to detect, but not the others.

11.3. Reproducibility

Reproducibility data (on a C_T value basis) were obtained by the analysis of the previously quantitated *Haemophilus influenzae, Neisseria meningitidis,* and *Streptococcus pneumoniae* DNA-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 425 copies/reactions for *Haemophilus influenzae:*

Variability (*Haemophilus influenzae*)	**Standard Deviation**	**Variance**	**Coefficient of Variation [%]**
Intra-assay (n=4)	0.05568	0.00310	0.28649
Inter-lot (n=3)	0.08902	0.00792	0.32126
Inter-operator (n=3)	0.01774	0.00031	0.06406
Total Inter-assay (n=5)	0.06501	0.00423	0.23469

The resulting data is given below for 1,520 copies/reactions for *Neisseria meningitidis:*

Variability (*Neisseria meningitidis*)	**Standard Deviation**	**Variance**	**Coefficient of Variation [%]**
Intra-assay (n=4)	0.03162	0.00100	0.32223
Inter-lot (n=3)	0.00500	0.00002	0.01821
Inter-operator (n=3)	0.03683	0.00136	0.13411
Total Inter-assay (n=5)	0.02642	0.00070	0.09623

The resulting data is given below for 9,000 copies/reactions for *Streptococcus pneumoniae:*

Variability (*Streptococcus pneumoniae*)	Standard Deviation	Variance	Coefficient of Variation [%]
Intra-assay (n=4)	0.03162	0.00100	0.11371
Inter-lot (n=3)	0.03819	0.00146	0.13715
Inter-operator (n=3)	0.02462	0.00061	0.08856
Total Inter-assay (n=5)	0.03941	0.00155	0.14162

12. REFERENCES

1. CDC (2019). Haemophilus influenzae. Centers for Disease Control and Prevention. https://www.cdc.gov/hai/organisms/hinfluenzae/hinfluenzae.html

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4. WHO (2019). Haemophilus influenzae. World Health Organization. https://www.who.int/immunization/topics/Haemophilus_influenzae/en/

5. WHO (2019). Neisseria meningitidis. World Health Organization. https://www.who.int/immunization/topics/meningococcal/en/

6. Todar, K. (2012). Todar's Online Textbook of Bacteriology. Haemophilus influenzae. https://textbookofbacteriology.net/Haemophilus.html

13. SYMBOLS

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

14. ORDERING INFORMATION

Catalog Number:

ABBMP9 (100 rxn/box)
ABBMP8 (50 rxn/box)
ABBMP7 (25 rxn/box)

15. CONTACT INFORMATION

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Document Revision History

Document Version No	Revision No	Date	Description
V1	01	16th May 2022	First Publishing
V2	02	22nd February 2023	The content has been updated and checked
V3	03	14th July 2023	The content has been updated and checked

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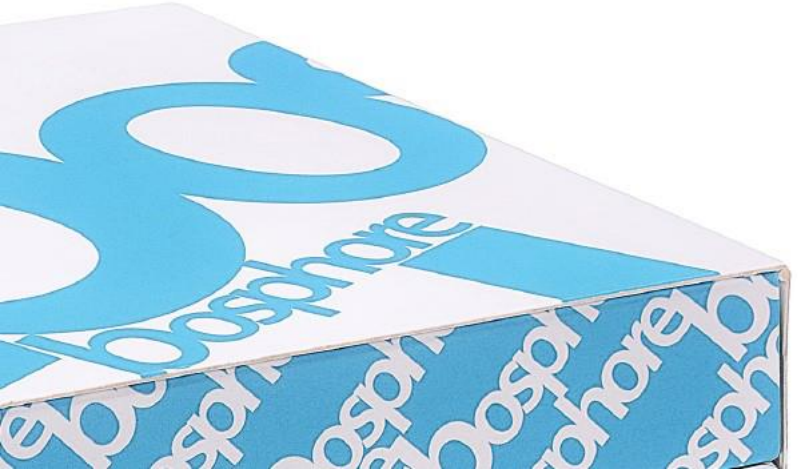
INSTRUCTIONS FOR USE

Brucella
Detection Kit v1

For *In Vitro* Diagnostic Use

MB12v7f
14th November 2024



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CONTENTS

1. PRODUCT DESCRIPTION

Bosphore *Brucella* Detection Kit v1 is a Real-Time PCR kit for *in vitro* diagnostics that detects and characterizes a region within the *BCSP31* gene of *Brucella* (*B. abortus, B. melitensis, B. canis, B. suis, B. ovis,* and *B. microti*) from serum, breast milk, and CSF samples. Fluorescence detection is performed using FAM and HEX filters. *Brucella* DNA is amplified, and fluorescence detection is performed using the FAM filter.

Component	FAM	HEX
PCR Master Mix	*Brucella* (*B. abortus, B. melitensis, B. canis, B. suis, B. ovis,* and *B. microti*)	Internal Control

Internal control has been integrated into the kit to check 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 the PCR step.

2. CONTENT

Bosphore *Brucella* Detection Kit v1 consists of the following dH_2O, Real-Time PCR Master Mix, positive control, 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)	(830 µL)	(415 µL)
3	Internal Control	(30 µL)	(15 µL)	(15 µL)
4	Positive Control	(88 µL)	(44 µL)	(44 µL)

3. STORAGE

PCR reagents for Bosphore *Brucella* 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 Bacterial DNA Extraction Kit, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Bacterial DNA Extraction Kit, Bosphore Bacterial DNA Extraction Spin Kit v2 or Bosphore Nucleic Acid Extraction Versatile Spin Kit or Magnesia 16 Nucleic Acid Extraction Instrument and Magnesia Genomic DNA Bacterial 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, coats, and caps

*For other Real-Time PCR devices that can be used with Bosphore *Brucella* 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

- 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

Brucella is a genus of bacteria belonging to the phylum *Proteobacteria,* class *Alphaproteobacteria,* order Rhizobiales*,* family *Brucellaceae.* It is the causative agent of Brucellosis, a zootonic disease that is a worldwide problem and can be transmitted to humans. Infection factors are aerobic, small, Gram-negative rods (Glowacka et al., 2018).

The genus *Brucella* has at least six species, where *Brucella abortus* (affecting primarily cattle), *Brucella suis* (affecting primarily swine) and *Brucella melitensis* (affecting primarily sheep and goats.) are of the major concern, which are all 'not host-specific' and may be transmit to other animal species and humans under appropriate conditions (Corbel, 1997; Robinson, 2003).

Brucellosis occurs worldwide, except in countries where bovine brucellosis (*B. abortus*) has been eliminated (Australia, Canada, Cyprus, Denmark, Finland, the Netherlands, New Zealand, Norway, Sweden, and the United Kingdom). The Mediterranean countries of Europe, northern and eastern Africa, Near East countries, India, Central Asia, Mexico and Central and South America are especially affected.

It is usually either an occupational or a food-borne infection. Sporadic and epidemics both occur in humans; however, the disease or infection is most often either unrecognized or, if diagnosed, not reported to the public authorities (WHO, 2005).

Transmission of infection to humans (with an incubation period of generally 1-2 months) occurs; through breaks in the skin, by direct contact with placental tissues or vaginal discharges from infected animals (lesser transmission degree of contact with blood or urine). Food-borne infection occurred via unpasteurized milk and other dairy products are rarely seen. Occupational airborne infection (laboratories and abattoirs) has also been reported. Also, cases of venereal and congenital infection in humans are reported (WHO, 2005).

8. METHOD

Bosphore *Brucella* 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 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 *Brucella* Detection Kit v1 uses multiplex qPCR and internal control is included in the system to control the PCR inhibition, and application issues. The reaction is performed in one PCR tube containing PCR Master Mix.

In PCR Master Mix, *Brucella* (*B. abortus, B. melitensis, B. canis, B. suis, B. ovis,* and *B. microti*) genome amplification is screened using FAM filter and the fluorescent signal produced by the internal control amplification is detected via the HEX filter.

9. PROCEDURE

9.1. DNA Extraction

We recommend that the Unio B24 Extraction System or Unio B48 Extraction System or Unio A24S Extraction & PCR Setup System and Unio Bacterial DNA Extraction Kit, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Bacterial DNA Extraction Kit, Bosphore Bacterial DNA Extraction Spin Kit v2 or Bosphore Nucleic Acid Extraction Versatile Spin Kit or Magnesia 16 Nucleic Acid Extraction Instrument and Magnesia Genomic DNA Bacterial Kit (Anatolia Geneworks) or other high-quality DNA extraction kits and systems are used with Bosphore *Brucella* Detection Kit v1. The DNA extraction should be performed according to the manufacturer's instructions.

9.2. Kit Components

9.3. Negative Control

The negative control is an essential component of Bosphore *Brucella* Detection Kit v1 for Real-Time PCR. It consists of double-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.

9.3.1. 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 *Brucella* (*B. abortus, B. melitensis, B. canis, B. suis, B. ovis,* and *B. microti*) and internal control.

9.3.2. Internal Control

The internal control included in the kit to check for PCR inhibition and application errors, the internal control is a synthetic DNA molecule. Internal control should add directly to the PCR Master Mix to control PCR inhibition as 0.2 µL.

Caution! The absence of internal control amplification in the HEX filter in negative samples may indicate a problem in application, or that the PCR reaction is inhibited. In this case, PCR should be repeated.

In samples with high bacterial 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.

9.3.3. Positive Control

The kit contains 1 positive control containing synthetic *Brucella* (*B. abortus, B. melitensis, B. canis, B. suis, B. ovis,* and *B. microti*) DNA. It must be included in the PCR reaction to test the reaction efficiency.

Positive Control: Synthetic *Brucella* (*B. abortus, B. melitensis, B. canis, B. suis, B. ovis,* and *B. microti*) 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.

9.4. 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	14.8 µL
Internal Control	0.2 µL
Sample DNA (Negative / Positive Control)	10 µL
Total Volume	25 µL

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). 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 *Brucella* Detection 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 on next page.

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	02:00 min	

Before starting to work with Bosphore *Brucella* 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,
- 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 *Brucella* Detection Kit v1 are essential for accurate result analysis. The cycle threshold acceptance criteria for the internal control, and positive control are listed on below:

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

In qualitative test results; examples 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 bacterial 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 PCR inhibition or application failure. In this case, PCR should be repeated. Please consider that in the samples that contain high bacterial 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:

	Brucella ***(B. abortus, B. melitensis, B. canis, B. suis, B. ovis,* and *B. microti)*** **(FAM)**	**Internal Control (HEX)**	**Result**
PCR Master Mix	+	+/-	The sample is *Brucella* (*B. abortus, B. melitensis, B. canis, B. suis, B. ovis,* and *B. microti*) 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 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.

The analytical detection limit for Bosphore *Brucella* Detection Kit v1 was found to be 7.5×10^2 IU/mL (p=0.05). The sensitivity was determined using serial dilutions of DNA calibrated with the previously quantitated *Brucella* DNA Control. The dilutions were tested in different runs in replicates. The results were analyzed by the probit method.

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; *Mycobacterium tuberculosis, Chlamydia trachomatis,* and *Helicobacter pylori* samples with known high positivity were tested and found negative. The experimental results indicated that the kit detects specifically and only *Brucella* pathogens that it intends to detect, but not the others.

11.3. Reproducibility

Reproducibility data (on a C_T value basis) were obtained by the analysis of the previously quantitated *Brucella* 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 10^4 copies/mL.

Variability (*Brucella*)	**Standard Deviation**	**Variance**	**Coefficient of Variation [%]**
Intra-assay (n=4)	0.043	0.0018	0.13
Inter-lot (n=3)	0.070	0.0052	0.21
Inter-operator (n=3)	0.017	0.0002	0.05
Total Inter-assay (n=5)	0.043	0.0018	0.13

12. REFERENCES

1. Głowacka, P., Żakowska, D., Naylor, K., Niemcewicz, M., & Bielawska-Drózd, A. (2018). *Brucella* - Virulence Factors, Pathogenesis and Treatment. Polish journal of microbiology, 67(2), 151–161. https://doi.org/10.21307/pjm-2018-029
2. Corbel M. J. (1997). Brucellosis: an overview. Emerging infectious diseases, 3(2), 213–221. https://doi.org/10.3201/eid0302.970219
3. Robinson, A. (2003). Guidelines for coordinated human and animal brucellosis surveillance (FAO animal production and health paper 156). Rome: Food and Agriculture Organization.
4. Brucellosis in humans and animals. WHO guidance. Geneva, World Health Organization, 2005.

13. SYMBOLS

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

14. ORDERING INFORMATION

Catalog Number:	**ABBRC3 (100 rxn/box)**
	ABBRC2 (50 rxn/box)
	ABBRC1 (25 rxn/box)

15. CONTACT INFORMATION

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Document Revision History

Document Version No	Revision No	Date	Description
V1	01	04th December 2014	First publishing
V2	02	12th October 2015	Partial content correction
V3	03	06th May 2017	The general content and type check
V4	04	05th April 2018	The content has been updated and checked
V5	05	04th December 2018	The general content and type check
V6	06	13th December 2023	The general content and type check
V7	07	14th November 2024	Breast milk has been added as a sample type

IVD CE

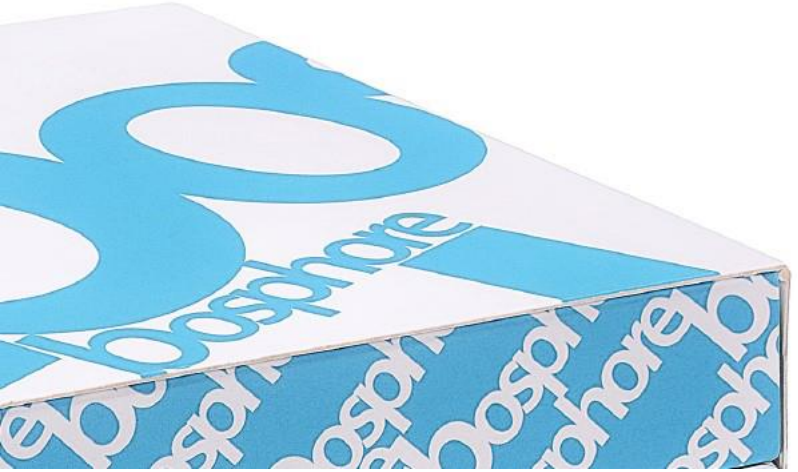
bosphore

INSTRUCTIONS FOR USE

Measles
Detection Kit v1

For *In Vitro* Diagnostic Use

MB346v6f
02nd October 2024



Anatolia geneworks

CONTENTS

1. PRODUCT DESCRIPTION

Bosphore Measles Detection Kit v1 is a Real-Time PCR kit for in vitro diagnostics that detects and characterizes the N gene of the Measles virus from a nasopharyngeal aspirate, throat swab, nasopharyngeal swab, serum, plasma, and urine samples. Fluorescence detection is performed using FAM and HEX filters. Measles virus RNA is amplified and fluorescence detection is performed using the FAM filter.

Component	**FAM** (Gene)	**HEX**
PCR Master Mix	Measles virus (N gene)	Internal Control

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 Measles Detection Kit v1 consists of the following dH_20, Real-Time PCR Master Mix, positive control, 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)	(830 µL)	(415 µL)
3	Internal Control	(550 µL)	(275 µL)	(275 µL)
4	Positive Control	(176 µL)	(88 µL)	(88 µL)

3. STORAGE

PCR reagents for Bosphore Measles 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, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Viral DNA/RNA Extraction Kit, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile 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 Measles 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

- 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

The measles virus (MeV), belonging to the Morbillivirus genus of the Paramyxoviridae family, is a single-stranded, negative-sense RNA virus that causes symptoms such as fever, coughing, conjunctivitis, coryza, and a characteristic rash. The virus's genome is composed of 15,894 nucleotides and can be divided into 24 genotypes based on 450 variable nucleotides (Maurice et al., 2013). Although there are many measles genotypes, only one dominant serotype exists (Simpson, 1952). The virus binds and invades host cells through two key envelope glycoproteins on its surface, Hemagglutinin (H) and Membrane Fusion Protein (F) (Lu et al., 2013).

According to recent studies, around 20 million people worldwide are affected by MeV each year, mainly in less developed Asian and African countries (Lozano et al., 2012). MeV is responsible for the highest number of deaths among vaccine-preventable diseases, with an estimated 158,000 deaths globally each year (WHO, 2014). The fatality rate from MeV is reported to be as low as 0.2% for those infected but can rise to 10% in cases of malnutrition (Kabra & Lodhra, 2013). Most deaths occur in children under five years of age [WHO, 2014]. Measles can cause a range of complications, from mild symptoms like diarrhea to serious cases such as pneumonia, bronchitis, and brain inflammation (Gardiner, 2007; Fisher, 2014). The severity of complications is linked to factors like malnutrition, underlying immunodeficiency, pregnancy, and vitamin A deficiency (Chen, 2011; National Institutes of Health Office of Dietary Supplements, 2013). Currently, there is no specific treatment for MeV, and most cases recover with supportive care (Gardiner, 2007).

MeV is present in the nose and throat mucus of infected individuals and is primarily transmitted through person-to-person contact, making it highly contagious with a transmission rate of 90% (Bloch, 1985).

The virus can survive for up to two hours in the air, making it possible to contract the disease without direct contact (Bloch, 1985). An infected individual can spread the disease from five days before and after the rash appears, with the period of highest contagion being during the febrile and respiratory symptoms of the late prodrome phase (Richardson, 2001). Measles is a human disease and is not spread by any other animal species (WHO, 2014).

8. METHOD

Bosphore Measles 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.

Bosphore Measles 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, Measles virus 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. RNA Extraction

We recommend that the 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, Magrev 24 Manual Magnetic Bead Nucleic Acid Extraction Stand and Magrev Nucleic Acid Extraction Versatile Kit or Magrev Viral DNA/RNA Extraction Kit, Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile 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 extraction kits and systems are used with Bosphore Measles Detection Kit v1. The RNA extraction should be performed according to the manufacturer's instructions.

9.2. Kit Components

9.2.1. Negative Control

The negative control is an essential component of Bosphore Measles 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), reverse transcriptase, PCR buffers, and dNTPs mix. PCR Master Mix also contains forward and reverse primers and dual-labeled probes specific for Measles virus and internal control.

9.2.3. 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, 0.2 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.

9.2.4. Positive Control

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

Positive Control: Synthetic Measles virus 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. Preparing the PCR

The positive and negative controls should be added to 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 RNA (Negative / Positive Control)	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). 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 Measles 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 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	
Hold	32 °C	02:00 min	

The following alternative short protocol can be applied if desired. Please refer to section 11.1 for the altered sensitivity values.

Steps	Temperature	Time	
Reverse Transcription	50 °C	17:00 min	
Initial Denaturation	95 °C	06:00 min	
Denaturation	97 °C	00:15 min	38 Cycles
Annealing (Data Collection)	55 °C	01:10 min	
Hold	32 °C	00:20 min	

Before starting to work with Bosphore Measles 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,
- 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 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 Measles Detection 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 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 on below shows the possible results and their interpretation:

	Measles virus (FAM)	Internal Control (HEX)	Result
PCR Master Mix	+	+/-	The sample is Measles virus positive
	-	+	The sample is Measles virus 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.

Montania 4896 Real-Time PCR Instrument:

- Cross Talk Option should be adjusted as shown below.

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

Figure 1: Montania 4896 Cross Talk Option

LightCycler 480 Instrument II – Roche: Please use a **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 the 'Cycle Range' option. **For LC480, 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** and turning "**Apply Fluorescence Drift Correction**" on in "**Baseline Setting**" for the analysis is recommended if necessary.

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. Please use "Outlier removal" up to 5% for Yellow (HEX) filter.

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.

Sensitivity data was obtained using the Montania 4896 Real-Time PCR Instrument, Magnesia 16 Nucleic Acid Extraction Instrument, and Magnesia Viral Nucleic Acid Extraction Kit with a starting volume of 400 µL and an elution volume of 60 µL.

The analytical detection limit for Bosphore Measles Detection Kit v1 was found 13 copies/reaction (p=0.05). The analytical detection limit for Bosphore Measles Detection Kit v1, using the shorter thermal protocol was found 23 copies/reaction (p=0.05). The dilutions were tested in different runs in replicates. The results were analyzed by the probit method.

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; Adenovirus, HBV, HCV, CMV, HSV-1, HSV-2, VZV, BKV, Mumps, Enterovirus, Rhinovirus, Influenza A and Influenza B samples with known high positivity were tested and found negative. The experimental results indicated that the kit detects specifically and only the Measles virus pathogen that it only intends to detect, but not the others.

11.3. Reproducibility

Reproducibility data (on a C_T value basis) were obtained by the analysis of the previously quantitated Measles virus-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 450 copies/mL.

Variability (Measles virus)	Standard Deviation	Variance	Coefficient of Variation [%]
Intra-assay (n=4)	0.06928	0.00480	0.23040
Inter-lot (n=3)	0.16600	0.02756	0.55428
Inter-operator (n=3)	0.10693	0.01143	0.35626
Total Inter-assay (n=5)	0.14405	0.02075	0.48058

12. REFERENCES

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13. SYMBOLS

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

14. ORDERING INFORMATION

Catalog Number:

ABMEA3 (100 rxn/box)
ABMEA2 (50 rxn/box)
ABMEA1 (25 rxn/box)

15. CONTACT INFORMATION

Anatolia Tanı ve Biyoteknoloji Ürünleri Araştırma Geliştirme Sanayi ve Ticaret A.Ş

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Document Revision History

Version	Revision No	Date	Description
V1	01	28th December 2018	First Publishing
V2	02	16th January 2020	Partial content correction
V3	03	07th May 2021	General content and type check
V4	04	12th February 2023	The content has been updated and checked
V5	05	04th March 2024	Added sample types
V6	06	02nd October 2024	Analysis settings for qPCR devices added

IVD CE

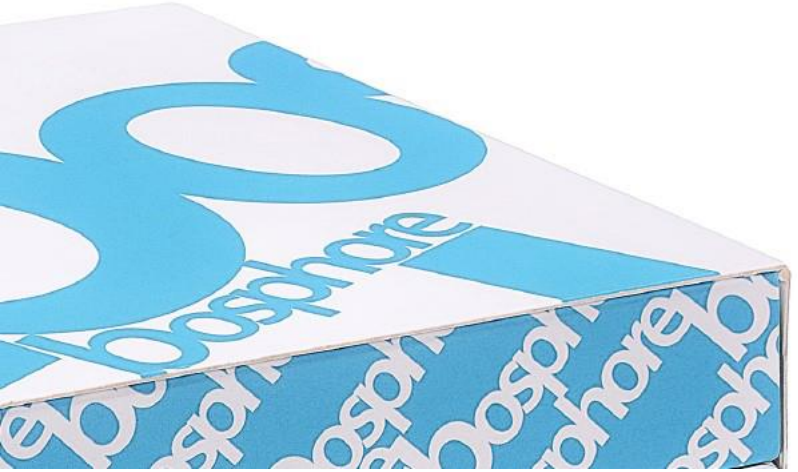
bosphore

INSTRUCTIONS FOR USE

Viral Meningitis
Panel Kit v2

For *In Vitro* Diagnostic Use

MB338v6f
25th July 2024



Anatolia geneworks

CONTENTS

1. PRODUCT DESCRIPTION

Bosphore Viral Meningitis Panel Kit v2 is a Real-Time PCR kit for *in vitro* diagnostics that detects and characterizes the Glycoprotein D of HSV-1, ORF38 gene of VZV, Glycoprotein D gene of HSV-2, 5′ UTR of Enterovirus (Coxsackie A and B, Echovirus, Poliovirus and Enterovirus 68 – 71), 5′ UTR gene of Parechovirus, and SH gene of Mumps virus from serum, plasma, tissue/biopsy samples, and CSF* samples. Fluorescence detection is performed using FAM, HEX, Texas RED, and Cy5 filters.

*CSF samples are the gold standard in the diagnosis of meningitis.

Component	**FAM** (Gene)	**HEX** (Gene)	**Texas RED**	**Cy5** (Gene)
PCR Master Mix 1	HSV-1 (Glycoprotein D)	VZV (ORF38 gene)	Internal Control	HSV-2 (Glycoprotein D)
PCR Master Mix 2	Enterovirus (5′ UTR)	Parechovirus (5′ UTR)	Internal Control	Mumps Virus (SH gene)

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

2. CONTENT

Bosphore Viral Meningitis Panel Kit v2 consists of the following dH_2O, Real-Time PCR Master Mixes, RT Mix, positive controls, 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	(1,660 µL)	(830 µL)	(415 µL)
3	PCR Master Mix 2	(1,640 µL)	(820 µL)	(410 µL)
4	RT Mix	(28 µL)	(14 µL)	(7 µL)
5	Internal Control	(550 µL)	(275 µL)	(275 µL)
6	Positive Control 1	(176 µL)	(88 µL)	(88 µL)
7	Positive Control 2	(176 µL)	(88 µL)	(88 µL)

3. STORAGE

PCR reagents for Bosphore Viral Meningitis Panel Kit v2 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 and Cy5 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 DNA Extraction Spin 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 DNA/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 Viral Meningitis Panel Kit v2, 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

HSV-1 (Herpes simplex virus 1) and HSV-2 (Herpes simplex virus 2) are both enveloped, double-stranded DNA viruses belonging to the Herpesviridae family. HSV-1 is primarily associated with oral lesions, while HSV-2 is primarily associated with genital lesions. However, both viruses can cause infection at either site. Additionally, they can cause meningitis, which is an inflammation of the meninges (the membranes covering the brain and spinal cord).

Meningitis caused by HSV-1 and HSV-2 is typically viral meningitis, which is less severe than bacterial meningitis but can still cause significant morbidity and mortality (Tyler, 2004).

VZV (Varicella-zoster virus) is also an enveloped, double-stranded DNA virus belonging to the Herpesviridae family. VZV causes two distinct clinical manifestations: chickenpox (varicella) and shingles (herpes zoster). Chickenpox is a common childhood disease characterized by a generalized vesicular rash, while shingles is a reactivation of VZV in the dorsal root ganglia and are characterized by a painful vesicular rash in a dermatomal distribution. VZV can also cause meningitis, particularly in immunocompromised individuals. VZV meningitis is usually self-limited and resolves spontaneously, but severe cases can occur (Pasedag et al., 2014).

Parechoviruses are small, non-enveloped, single-stranded RNA viruses belonging to the Picornaviridae family. There are currently 18 recognized types of human parechovirus (HPeV), which are divided into three genetic groups.

HPeV infections are common in young children and usually cause mild illness, such as gastrointestinal and respiratory symptoms. However, severe infections, including meningitis and sepsis, can occur, particularly in neonates and young infants. HPeV meningitis is typically self-limited and resolves without complications, but severe cases can occur (Rotbart et al., 1998).

Enteroviruses are also small, non-enveloped, single-stranded RNA viruses belonging to the Picornaviridae family. There are more than 100 types of enteroviruses, including coxsackieviruses, echoviruses, and polioviruses. Enteroviruses are highly infectious and are transmitted primarily through fecal-oral or oral-oral routes. Enteroviral infections are common in young children and usually cause mild illness, such as hand, foot, and mouth disease, as well as respiratory and gastrointestinal symptoms. However, enteroviruses can also cause severe diseases, including meningitis and encephalitis. Enteroviral meningitis is typically self-limited and resolves without complications, but severe cases can occur (Kadambari et al., 2019).

The mumps virus is a contagious virus that causes swelling of the salivary glands, giving it the nickname "the swelling disease." It spreads through respiratory droplets and can lead to complications such as meningitis or orchitis. Vaccination with the MMR vaccine is highly effective at preventing the virus (Hviid et al., 2008).

In summary, HSV-1, HSV-2, VZV, parechoviruses, Mumps virus and enteroviruses are all capable of causing meningitis, either as a primary manifestation or as a complication of a more systemic infection. While meningitis caused by these pathogens is usually self-limited and resolves without complications, severe cases can occur, particularly in immunocompromised individuals or young infants (Logan & MacMahon, 2008).

8. METHOD

Bosphore Viral Meningitis Panel Kit v2 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 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 DNA/RNA and its threshold cycle.

Bosphore Viral Meningitis Panel Kit v2 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 two PCR tubes containing PCR Master Mix 1 and PCR Master Mix 2.

In PCR Master Mix 1, HSV-1 genome amplification is screened using FAM filter, VZV genome amplification is screened using HEX filter, and HSV-2 genome amplification is screened using Cy5 filter.

In PCR Master Mix 2, Enterovirus genome amplification is screened using FAM filter, Parechovirus genome amplification is screened using HEX filter, and Mumps virus genome amplification is screened using Cy5 filter.

The fluorescent signal produced by the internal control amplification is detected via the Texas RED filters in both PCR Master Mix 1 and PCR Master Mix 2.

9. PROCEDURE

9.1. DNA/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 DNA Extraction Spin 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 extraction kits and systems are used with Bosphore Viral Meningitis Panel Kit v2. The DNA/RNA extraction should be performed according to the manufacturer's instructions.

9.2. Kit Components

9.2.1. Negative Control

The negative control is an essential component of Bosphore Viral Meningitis Panel Kit v2 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 HSV-1, VZV, HSV-2 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 Enterovirus, Parechovirus, Mumps virus and internal control.

9.2.4. RT Mix

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

9.2.5. Internal Control

The internal control included in the kit to check for DNA/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 DNA/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.

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 Texas RED 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.

9.2.6. Positive Control

The kit contains 2 positive control containing synthetic DNAs. It must be included in the PCR reaction to test the reaction efficiency.

Positive Control 1: Synthetic HSV-1, HSV-2 and VZV DNAs

Positive Control 2: Synthetic Enterovirus, Parechovirus, and Mumps virus 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 to the PCR reaction together with the samples. Ensure that all kit components are dissolved before use and refer to the table on the next page 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	15 µL
Internal Control*	0.2 µL*
Sample DNA (Negative / Positive Control)	10 µL
Total Volume	25 µL

Components	Volume
PCR Master Mix 2	14.75 µL
RT Mix	0.25 µL
Internal Control*	0.2 µL*
Sample RNA (Negative / Positive Control)	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 Mixes (PCR Master Mix - RT Mix mix for PCR Master Mix 2) into the PCR tubes or strips and add 10 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 Viral Meningitis Panel Kit v2 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 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)	58 °C	01:30 min	
Hold	32 °C	02:00 min	

The following alternative short protocol can be applied if desired. Please refer to section 11.1 for the altered sensitivity values.

Steps	Temperature	Time	
Reverse Transcription	50 °C	17:00 min	
Initial Denaturation	95 °C	06:00 min	
Denaturation	97 °C	00:15 min	38 Cycles
Annealing (Data Collection)	58 °C	01:10 min	
Hold	32 °C	00:20 min	

Before starting to work with Bosphore Viral Meningitis Panel Kit v2, 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 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 and positive control of Bosphore Viral Meningitis Panel Kit v2 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 Controls	≤28
Internal Control	≤32

In qualitative test results; samples that cross the threshold in the FAM, HEX and Cy5 filters; 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 Texas RED 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 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:

	HSV-1 (FAM)	VZV (HEX)	Internal Control (Texas RED)	HSV-2 (Cy5)	Result
PCR Master Mix 1	+	-	+/-	-	The sample is HSV-1 positive
	-	+	+/-	-	The sample is VZV positive
	-	-	+/-	+	The sample is HSV-2 positive
	-	-	+	-	Sample is negative
	+	+	+/-	-	The sample is HSV-1 and VZV positive
	-	+	+/-	+	The sample is HSV-2 and VZV positive
	+	-	+/-	+	The sample is HSV-1 and HSV-2 positive
	+	+	+/-	+	The sample is HSV-1, VZV and HSV-2 positive
	-	-	-	-	The test should be repeated!

PCR Master Mix 2	Enterovirus (FAM)	Parechovirus (HEX)	Internal Control (Texas RED)	Mumps virus (Cy5)	Result
	+	-	+/-	-	The sample is Enterovirus positive
	-	+	+/-	-	The sample is Parechovirus positive
	-	-	+/-	+	The sample is Mumps virus positive
	-	-	+	-	Sample is negative
	+	+	+/-	-	The sample is Enterovirus and Parechovirus positive
	-	+	+/-	+	The sample is Parechovirus and Mumps virus positive
	+	-	+/-	+	The sample is Enterovirus and Mumps virus positive
	+	+	+/-	+	The sample is Enterovirus, Parechovirus and Mumps virus positive
	-	-	-	-	The test should be repeated!

CFX96 Real-Time PCR Detection System- Bio-Rad: Use of a **white plate** and turning "**Apply Fluorescence Drift Correction**" on in "**Baseline Setting**" for the analysis is recommended if necessary.

LightCycler 480 Instrument II – Roche: Please use a **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 the '**Cycle Range**' option. **For LC480, color compensation protocol must be performed. Bosphore Color Compensation Plate Set B could be used for PCR Master Mix 1 and Bosphore Color Compensation Plate Set D could be used for PCR Master Mix 2.**

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. Please use "**Outlier removal**" up to **10%** for **PCR Master Mix 2**.

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/RNA 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. Sensitivity data was obtained using the Montania 4896 Real-Time PCR Instrument, Magnesia 16 Nucleic Acid Extraction Instrument, and Magnesia Viral DNA/RNA Extraction Kit EP with a starting volume of 400 µL and an elution volume of 60 µL.

The analytical detection limit for Bosphore Viral Meningitis Panel Kit v2 was found to 11 copies/reaction for HSV 1, 7 copies/reaction for HSV 2, 9 copies/reaction for VZV, 101 copies/reaction for Enterovirus, 99 copies/reaction for Parechovirus, and 162 copies/reaction for Mumps virus. The dilutions were tested in different runs in replicates. The results were analyzed by the probit method.

The analytical detection limit for Bosphore Viral Meningitis Panel Kit v2, using the shorter thermal protocol was found to be 20 copies/reaction for HSV 1, 12.5 copies/reaction for HSV 2, 15 copies/reaction for VZV, 140 copies/reaction for Enterovirus, 188 copies/reaction for Parechovirus, and 260 copies/reaction for Mumps virus. The dilutions were tested in different runs in replicates. The results were analyzed by the probit method.

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; JCV, CMV, EBV, HBV, 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 pathogens that it only intends to detect, but not the others.

12. REFERENCES

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2. Pasedag, T., Weissenborn, K., Wurster, U., Ganzenmueller, T., Stangel, M., & Skripuletz, T. (2014). Varicella zoster virus meningitis in a young immunocompetent adult without rash: a misleading clinical presentation. Case reports in neurological medicine, 2014.

3. Kadambari, S., Braccio, S., Ribeiro, S., Allen, D. J., Pebody, R., Brown, D., ... & Ladhani, S. (2019). Enterovirus and parechovirus meningitis in infants younger than 90 days old in the UK and Republic of Ireland: a British Paediatric Surveillance Unit study. Archives of Disease in Childhood, 104(6), 552-557.

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13. SYMBOLS

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

14. ORDERING INFORMATION

Catalog Number:	**ABVMP6 (100 rxn/box)**
	ABVMP5 (50 rxn/box)
	ABVMP4 (25 rxn/box)

15. CONTACT INFORMATION

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Document Revision History

Document Version No	Revision No	Date	Description
V1	01	27th December 2019	First Publishing
V2	02	02nd January 2020	Partial content correction
V3	03	06th January 2020	Partial content correction
V4	04	12th May 2023	The content has been updated and checked
V5	05	25th July 2024	RT Mix added
V6	06	26th August 2024	Other PCR Instruments adjustments added

IVD CE

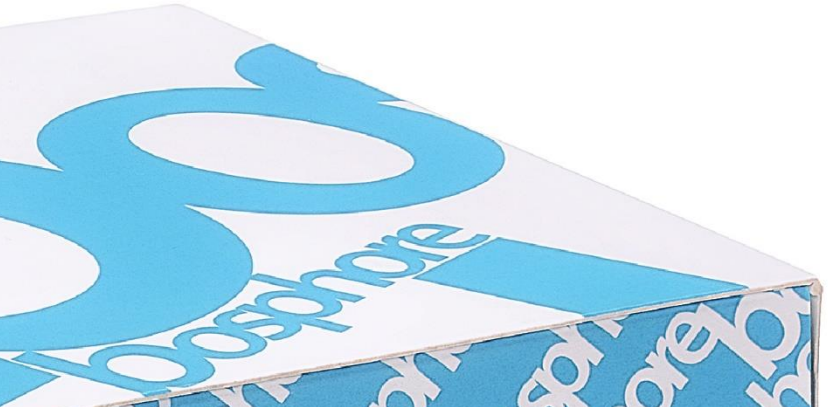
bosphore

INSTRUCTIONS FOR USE

West Nile Virus
Quantification Kit v2

For *In Vitro* Diagnostic Use

MB100v9f
14th November 2024



Anatolia geneworks

CONTENTS

1. PRODUCT DESCRIPTION

Bosphore West Nile Virus Quantification Kit v2 is a Real-Time PCR kit for *in vitro* diagnostics that detects and characterizes the 3' UTR region of West Nile virus RNA from serum, plasma, urine, whole blood, and CSF samples. Fluorescence detection is performed using FAM, and HEX filters. West Nile virus RNA is amplified and fluorescence detection is performed using the FAM filter.

Component	**FAM** (Gene)	**HEX**
PCR Master Mix	West Nile virus (3' UTR)	Internal Control

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 West Nile Virus Quantification Kit v2 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	(1,640 µL)	(820 µL)	(410 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 (1,000 copies/µL)	(88 µL)	(44 µL)	(44 µL)
7	Standard 2 (100 copies/µL)	(88 µL)	(44 µL)	(44 µL)
8	Standard 3 (10 copies/µL)	(88 µL)	(44 µL)	(44 µL)
9	Standard 4 (2 copies/µL)	(88 µL)	(44 µL)	(44 µL)

3. STORAGE

PCR reagents for Bosphore West Nile Virus Quantification Kit v2 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

* See section 9.4 for the thermal protocol suitable for LightCycler 480 Instrument II – Roche.

** For other Real-Time PCR devices that can be used with Bosphore West Nile Virus Quantification Kit v2, 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

West Nile virus (WNV) is a *Flavivirus* belonging to the *Flaviviridae* family that was first isolated in 1937 in the "West Nile" province of Uganda/Africa and was named after this region. WNV virions are approximately 50 nm in diameter and are composed of a single-stranded RNA genome of length between 11 kb and 12 kb (Brinton, 2002).

West Nile virus was first isolated in a woman in the West Nile district of Uganda in 1937. It was identified in birds (crows and Columbiformes) in the Nile Delta region in 1953. Before 1997 WNV was not considered pathogenic for birds, but at that time in Israel, a more virulent strain caused the death of different bird species presenting signs of encephalitis and paralysis. Human infections attributable to WNV have been reported in many countries in the World for over 50 years.

In 1999 a WNV circulating in Israel and Tunisia was imported in New York producing a large and dramatic outbreak that spread throughout the continental United States of America (USA) in the following years.

The WNV outbreak in the USA (1999-2010) highlighted that the importation and establishment of vector-borne pathogens outside their current habitat represent a danger to the world. The largest outbreaks occurred in Greece, Israel, Romania, Russia, and the USA. Outbreak sites are on major birds' migratory routes.

In its original range, WNV was prevalent throughout Africa, parts of Europe, the Middle East, West Asia, and Australia. Since its introduction in 1999 into the USA, the virus has spread and is now widely established from Canada to Venezuela (WHO, 2017).

Most of the WNV infections are asymptomatic without any symptoms in people. The second type of WNV infection results in mild fever (West Nile Fever) with recovery from the illness, but however, third type of WNV infection with the virus crossing the blood-brain barrier causes severe outcomes such as inflammation of the brain (encephalitis) and the tissue surrounding the brain and spinal cord (meningitis) that all resulting in long-term effects like memory loss, depression, etc. The common route of WNV transmission is shown to be the mosquitos as vectors carrying the virus across vertebrate hosts. Mosquitos receive the virus upon feeding on infected birds, and the virus is transmitted to other hosts via mosquito bites. Humans, horses, and most other mammals are widely accepted as incidental hosts as they don't transmit the virus to others. However, with increasing research after WNV outbreaks, new WNV transmission routes are transmitted via blood transfusion, organ transplantation, intrauterine exposure, and breastfeeding (Hayers et al., 2005).

8. METHOD

Bosphore West Nile Virus Quantification Kit v2 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 RNA 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 number of unknown templates can be determined using standard curves constructed using C_T values of the known starting amounts of target templates.

Bosphore West Nile Virus Quantification Kit v2 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, West Nile virus genome amplification is screened using 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. RNA Extraction

We recommend that the 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 or other high-quality RNA extraction kits and systems are used with Bosphore West Nile Virus Quantification Kit v2. The RNA extraction should be performed according to the manufacturer's instructions.

9.2. Kit Components

9.2.1. Negative Control

The negative control is an essential component of Bosphore West Nile Virus Quantification Kit v2 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 West Nile virus 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, 0.2 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, 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. Positive Control

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

Positive Control: Synthetic West Nile virus 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.

9.2.6. Quantitation Standards

The quantitation standards are calibrated plasmid standards of $1x10^3$, $1x10^2$, $1x10^1$, and 2 copies/µL.

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	14.75 µL
RT Mix	0.25 µL
Internal Control*	0.2 µL*
Sample RNA (Standards/Positive Control/ Negative Control)	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/standards/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 West Nile Virus Quantification Kit v2 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 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)	55.5 °C	01:10 min	
Hold	32 °C	02:00 min	

The thermal protocol suitable for LightCycler 480 Instrument II – Roche is given 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	01:10 min	
Hold	32 °C	02:00 min	

Before starting to work with Bosphore West Nile Virus Quantification Kit v2, 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. The standard curve is plotted using the data obtained from the defined standards, with the axes C_T-Threshold Cycle and Log Starting Quantity.

Caution! Since the quantitation standards are provided as plasmid samples and as copies/µL, the following formula should be applied to the resulting copies/µL values obtained for the samples, to assess the quantitation values of the samples in copies/mL.

This mathematical factor takes the starting volume of RNA extraction and the elution volume into consideration, to ensure 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 µL and the elution volume is 60 µL, applying the formula; 1,000 x 60 ÷ 0.4 = 150,000 copies/mL is calculated as the West Nile virus RNA that the sample material contains.

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 West Nile Virus Quantification Kit v2 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)**
Positive Control	30±4
Internal Control	≤32
Standard 1	23.5±2
Standard 2	26.5±2
Standard 3	30±2
Standard 4	32.5±2
Correlation Coefficient	>0.970
PCR Efficiency	>80%

In quantitative test results, examples that cross the threshold in the FAM 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 pathogen 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:

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

LightCycler 480 Instrument II – Roche: Please use a **white plate** for Roche LightCycler 480. "**Abs Quant/Fit Points**" should be chosen as the analysis type for the appropriate threshold level selection. "**Abs Quant/2nd Derivative Max**" should be chosen as the analysis type for the quantitation results. Analysis should be performed with the '**Cycle Range**' option. **For LC480, color compensation protocol must be performed Bosphore Color Compensation Plate Set F could be used for PCR Master Mix.**

CFX96 Real-Time PCR Detection System - Bio-Rad: Use of a **white plate** and turning "**Apply Fluorescence Drift Correction**" on in "**Baseline Setting**" for the analysis is recommended.

Rotor-Gene Q – QIAGEN: Please use "**Outlier removal**" up to **15%** if necessary.

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 considered 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 West Nile virus Quantification v2 was found to be 50 copies/mL.

The sensitivity was determined using serial dilutions of RNA calibrated with the previously quantitated West Nile virus RNA Control. 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 West Nile Virus Quantification Kit v2 was determined to be $1.2x10^2$ - $4x10^9$ copies/mL. The standard curve correlation coefficient was found to be 0.999 for the linearity run with a dilution series of the West Nile virus Positive Control.

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; Zika virus, Dengue virus, Chikungunya virus, HSV1 (Herpes Simplex Virus 1), HSV2 (Herpes Simplex Virus 2), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Immunodeficiency Virus (HIV), *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Gardnerella vaginalis*, *Mycoplasma genitalium*, *Chlamydia trachomatis*, *Plasmodium malariae*, *Salmonella enterica*, *Plasmodium ovale*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, and *Haemophilus influenzae* samples with known high positivity were tested and found negative. The experimental results indicated that the kit detects specifically and only Bosphore West Nile Virus Quantification Kit v2 pathogen that it 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 West Nile virus 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 37,500 copies/mL.

Variability (West Nile virus)	Standard Deviation	Variance	Coefficient of Variation [%]
Intra-assay (n=4)	0.01708	0.00029	0.18624
Inter-lot (n=3)	0.02622	0.00069	0.10320
Inter-operator (n=3)	0.03126	0.00098	0.12308
Total Inter-assay (n=5)	0.03008	0.00090	0.11841

12. REFERENCES

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2. World Health Organization. (3 October 2017) West Nile virus. https://www.who.int/news-room/fact-sheets/detail/west-nile-virus
3. Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL (2005). "Epidemiology and transmission dynamics of West Nile virus disease". Emerging Infect. Dis. 11 (8): 1167–73.

13. SYMBOLS

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

14. ORDERING INFORMATION

Catalog Number:

ABWNV6 (100 rxn/box)
ABWNV5 (50 rxn/box)
ABWNV4 (25 rxn/box)

15. CONTACT INFORMATION

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Document Revision History

Document Version No	Revision No	Date	Description
V1	01	04th December 2018	First Publishing
V2	02	12th January 2020	Partial content correction
V3	03	06th April 2021	The general content and type check
V4	04	15th February 2022	The content has been updated and checked
V5	05	06th September 2022	The general content and type check
V6	06	17th November 2023	The content has been updated and checked
V7	07	30th November 2023	The general content and type check
V8	08	17th April 2024	Positive control acceptance criteria changed
V9	09	14th November 2024	PCR Master Mix volume changed