

bosphore[®]

FV H1299R Detection Kit v1 USER MANUAL

For *in vitro* Diagnostic Use

Anatolia[®]
geneworks

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1. PRODUCT DESCRIPTION

Bosphore® FV H1299R Detection Kit v1 detects the A4070G mutation (H1299R a change of histidine to arginine at position 1299) of the F5 gene in human biological samples. Wild type F5 allele is amplified and fluorescence detection is accomplished using the HEX filter. Mutant F5 allele is amplified and fluorescence detection is accomplished using the FAM filter.

2. CONTENT

Bosphore® FV H1299R detection Kit v1 is composed of Real-Time PCR reagents.

Component	REAGENT	100 Reactions	50 Reactions	25 Reactions
1	dH ₂ O	(1000 µl)	(1000 µl)	(500 µl)
2	PCR Mix	(1100 µl)	(550 µl)	(275 µl)
3	Detection Mix 1	(110 µl)	(55 µl)	(28 µl)
4	Positive Control	(88 µl)	(44 µl)	(22 µl)

3. STORAGE

Bosphore® FV H1299R 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 component should not be exposed to light and air more than necessary, vials must be kept closed except during pipetting. We recommend preparing the PCR on a cooling block and keeping the detection mixes within a closed container.

The components maintain their stability until the expiry dates on the labels, if they are stored at 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 or strips
- Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Genomic DNA Whole Blood Kit/ Magrev® 24 and Magrev® Whole Blood Genomic DNA Extraction Kit/ Bosphore®

Genomic DNA Extraction Spin Kit (Anatolia Geneworks), or other high quality genomic DNA extraction kits/systems

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

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

Important!:

- The product should be delivered on dry ice. Check for presence of dry ice upon arrival.
- Check for the expiry dates on the box and tube labels, upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNase, RNase, pyrogen free micropipette tips with filters, and DNase, RNase, pyrogen free microcentrifuge tubes should be used.
- Before starting a test procedure, all components should be thoroughly thawed. After thawing, all components should be centrifuged briefly (spin-down for 3-5 seconds), and mixed well to ensure homogeneity prior to use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared, and they should be quickly returned to -20°C.
- PCR and nucleic acid isolation must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- All the biological wastes produced during the nucleic acid isolation step; including the blood samples and material contacted with them, should be discarded into medical waste and disposed safely.

6. PRODUCT USE LIMITATIONS

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

7. MUTATION

FV His1299Arg mutation, is one of the 13 polymorphisms that occur within the HR2 which is a complex haplotype of the FV (F5) gene, and is located in exon 13. It is caused by the adenine-guanine polymorphism in the 4070th nucleotide of the gene. The single base change results in the conversion of histidine to arginine in the 1299th amino acid. This change and the R2 haplotype leads to an increase in both the Protein C resistance and the risk of venous thrombosis, and affects plasma F5 levels. This condition results in an increased tendency for developing blood clots. [1], [2]

Factor V H1299R polymorphism which is one of the reasons of Thrombophilia, is observed frequently (0.08-0.21) in healthy individuals especially in Italy, India, Somalia and Turkey. The prevalence of the R2 haplotype in Asia, Europe and Africa is between 5% and 17% [3]. Prevalence of the mutation within Turkish population was reported as 8.5%[4].

8. METHOD

Bosphore® FV H1299R Detection Kit v1 is based on the Real-Time PCR method. 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. 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 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 test utilizes **the 5' exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent hydrolysis probe during the extension phase. The probe is labeled at the 5' end with a fluorescent reporter, 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, even if the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step, Taq Polymerase encounters and cleaves the probe bound to the template. As the reporter is relieved from the suppressing effect of the quencher, fluorescence signal can be detected.

As the PCR product accumulates, the fluorescence generated by the reporter increases. The point at which the signal rises above background level and becomes detectable, is called the threshold cycle (C_T).

Bosphore® FV H1299R Detection Kit v1 employs multiplex PCR. F5 DNA, whether wild-type or mutant, is amplified in a single reaction, using sequence-specific primers against mutant and wild-type alleles. The fluorescent signal generated by the mutant-type F5 gene amplification is detected by a probe labeled at the 5' end with FAM, through the FAM channel. In contrast, the fluorescent signal generated by the wild-type allele 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. DNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Genomic DNA Whole Blood Kit/ Magrev® 24 - Magrev® Whole Blood Genomic DNA Extraction Kit/ Bosphore® Genomic DNA Extraction Spin Kit (Anatolia Geneworks) isolation system is used with Bosphore® FV H1299R Detection Kit v1. **The DNA isolation should be performed according to the manufacturers' instructions.**

9.2. Kit Components

9.2.1. PCR Mix

HotStarTaq DNA Polymerase: HotStarTaq DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*, cloned into E.Coli. The enzyme is provided in an inactive form. It is activated by a 15-minute 95 °C incubation step. This prevents the formation of misprimed products and primer-dimers during reaction setup and the first denaturation step, leading to high PCR specificity and accurate quantification. PCR Buffer: contains Tris-Cl, KCl, $(NH_4)_2SO_4$, 8 mM $MgCl_2$, pH 8.7 (20°C). dNTP Mix: Contains ultrapure quality dATP, dGTP, dCTP ve dTTP/dUTP.

9.2.2. Detection Mix 1

Detection Mix 1 contains F5 gene -specific forward and reverse primers and two dual-labeled probes against wild-type and mutant F5 allele.

9.2.3. Positive Control

The positive control contains heterozygous F5 gene containing one copy of the wild-type and mutant F5 allele each. It should be included in the PCR to efficiently test whether the samples being analyzed are positive or negative.

9.3. Preparing the PCR

Positive control should be added into the PCR reaction together with the samples and the negative control (PCR-grade water). 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 3 samples, an extra 10% should be added to the total sample number.

PCR Mix	10 µl
Detection Mix 1	1 µl
dH ₂ O	4 µl
Sample DNA	
Positive/Negative Control	5 µl
Total Volume	20 µl

Pipette 15 µl of the master mix into the PCR tubes or strips, and add 5 µl of DNA (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® FV H1299R Detection Kit v1 is composed of 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.

Initial denaturation	95°C	14:30 min.	
Denaturation	95°C	00:30 min.	} 35 cycles
Annealing and Synthesis (Data Collection)	68°C	00:20 min.	
Hold	22°C	1:00 min.	

Before starting 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.

10. ANALYSIS

By the end of the thermal protocol, the Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold. For Montania® 483 and Montania® 484, crosstalk calibration option from the settings menu must be selected and the values must be entered as follows:

		Channel 1/FAM	Channel 2/HEX
FVH1299R	Channel 1/ FAM	-	7,0 to 10,0
	Channel 2/ HEX	7,0 to 10,0	-

For Montania® 4896, crosstalk calibration option from the settings menu must be selected and the values must be entered as follows:

		Channel 1/FAM	Channel 2/HEX
FVH1299R	Channel 1/ FAM	-	3,0 to 5,0
	Channel 2/ HEX	3,0 to 5,0	-

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

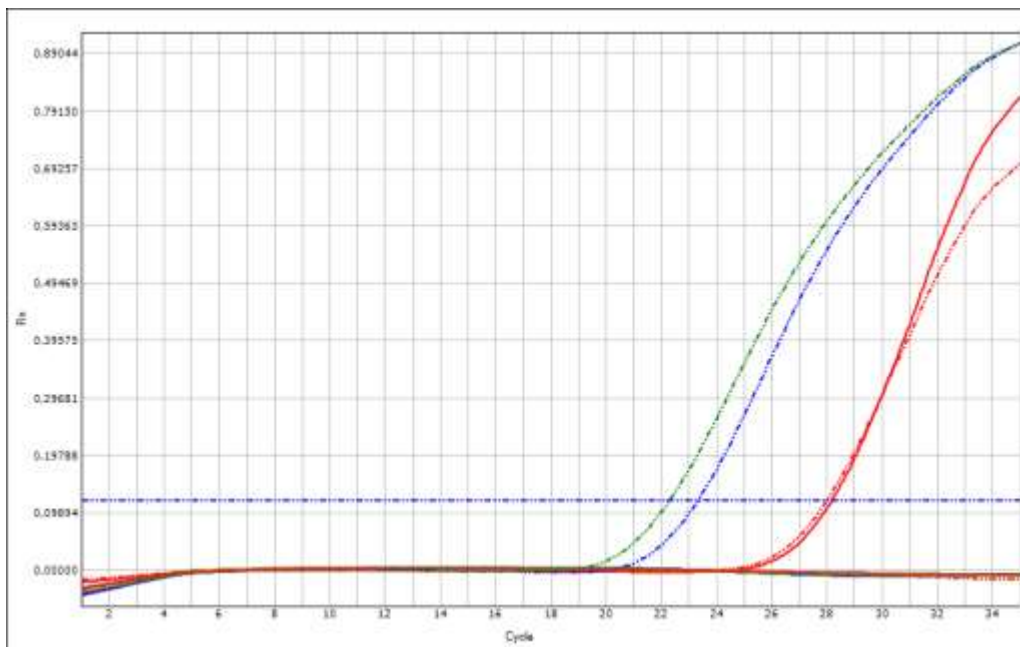


Fig. 1: Amplification Curve of a Bosphore® FV H1299R detection Detection v1 Test

Analysis of the results should be performed by trained personnel who have received the required training for analyzing Real-Time PCR data. We recommend that the test results must be **evaluated by an expert clinician, taking the patient's clinical findings** and the results of other tests into consideration.

Amplification should be observed from both FAM and HEX filters of the heterozygous positive control during the test. The amplification of both filters from the tested samples should be compared with that of the positive control. Test results should not be reported unless the assay results meet the criteria stated above. Please contact the manufacturer if an impairment in the **product's performance is observed (See the last page for contact information)**.

The qualitative **results of the test are displayed on the "Report Mode" screen. The samples that cross the threshold in FAM channel and HEX channel are displayed as positive, for mutant and wild-type alleles respectively.**






The following table shows the possible results and their interpretation:

FV H1299R mutant (FAM)	FV H1299R wild type(HEX)	Result
+	+	FV H1299R positive-heterozygous mutant
-	+	FV H1299R negative-homozygous wild type
+	-	FV H1299R positive-homozygous mutant
-	-	Test must be repeated!

11. REFERENCES

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12. SYMBOLS

-  Use by
-  Lot/Batch
-  Catalog number
-  Temperature limitation
-  Caution, consult accompanying documents



Manufacturer



In Vitro Diagnostic Medical Device

13. CONTACT INFORMATION



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