Enterovirus Detection Kit v1

USER MANUAL

For *in vitro* Diagnostic Use

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Contents

1. PRODUCT DESCRIPTION

Bosphore® Enterovirus Detection Kit v1 detects Enterovirus RNA in human biological samples including serum and plasma, cerebrospinal fluid, tissue, swab, feces, encompassing all Enteroviruses (coxsackie A and B, echoviruses, polioviruses and enteroviruses 68 - 71). The analytic sensitivity is 0.95 copies/µl. A region within the 5′UTR is amplified and fluorescence detection is accomplished using the FAM filter.

An internal control has been integrated into the kit in order to check PCR inhibition. The amplification data of the internal control is detected with the HEX filter depending on the Real-Time PCR system used. The internal control is added in the PCR step.

2. CONTENT

Bosphore® Enterovirus Detection Kit v1 is composed of Real-Time RT PCR reagents:

Component	REAGENT	100 Reactions	50 Reactions	25 Reactions
1	dH_2O	(1000 µl)	(1000 µl)	(500 µl)
2	PCR Master Mix	(1608 µl)	(804 µl)	(402 µl)
3	Internal Control	(44 µl)	(22 µl)	(15 µl)
4	Positive Control 1	(88 µl)	(44 µl)	(22 µl)

3. STORAGE

Bosphore® Enterovirus Detection Kit v1 PCR reagents should be stored at -20°C. Repeated thawing and freezing (>3x) should be avoided since it may reduce sensitivity. If the components are to be used in small amounts, they should be frozen in aliquots.

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

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

4. REQUIRED MATERIALS AND DEVICES

- Montania® 483, Montania® 484 or Montania® 4896 Real-Time PCR Instrument (Anatolia Geneworks), or another Real-Time PCR system with FAM and HEX filters (iCycler, iQ5, CFX–BioRad, LightCycler 480-Roche, 7500 Real-Time PCR System, ABI, Stratagene Mx3005P, Mx3000P-Agilent, LineGeneK, LineGene 9600-Bioer, Rotorgene 6000, Q-Qiagen)
- 0.2 ml Thin-Wall PCR tubes, PCR plates or strips
- Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Viral Nucleic Acid Extraction Kit/ Magrev® 24 stand and Magrev® Viral DNA/RNA Extraction Kit/Bosphore® Viral RNA Extraction Spin Kit (Anatolia Geneworks) or other high quality viral RNA extraction kits and systems

- Deep freezer (-20°C)
- Desktop centrifuge with rotor for 2 ml. microcentrifuge tubes
- Calibrated adjustable micropipettes
- DNAse, RNAse, pyrogen free micropipette tips with filters
- DNAse, RNAse, pyrogen free 1.5 or 2 ml. microcentrifuge tubes
- Disposable laboratory gloves

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

Important!:

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

6. PRODUCT USE LIMITATIONS

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

7. PATHOGEN

Causative Agents

Human Enteroviruses are icosahedral non-enveloped, small, single stranded RNA (about 7.5kb in long enclosed by capsis) viruses, belonging to the 'Picornaviridae' family. They include the polioviruses and human enterovirus groups A to D which are associated with a spectrum of syndromes such as respiratory infections, meningitis, encephalitis, etc. They can also be classified into four major groups/subgeneras; polioviruses, Coxsackie A viruses (CA), Coxsackie B viruses (CB), and Echoviruses according to their pathogenesis in humans and animals [1],[2],[3] .

Epidemiology

Human Enteroviruses are distributed worldwide affecting millions of people each year. These viruses are influenced by season and climates. Although most infections occur silently or trivial in severity, epidemics in tropical countries during hot and rainy season are reported such as in 1969 in Ghana, then in Indonesia, India and the Far East. Although the worldwide prevalence has been recently decreasing due to improved economic conditions and availability of vaccines, there are recent cases reported (Haiti, Dominican Republic, Afghanistan, Egypt, India, Niger, Nigeria, Pakistan,) considering the mutations in polio strains. It is endemic in 4 countries: Afghanistan, India, Nigeria, and Pakistan, and 14 other previously polio-free countries (Angola, Burkina Faso, Benin, Central Africa Republic, Chad, Côte d'Ivoire, The Democratic Republic of Congo, Ghana, Ethiopia, Nepal, Niger, Sudan, Tango) have reported some cases [4], [5].

Modes of Transmission:

Enteroviruses are transmitted mostly via the fecal-oral route. However, there are some exceptions, including coxsackievirus A21, which spread mainly by respiratory secretions, and enterovirus 70, found in tears and spread via fingers and fomites. Upon entry, virus replicates and viral particles are shed in the feces and in upper respiratory tract secretions for days, prior to the symptom onset with an average incubation period of 3-10 days. Minor Viremia occurs with the onset of symptoms and virus spreads to the spleen, liver and bone marrow. Dissemination to the target organs follows, and viral replication in target organs produces the major viremia skin and CNS. [6], [7].

8. METHOD

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

Polymerase chain reaction is a technique that is used for amplification of a DNA region. The reaction occurs by the repeating cycles of heating and cooling. The main components of PCR are primers, dNTPs, Taq polymerase enzyme, buffer solution and template. As a brief explanation, primers are small synthetic

DNA those anneal to the specific regions of the template in order to start the synthesis. dNTPs are the building blocks of the amplified products. Taq polymerase amplifies the DNA template. Buffer solution provides the pH adjustment required for the reaction and template, as referred, is the target region for synthesis. In addition to these components, in RT PCR reverse transcriptase is added to the reaction and cDNA synthesis from the RNA template is acquired.

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

The assay utilizes the 5′ exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent hybridization probe during the extension phase of PCR.

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

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

Bosphore Enterovirus Detection Kit v1 employs multiplex PCR, and an internal control is incorporated into the system in order to control the isolation procedure and to check for possible PCR inhibition. Enterovirus RNA (cDNA) and an internal control are co-amplified in a single reaction, using sequence-specific primers. The fluorescent signal generated by the enterovirus amplification is detected by a probe labeled at the 3′ end with FAM, through the FAM channel. The fluorescent signal generated by the internal control amplification, is detected by a second probe (labeled at the 5′ end with a different reporter molecule, HEX) through the HEX channel.

9. PROCEDURE

9.1. RNA Isolation

For extraction from human serum and plasma, we recommend that the Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Viral Nucleic Acid Extraction Kit/ Magrev® 24 stand and Magrev® Viral DNA/RNA Extraction Kit/ Bosphore® Viral RNA Extraction Spin Kit (Anatolia Geneworks) or other high quality viral RNA extraction kits and systems are used with Bosphore® Enterovirus Detection Kit v1. The viral RNA isolation should be performed according to the manufacturers' instructions. The starting volume is 400 µl and the elution volume is 60 µl.

9.2. Kit Components

9.2.1. PCR Master Mix

PCR master mix contains; HotStarTaq DNA Polymerase, RT-PCR Buffer, Enterovirus-specific forward and reverse primers and dual-labeled probes, internal control-specific forward and reverse primers and a dual-labeled probe, RT-Mix and dNTPs.

9.2.2. Internal Control

An internal control is included in the kit to control PCR inhibition. The internal control is a synthetic DNA molecule. It is added directly into the PCR master mix to control the PCR inhibition exclusively. For this purpose, 0.4 µl of internal control should be added for each reaction into the master mix. Lack of internal control amplification in the FAM negative samples, may indicate a problem in isolation or PCR inhibition. In this case, isolation and PCR should be repeated. In samples that contain a high viral load, internal control can be suppressed and no increase of the signal is detected. Please use the table below for the interpretation of internal control data:

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

9.2.3. Positive Control

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

9.3. Preparing the RT-PCR

The positive and the negative control (Extracted Enterovirus negative serum/plasma) should be added into the PCR reaction together with the samples. Make sure that all the kit components are thawed before use. Refer to the table below for preparing the PCR. It is for only one reaction, multiply these values with the sample number to find the values required for the master mix. While preparing master mixes for more than 5 samples, an extra 10% should be added to the total sample number.

PCR Master Mix	14.6 µl
Internal Control	0.4 µl
Sample RNA (Negative/Positive Control)	10 µl
Total Volume	25 µl

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

9.4. Programming the Real-Time PCR Instrument

The thermal protocol for Bosphore® Enterovirus Detection Kit v1 is composed of two-steps; firstly a reverse transcription step and secondly Real Time PCR steps; an initial denaturation for activation the HotStarTaq DNA Polymerase, a two-step amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle.

Reverse Transcription	50°C	30:00 min.	
Initial denaturation	95°C	14:30 min.	
Denaturation	97°C	00:30 min.	50 cycles
Annealing and Synthesis (Data Collection)	55°C	01:20 min.	
Hold	22°C	05:00 min.	

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

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

10. ANALYSIS

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

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

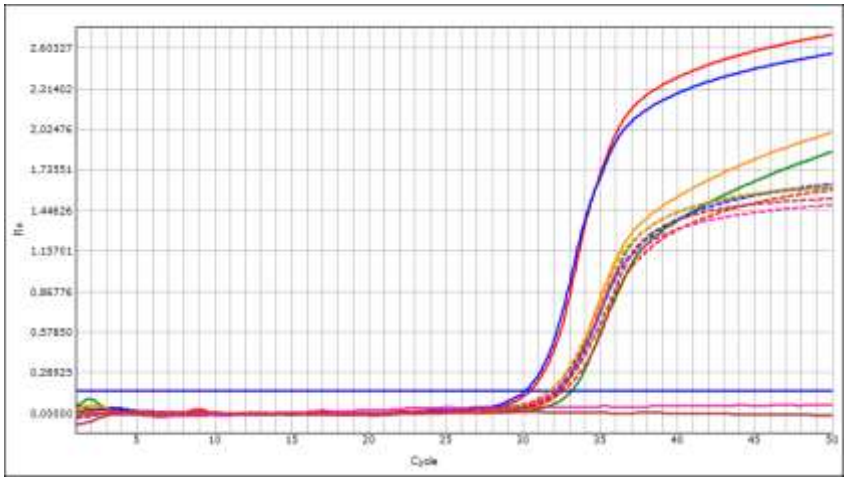

Fig. 1: Amplification Curve of a Bosphore® Enterovirus test

Analysis of the results should be performed by trained personnel who have received the required training for analyzing Real-Time PCR data.

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

Positive control of Bosphore® Enterovirus Detection Kit v1 is essential for accurate result analysis. The cycle threshold acceptance criteria for the positive control is 32±2. Test should be repeated unless the assay results meet the criteria stated above.

The qualitative results of the test are displayed on the "Report Mode" screen. The samples that cross the threshold in FAM channel are displayed as positive whereas samples that do not cut the threshold are displayed as "Negative" or "No Ct". These samples are regarded as negative or having a bacterial load below the detection limit of the assay. For these undetectable samples, the HEX data of the internal control should also be checked to avoid false negative results.

The following table shows the possible results and their interpretation:

Signal detected in FAM filter pair	The sample contains Enterovirus RNA,	No need to check the internal control since the sample is positive (high positive samples may suppress the signal from the internal control)
No signal in FAM, signal in HEX	The Enterovirus RNA in the sample is not detectable	Signal from HEX filter pair rules out the possibility of PCR inhibition
No signal in FAM and HEX	Inconclusive/must be repeated	No signal in HEX points out to PCR inhibition or to a problem in RNA isolation

11. SPECIFICATIONS

11.1. Sensitivity

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

The analytical detection limit for Bosphore® Enterovirus Detection Kit v1 was found to be 0.95 copies/µl. The sensitivity was determined using serial dilutions of RNA calibrated with the previously quantitated Enterovirus RNA Control. The dilutions were tested in different runs in replicates. The results were analyzed by probit method.

11.2. Reproducibility

Reproducibility data (on CT value basis) were obtained by the analysis of positive control of the Bosphore® Enterovirus Detection Kit v1. Test was performed in at least 4 replicates by 3 different operators, on multiple days, using 3 different lots. The resulting data is given in Table 1:

Table 1: Reproducibility Data.

Enterovirus (14 copies/µl)	Standard deviation	Variance	Coefficient of variation [%]
Intra-assay Variability N=4	0,045	0,002	0,143
Inter-lot Variability N=3	0,052	0,003	0,165
Inter-operator Variability N=3	0,127	0,016	0,400
Total Inter-assay Variability N=5	0,111	0,012	0,351

11.3. Cross-Reactivity

To eliminate potential cross-reactivity, both assay design evidence and experimental studies were employed. Primer and probe sequences were checked for possible homology to other known pathogen sequences by sequence comparison analysis using database alignment. Samples of HIV, HCV, HDV, HBV, JCV, BKV, West Nile Virus, Dengue Virus with known high positivity were tested, and found negative.

12. REFERENCES

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13. SYMBOLS

 Use by

 Lot/Batch

 Catalog number

 Temperature limitation

 Caution, consult accompanying documents

 Manufacturer

 In Vitro Diagnostic Medical Device

14. CONTACT INFORMATION

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