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USER MANUAL

ASFV Detection Kit v2

For Research Use Only

MB558v3f
23rd May 2024



Anatolia
geneworks

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1. PRODUCT DESCRIPTION

Bosphore ASFV Detection Kit v2 is a Real-Time PCR kit for research use that detects and characterizes the major capsid protein p72 gene of African Swine Fever Virus (ASFV) in samples including tissue (including tonsils, spleen, lymph nodes, kidneys, liver, lungs, bone marrow), swab, serum, and whole blood (with EDTA) from pigs and wild boars. Fluorescence detection is performed using FAM and HEX filters. ASFV DNA is amplified, and fluorescence detection is performed using the FAM filter.

Component	FAM (gene)	HEX (gene)
PCR Master Mix	ASFV (major capsid protein p72 gene)	Internal Control (GAPDH)

An internal control based on the detection of swine endogenous nucleic acid sequence (GAPDH) present in the swine genome has been employed in order to check DNA extraction, PCR inhibition, and sampling or application errors. Internal control is detected by the HEX channel.

2. CONTENT

Bosphore ASFV Detection Kit v2 consists of the following dH₂O, Real-Time PCR Master Mix, and positive control.

Component	Reagent	100 Reactions	50 Reactions	25 Reactions
1	dH ₂ O	(1000 µL)	(1000 µL)	(1000 µL)
2	PCR Master Mix	(1650 µL)	(830 µL)	(415 µL)
3	Positive Control	(88 µL)	(44 µL)	(44 µL)

3. STORAGE

The PCR reagents for the Bosphore ASFV Detection 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 Cyclers - Quantabio, Rotor-Gene Q – QIAGEN, Mx3000P-Agilent or another Real-Time PCR system with FAM and HEX filters*
- 0.2 mL thin-wall PCR tubes, PCR plates or strips
- UNIO B24 Extraction Systems and UNIO Whole Blood Genomic DNA Extraction Kit or UNIO Viral DNA/RNA Extraction Kit, Magrev 24 Stand and Magrev Viral DNA/RNA Extraction Kit or Magrev Whole Blood Genomic DNA Extraction Kit or Magrev Tissue Genomic DNA Extraction Kit, UNIO 96 Extraction and PCR Setup System and UNIO 96 Nucleic Acid Extraction Versatile Kit, Magnesia 16 Nucleic Acid Extraction System and Magnesia Genomic DNA Whole Blood Extraction Kit or Magnesia Viral Nucleic Acid Extraction Kit, Bosphore Nucleic Acid Extraction Versatile Spin Kit (Anatolia Geneworks) or other high-quality DNA extraction kits and systems
- Deep freezer (-20 °C)
- Desktop centrifuge with rotor for 2 mL or 1.5 mL microcentrifuge tubes
- DNase, RNase, pyrogen-free 1.5 mL or 2 mL microcentrifuge tubes
- Calibrated, adjustable micropipettes
- DNase, RNase, pyrogen-free micropipette tips with filters
- Disposable laboratory gloves

* For other Real-Time PCR devices that can be used with Bosphore ASFV Detection 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 °C.
- PCR and nucleic acid extraction must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- Pathogen information should be reviewed to be aware of the health-related risks.
- Biological samples should be handled with extreme caution and in a microbiological safety cabinet of the appropriate class. Physical contact with pathogens should be avoided by wearing lab coats and gloves, making no allowance for eating or drinking within the workspace, and preventing unauthorized individuals' access to the working area.
- All the pathogenic wastes produced during the nucleic acid extraction step, including the serum and plasma samples and materials contacted with them, should be discarded into medical waste and disposed of safely.

6. PRODUCT USE LIMITATIONS

- This product is for research use only.
- This product should be used in accordance with this user manual, by personnel specially trained to perform molecular biology laboratory techniques.

7. INFECTION

Causative Agents

African Swine Fever Virus (ASFV) which belongs to the family '*Asfarviridae*' is the only virus with a double-stranded DNA genome known to be transmitted by arthropods. It replicates itself within the cytoplasm of infected cells and causes African Swine Fever (ASF) in the swine population. It is a serious disease that can result in widespread outbreaks among the swine population (Sánchez-Cordón, 2018).

ASF has some symptomatic signs such as high fever, depression, anorexia and loss of appetite, hemorrhages within the skin (redness of skin on ears, abdomen, and legs), abortion in pregnant sows, cyanosis, vomiting, diarrhea, and death, which is the final stage, are seen. Death occurs within 6-13 days with mortality rates of up to 100% (World Organization for Animal Health, 2023).

Epidemiology

ASFV is widespread in Asia, China, and an enormous part of Europe (Yang et al., 2023). In 2018, the ASFV had spread in Asia, and affected over 10 percent of the entire swine population, leading to severe economic losses within the pig sector (Food and Agriculture Organization of the

United Nations, 2023). Most of the countries in the European Economic Community affected by this epidemic must act and manage against this ASF virus, which is having a negative and devastating impact on the swine industry.

Modes of Transmission

ASFV is, a contagious viral infection among pigs and wild boar and it is a highly contagious hemorrhagic viral disease. The virus can be spread by ticks, but also by swine eating pork products that contain the virus (Frant et al., 2017). The National Pig Association, states that the virus can also be transmitted by direct or indirect contact with infected pigs, feces, or body fluids (National Pig Association, 2023).

8. METHOD

Bosphore ASFV Detection 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 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 ASFV Detection Kit v2 uses multiplex qPCR and endogenous internal control incorporated into the system in order to check for check PCR inhibition, DNA extraction, sampling, or application errors.

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

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

9. PROCEDURE

9.1. DNA Extraction

We recommend that UNIO B24 Extraction Systems and UNIO Whole Blood Genomic DNA Extraction Kit or UNIO Viral DNA/RNA Extraction Kit, Magrev 24 Stand and Magrev Viral DNA/RNA Extraction Kit or Magrev Whole Blood Genomic DNA Extraction Kit or Magrev Tissue Genomic DNA Extraction Kit, UNIO 96 Extraction and PCR Setup System and UNIO 96 Nucleic Acid Extraction Versatile Kit, Magnesia 16 Nucleic Acid Extraction System and Magnesia Genomic DNA Whole Blood Extraction Kit or Magnesia Viral Nucleic Acid Extraction Kit, Bosphore Nucleic Acid Extraction Versatile Spin Kit (Anatolia Geneworks) or other high-quality extraction kits and systems are used with Bosphore ASFV Detection Kit v2. The DNA extraction should be performed according to the manufacturer's instructions.

9.2. Kit Components

9.2.1. 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 African Swine Fever virus and endogenous internal control.

9.2.2. Internal Control

The internal control (IC), also known as the internal sample control, is designed to detect a specific swine endogenous nucleic acid sequence (GAPDH) present in swine clinical samples. The kit includes primers and probes specifically for this purpose. The IC serves multiple functions, including acting as a control for DNA extraction, PCR inhibition, and application errors.

In samples, the absence of amplification of the internal control in the target channels may indicate potential issues with extraction, PCR inhibition, sampling, or application errors. If this occurs, it is recommended to repeat the steps involving extraction, sample collection, and PCR.

To evaluate internal control amplification, please refer to the table provided in Section 10.

9.2.3. Positive Control

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

Positive Control: Synthetic ASFV 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 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.

Components	Volume
PCR Master Mix	15 μ L
Sample DNA (Negative / Positive Control)	5 μ L
Total Volume	20 μ L

Pipette 15 μ L of the PCR Master Mix into the PCR tubes or strips and add 5 μ L of DNA (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 ASFV Detection Kit v2 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 the next page.

Steps	Temperature	Time	
Initial Denaturation	95 °C	10:00 min	
Denaturation	97 °C	00:20 min	} 40 Cycles
Annealing (Data Collection)	58 °C	00:30 min	
Hold	32 °C	02:00 min	

Before starting to work with Bosphore ASFV Detection 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,
- 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 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 in FAM and HEX filters. If the negative control has a signal in FAM and HEX filters, 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 ASFV Detection 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 Control	≤30
Internal Control	≤35

In qualitative 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, sampling, 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, also in consideration of the clinical findings, and the guidelines of the relevant animal health authorities. The table below shows the possible results and their interpretation:

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

In rare cases of PCR inhibition due to medication or other PCR inhibitors in the sample, we recommend repeating the test of inhibited samples, by freezing and thawing the DNA samples and using them in the PCR after diluting them 1:2 with dH₂O.

Light Cycler 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 Universal CC FAM (510)-VIC (580) 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 **10%** if necessary. It is recommended to use the "Gain" settings as **10** for **FAM** and **HEX** filters.

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.

The analytical detection limit for Bosphore ASFV Detection Kit v2 was found to be 8 copies/reaction. The sensitivity was determined using serial dilutions of with the quantitated synthetic ASFV 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; SARS-CoV-2, Influenza H1N1 viruses also bovine, donkey, horse, chicken, and turkey samples with known high positivity were tested and found to be negative. The experimental results indicated that the kit detects specifically and only ASFV pathogen 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 synthetic ASFV DNA. 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 833 copies/reactions.

Variability (ASFV)	Standard Deviation	Variance	Coefficient of Variation [%]
Intra-assay (n=4)	0.04761	0.00227	0.18048
Inter-lot (n=3)	0.16927	0.02865	0.64126
Inter-operator (n=3)	0.05375	0.00289	0.20376
Total Inter-assay (n=5)	0.12586	0.01584	0.47692

12. VALIDATION STUDY

Bosphore ASFV Detection Kit v2 has been validated by The European Union ASF reference laboratory (EURL-ASF) Centro de Investigación en Sanidad Animal (CISA-INIA).

Validation of the Bosphore ASFV Detection Kit v2 was performed with 552 reference samples. 255 ASFV-positive samples were obtained affected ASF European countries and 92 blood samples from domestic pigs in Spain served as negative samples for diagnostic specificity assessment.

HOST	Affected ASF Europea countries	Free ASF European countries	Total
Domestic pig	146	92	238
Wild boar	314	0	314
			552

A total of 460 field samples from both domestic pigs and wild boars collected in European countries affected by genotype II ASFV between 2018 and 2022. These samples included 185 tissue samples, 144 EDTA-blood samples, and 131 serum samples. Among the tissues tested, the distribution was as follows: 72 spleen samples, 34 bone marrow, 31 kidney samples, 16 lymph nodes, 14 lung samples, 4 tonsil samples, 4 liver and smaller numbers for tonsils, joint tissue, muscle, skin and heart sample.

Additionally, a set of 92 blood samples from domestic pigs in Spain served as negative samples for diagnostic specificity assessment.

The Bosphore ASFV Detection Kit v2 demonstrated a diagnostic specificity of 100% and diagnostic sensitivity of 94,7%. And also The Kappa value (κ) of 0.87 [95% CI] suggests almost perfect agreement between the UPL reference method and the Bosphore ASFV Detection Kit v2.

Analytical sensitivity was initially assessed by analyzing 132 experimental EDTA-blood and 32 tissue samples from domestic pigs experimentally infected with ASFV isolates belonging to diverse genotypes, including I, II, IX, X, and XXIII.

Out of the 132 blood samples, the Bosphore ASFV Detection Kit v2 identified ASFV in **118** of them, accounting for **89.4%** of the samples. This information reflects the analytical sensitivity of testing method and demonstrate its ability to detect ASFV in a significant proportion of the experimental blood samples collected from infected pigs with diverse ASFV isolates and genotypes. The results showed a high level of agreement, with a κ index of **1 (95% CI)**. 23 positive samples were detected from 32 samples, thus ASFV genome detection was achieved in 90.6% of the tissue samples.

To assure the detection range of the real time PCR kit, an initial evaluation was carried out by the analysis of a collection of 23 ASFV reference DNAs belonging to 22 genotypes. The PCR Bosphore ASFV Detection Kit v2 was able to detect the **22 genotypes**.

For Validation and Assesment Reports please contact Anatolia Geneworks from the information in Section 16.

13. REFERENCES

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4. *Food and Agriculture Organization of the United Nations* (9 August 2019). “One year on, close to 5 million pigs lost to Asia's swine fever outbreak” <https://www.fao.org/news/story/en/item/1204563/icode/#:~:text=FAO%20urges%20Asian%20countries%20to,one%20year%20ago%20this%20month.>
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14. SYMBOLS

Use-by Date



Caution, consult accompanying documents.



Batch Code



Manufacturer



Catalog Number



Research use only

15. ORDERING INFORMATIONCatalog Number:**ABASF6 (100 rxn/box)****ABASF5 (50 rxn/box)****ABASF4 (25 rxn/box)****16. CONTACT INFORMATION****Anatolia Tanı ve Biyoteknoloji Ürünleri Araştırma
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Document Revision History

Document Version No	Revision No	Date	Description
V1	01	28 th June 2022	First Publishing
V2	02	23 rd November 2023	The general content and type check
V3	03	23 rd May 2024	The content has been updated and checked