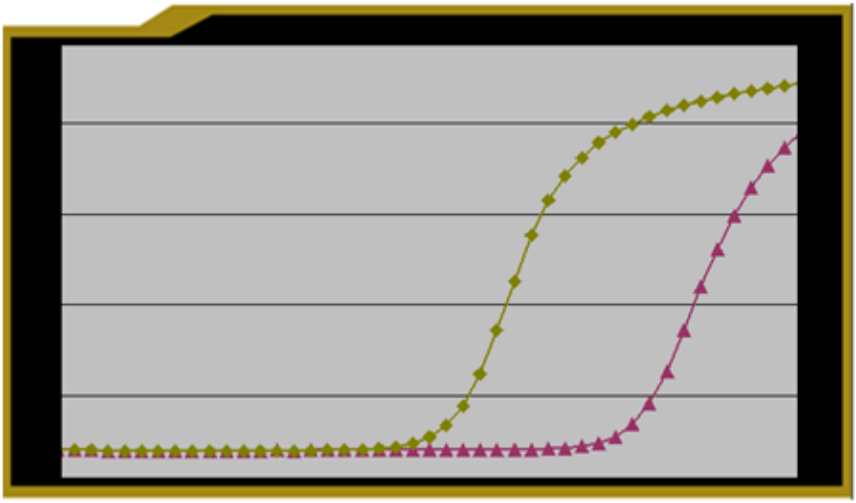
African Swine Fever Virus DNA Test Kit

VetAlert™ ASFV Real-Time PCR



A Diagnostic PCR Test Kit for the Qualitative Detection of African Swine Fever Virus DNA extracted from spleen and whole blood collected in EDTA from swine.

NAME AND INTENDED USE

The ASFV DNA Test Kit is intended for the rapid *in vitro* qualitative detection of African Swine Fever (ASF) viral DNA by real-time polymerase chain reaction (rPCR). The reagents should be used to detect viral DNA extracted from spleen and whole blood collected in EDTA from swine. The test is intended for use by veterinary or other laboratory scientists for the presumptive identification of ASFV. The test should be performed on the ABI 7500 or an equivalent real-time PCR instrument.

SUMMARY AND EXPLANATION

African Swine Fever (ASF) is a highly contagious and often fatal disease of swine. It affects domestic and wild pig populations. ASF Virus (ASFV), the causative agent of ASF is a double-stranded DNA virus and a member of the genus Asfivirus belonging to the Asfarviridae family. It is transmitted through direct and indirect contact. Transmission can also happen through certain tick vector species (soft ticks) therefore ASFV is an Arbovirus.

PRINCIPLES OF THE PROCEDURE

The ASFV PCR test is a real-time PCR method that utilizes a specific set of forward and reverse primers and fluorogenic probe hydrolysis chemistry for the detection of ASF viral DNA in samples. The test has specific sets of oligonucleotide primers and FAM probes that target the VP72 gene of the ASFV. The assay also includes specific oligonucleotide primers and Cy5 probe for the detection of an Inhibition Control (IC). In order to harmonize the PCR mastermix and cycling conditions between USDA licensed products that include both DNA and RNA viruses, the cycling conditions include a reverse transcription (RT) step, and the IC is an in vitro transcript (RNA). The RT step does not impact the performance of the ASFV DNA Test Kit, and the IC performs as expected for either DNA or RNA assay targets.

PRODUCT DESCRIPTION

ASFV DNA Test Kit

Materials Provided: Single-Unit Kit

Component	Contents
Mastermix	6 Tubes of ASFV Mastermix (16 reactions each)
Enzyme	1 Tube of Enzyme
Positive Control	1 Tube of ASFV Positive Control
Inhibition Control	1 Tube of ASFV Inhibition Control
Product Insert	1 Product Insert

Materials Provided: 10-Unit Kit

Box	Component	Contents
1	Mastermix	60 Tubes of ASFV Mastermix (16 reactions each)
	Enzyme	10 Tube of Enzyme
2	Positive Control	10 Tubes of ASFV Positive Control
	Inhibition Control	10 Tubes of ASFV Inhibition Control
	Product Insert	1 Product Insert

Materials and Equipment Required, but Not Provided:

- DNA extraction materials
- Real-time PCR instrument: *e.g.* ABI 7500
- Micropipettes and sterile pipette tips with aerosol barriers
- 1xTE Buffer (10mM Tris-HCL, pH 8.0; 1mM EDTA) or molecular biology grade water, for use as a “no template” control (NTC)

STORAGE AND STABILITY

Store the Mastermix and Enzyme between -15°C and -25°C. Avoid repeated freeze-thaws of the reagents as this can negatively affect their performance.

Store the Inhibition Control (IC) and the Positive Control (PC) between -15°C and -25°C. To avoid repeated freeze-thaws and subsequent degradation of the IC and PC:

a. Once thawed, make small working aliquots (≥ 20 µL per tube). Use 0.5 mL sterile microcentrifuge tubes with O-rings to avoid evaporation. Store both the PC and IC aliquots between -15°C and -25°C. Limit each aliquot to no more than 2 freeze-thaws.

PRECAUTIONS

Real-time PCR is an extremely sensitive method for amplifying and detecting small quantities of DNA. Due care should be taken to prevent any carryover contamination from previous PCR amplifications coming in contact with the reagents and positive control components in the kit.

Recommendations to prevent carryover contamination:

- Store and extract positive or suspected positive material (such as test specimens, positive extraction controls, or previously amplified material) separately from all PCR kit components, e.g., in separate rooms.

- Prepare and set-up the PCR Mastermix in a BSL-2 cabinet in a DNA/RNA-free room.

- Add extracted DNA and Positive Control to reaction tubes or wells in a room separate from the Mastermix set-up.

- Use sterile pipette tips with aerosol barriers to avoid potential sample-to-sample contamination.

- Proceed with testing immediately after the addition of Enzyme to the Mastermix. Do not allow the Mastermix to sit for prolonged periods before use.

LIMITATIONS

This test is used for testing extracted DNA from spleen and whole blood collected in EDTA from swine. No claims are made for other samples or for organisms derived from other animal species.

SPECIMEN COLLECTION AND STORAGE

Materials should be processed quickly and maintained as cold as possible without freezing during transport and laboratory accessioning to prevent loss of any ASF viral DNA that might be present. DNA must be extracted using standard methods before testing.

The choice of the nucleic acid extraction method is determined by laboratories authorized to perform ASFV testing. Extraction methods that are known to be effective for purifying DNA from ASFV include: The Qiagen QIAamp Viral RNA Mini Kit (1,2), and the Thermo Fisher Scientific MagMAX™ Pathogen RNA/DNA Kit (3).

1. Elnagar A, Pikalo J, Beer M, Blome S, Hoffmann B. Swift and Reliable "Easy Lab" Methods for the Sensitive Molecular Detection of African Swine Fever Virus. Int J Mol Sci. 2021;22(5):2307. Published 2021 Feb 25. doi:10.3390/ijms22052307.
2. Haines FJ, Hofmann MA, King DP, Drew TW, Crooke HR. Development and validation of a multiplex, real-time RT PCR assay for the simultaneous detection of classical and African swine fever viruses. PLoS One. 2013;8(7):e71019. Published 2013 Jul 26. doi:10.1371/journal.pone.0071019.00.
3. NVSL SOP-DS-0071. Preparation, Performance, and Interpretation of the African Swine Fever rPCR Assay on the Applied Biosystems 7500 Real-time PCR System.

POSITIVE AND NEGATIVE CONTROLS

One PC and one “no template” control (NTC) reaction should be included with each run. The NTC is not provided, however it is recommended that a 1xTE Buffer (10mM Tris-HCL, pH 8.0; 1mM EDTA) or molecular biology grade water be used as a NTC.

Procedures

1. Preparing the Mastermix:

   a. Determine the number of reactions required.

      Note: To ensure that there is enough Mastermix prepared, an additional reaction should be added to the number of reactions required.

   b. Option A: IC is used as an extraction control

      Note: Positive and NTC amplification controls must follow Option B.

      Note: Results for the IC used as an extraction control will vary. Each laboratory must validate their own IC performance range based on the Negative Extraction Control, PCR instrument platform, and extraction procedure used.

      i. Incorporate the IC into the DNA extraction process by adding 6.0 µL per sample of the IC to the extraction kit's lysis buffer (extraction kit is not provided). The product at the end of the extraction process will contain the extracted IC.

      ii. If less than 16 reactions are required, use the following table to calculate the volume of Mastermix and Enzyme needed.

	1 reaction
Mastermix	19.25 µL
Enzyme	0.75 µL
Total Mastermix volume	20.00 µL

      iii. If 16 reactions are required, one Mastermix vial (16 reactions per vial) can be prepared by adding 13.5 µL of Enzyme directly to the reagent vial.

      iv. Remove Mastermix vial(s) from the freezer. Thaw at room temperature. Once thawed, keep the Mastermix cold at all times. Gently vortex and briefly centrifuge the vials before use.

      v. Remove Enzyme vial from the freezer and briefly centrifuge (do not vortex). Return Enzyme vial to the freezer immediately after use.

Important: Once the Enzyme is added to the Mastermix, the prepared Mastermix must be kept on ice and used within 2 hours.

vi. Continue to Step 2.

c. Option B: IC is used as an inhibition control for the PCR reaction

i. If less than 16 reactions are required, use the following table to calculate the volume of Mastermix, IC, and Enzyme needed.

1 reaction

Mastermix	19.00 µL
IC	0.25 µL
Enzyme	0.75 µL
Total Mastermix volume	20.00 µL

ii. If 16 reactions are required, one Mastermix vial (16 reactions per vial) can be prepared by adding 4.5 µL of IC and 13.5 µL of Enzyme directly to the Mastermix vial.

iii. Remove Mastermix vial(s) and the IC vial(s) from the freezer. Thaw at room temperature. Once thawed, keep the Mastermix and IC cold at all times. Gently vortex and briefly centrifuge the vials before use.

iv. Remove Enzyme vial from the freezer and briefly centrifuge (do not vortex). Return Enzyme vial to the freezer immediately after use.

Important: Once the Enzyme is added to the Mastermix, the prepared Mastermix must be kept on ice and used within 2 hours.

v. Continue to Step 2.

2. Aliquot 20.0 µL of the prepared Mastermix into each PCR reaction tube or well of a 96-well plate.

Note: Keep the Mastermix cold at all times to ensure optimal performance of the reagents. Use cold blocks for PCR tubes or for 96-well plates.

3. Prepare the negative control, extracted sample, and positive control reactions.

Note: Always prepare and cap the negative control first before handling either an extracted sample or the positive control.

a. Negative Control:

Add 5.0 µL of the negative control (not provided) to a PCR reaction tube or well that contains 20.0 µL of the prepared Mastermix.

Note: Molecular biology grade water or 1X Tris-EDTA (pH 8.0) can be used as a negative control.

b. Extracted Sample(s):

Add 5.0 µL of the extracted sample to a PCR reaction tube or well that contains 20.0 µL of the prepared Mastermix.

c. Positive Control:

Add 5.0 µL of the ASF Positive Control to a PCR reaction tube or well that contains 20.0 µL of the prepared Mastermix.

4. Cap and briefly centrifuge the PCR reaction tube(s) or 96-well plate.

Note: After centrifuging, ensure that all of the mix is at the bottom of each tube or well. Centrifuge again if needed.

5. Load the PCR reaction tube(s) or 96-well plate into the appropriate real-time PCR instrument and follow the thermal cycling protocol below.

Real-Time PCR Set Up:

The assay utilizes different reporter dyes to distinguish between targets (see table below). Select the appropriate channel on the desired real-time PCR instrument to view the amplification signal for each target.

Target	Reporter Dye
ASFV	FAM
IC	Cy5 equivalent

Please contact Tetracore with any questions regarding instrument compatibility and analysis settings.

THERMAL CYCLING PROTOCOL:
Stage 1: 48ºC for 15 minutes
Stage 2: 95ºC for 2 minutes
Stage 3: 45 cycles (2-step PCR):
Step 1: 95ºC for 10 seconds
Step 2: 60ºC for 40 seconds (collect data step)

REACTION VOLUME: 25 µL

ESTIMATED RUN TIME: 1 hour and 26 minutes (on the ABI 7500 instruments. Time may vary depending on instrument used).

ABI 7500 Settings:
Use ABI 7500 software version 1.4 or 1.5.

- If using the ABI 7500 Fast System, select Run Mode: Standard 7500.
- Select ROX as Passive Reference Dye.
- Assay: Standard Curve (Absolute Quantification)
- ASFV Target: Select detector for FAM as reporter dye and None as quencher.
- Inhibition Control: Select detector for Cy5 as reporter dye and None as quencher.
- Sample Volume: 25 µL
- Data Collection: 60°C for 40 seconds step.

Analysis Settings for the ABI 7500®

- When the run is complete, view each well individually to verify true amplification.
- Select the Amplification Plot tab to view the amplification curves. From the Tools menu, click on Graph Settings. Change Post Run Settings to Linear Y-axis and select Auto Scale.
- Use Auto Baseline for initial analysis. If necessary, adjust baseline manually for each channel.
- For the FAM channel, set Threshold at 3% of the final normalized fluorescence of the Tetracore Positive Control amplification curve.
- For the Cy5 channel, set Threshold at 3% of the final normalized fluorescence of the IC amplification curve for the NTC.

  Example: In the FAM channel, the final PC fluorescence at cycle 45 is approximately 1.8 and 3% of 1.8 is 0.054. Therefore, set the threshold for the FAM channel at 0.054. Round to the nearest hundredth or thousandth as appropriate.

  Important: For all PCR instruments, Follow the instrument user guide for data analysis to verify that all positive PCR signals are true amplification signals with, at the minimum, a positive inflection point.

Verify the following prior to interpretation of the results:

1. The amplification curve of the Positive Control provided has a FAM cycle threshold (Ct) value ≤ 28.
2. The NTC does not cross the FAM threshold and remains negative through PCR cycle 38.
3. The NTC reaction(s) must have a Cy5 Ct value ≤ 34.

Interpretation of the results

The test is a fluorogenic probe hydrolysis assay that produces a characteristic fluorescent signal with a positive test. The ideal shape of a positive amplification curve is visualized on a linear scale as a sigmoidal curve on a two-dimensional linear grid where the x-axis represents the PCR cycle number and the y-axis represents the relative fluorescence of

the signal. True positive amplification signals must have, at a minimum, a positive inflection point.

1. A FAM Ct value is present:

    - The result is positive for ASFV if it has a FAM Ct ≤ 38

    - Here, a Cy5 Ct value may be dispensable, because very high concentrations of ASFV DNA may compete with the IC, producing a late Cy5 Ct value or no Cy5 Ct value at all.

    - Positive samples must be confirmed by USDA-APHIS authorities.

2. A positive Cy5 (IC) Ct value and a FAM Ct of > 38:

    - This is a Negative Result.

    - If the IC is used as an Extraction Control (option A), PCR inhibition may be present if the IC Ct value is outside of the validated range. If PCR inhibition is present, it is recommended that DNA extraction be repeated, or repeat the test with a diluted sample (e.g. 1:5 dilution with molecular biology grade water).

    - If the IC is used as an Inhibition Control (option B), PCR inhibition may be present if the IC Ct value is >34. If PCR inhibition is present, it is recommended that DNA extraction be repeated, or repeat the test with a diluted sample (e.g. 1:5 dilution with molecular biology grade water).

3. Neither FAM nor Cy5 Ct values are present:

    - The test is not valid and no diagnosis is possible. The test should be repeated.

    - Please contact Tetracore with any questions regarding the use or interpretation of this test. 1-240-268-5400.

Symbol Key

Symbol	Meaning	Symbol	Meaning
	Serial number		Expiration Date
	Consult Instructions for Use		Manufacturer
	Contains Sufficient for <n> Tests		Temperature Limit

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VLN/PCN 644/57A5.80

Revision: 08 June 2022
Part number: PLM-0114-6