Date of the manual: 16.11.2022

For laboratory/veterinary use only, for *in vitro* diagnostic

Triplex ASFV Fast qPCR Kit

Kit contents

Składnik	Cat. no. E0472-01 24 reactions of 25 µl	Cat. no. E0472-02 96 reactions of 25 µl
Triplex ASFV Fast Master Mix	1 x 480 µl	4 x 480 µl
PC — positive control black cap	1 x 40 µl	1 x 150 µl
NC — negative control clear cap	1 x 40 µl	1 x 150 µl
Ex-DNA blue cap	1 x 120 µl	1 x 480 µl

Storage:

The kit should be stored in the dark at −20° C. Avoid repeated thawing and freezeing (>2x), due to possible reduced sensitivity.

Description

- Triplex ASFV Fast qPCR Kit is intended for the detection of DNA from African swine fever virus (ASFV) in blood, serum, plasma, swab samples (from mouth, nose, nasopharynx), tissues and organs of pigs and wild boars.
- The high sensitivity of the kit allows the detection of the ASF virus in both single samples and mixed (pooled) samples consisting of a maximum of 5 individual samples.
- Triplex ASFV Fast qPCR Kit allows to use a standard or fast PCR amplification program, depending on the user's preferences and the type of real-time PCR device used.
- The kit contains specific primers for the highly conserved fragment of the p72 gene of the ASF virus. The amplified p72 gene fragment is detected using a probe labeled with FAM dye.
- Additionally, the kit includes two internal controls, which contains:
  a. primers and a probe labeled with HEX dye for a fragment of the porcine ACTB gene encoding beta-actin. (endogenous control),
  b. primers and a probe labeled with Cy5 and exogenous fragment of DNA - Ex-DNA (exogenous control).

  Internal controls allow both extraction and amplification to be monitored. The use of an exogenous control allows for the additional assessment of sample quality and possible inhibition of the PCR reaction.
- Triplex ASFV Fast qPCR Kit allows the analysis of samples both with the use of the Ex-DNA (three-channel analysis: FAM, HEX, Cy5) and without it (two-channel analysis: FAM and HEX). However, it is highly recommended to perform analyzes using an exogenous internal control. To analyze samples this way, add Ex-DNA to the lysis buffer or to the collected sample at an amount of 0.1 µl for each 1 µl of elution volume. For example, when eluting with a 50 µl of elution buffer, 5 µl of Ex-DNA should be used in the DNA isolation process.
- DNA used for PCR-based diagnostics should be of good quality and purified using dedicated, commercial kits using silica or magnetic bead technology.
- Triplex ASFV Fast Master Mix contains all of the necessary reagents, including Perpetual Taq DNA polymerase, reaction buffer, deoxynucletide triphosphates (dNTPs), primers and probes combination and passive reference dye (ROX).
- Perpetual Taq DNA Polymerase is a modified "hot start" enzyme that is blocked at moderate temperatures by antibodies and allows room temperature reaction setup. The polymerase activity is restored during the initial 2-minutes denaturation step.
- The passive ROX reference dye included in the master mix enables fluorescence normalization in particular cyclers. The use of ROX dye is required for all Applied Biosystems real-time PCR cyclers and optional for Agilent cyclers. ROX compensates for differences in fluorescence signal between wells caused by small differences in reaction volume and fluorescence fluctuations. ROX is not involved in the PCR reaction and does not interfere with real-time PCR on any instrument.
- Positive control - PC contains all three DNA fragment: ASF virus, the porcine ACTB gene and exogenous contol Ex-DNA and serves to prove of the kit's operation, including the correct preparation of the PCR reaction. The fluorescence signal for ASF virus DNA in the FAM channel has CT= 29±2, for the ACTB gene in the HEX channel has CT= 29±2 and for the Ex-DNA in Cy5 channel is C_T= 28±3.

Equipment and reagents to be supplied by user

1. Pipets
2. Sterile pipet tips with filters
3. Sterile 1.5 ml tubes
4. PCR tubes, strip tubes or 96-well optical microplates
5. Lab coat, disposable gloves, protective goggles
6. Centrifuge with rotor for 1.5 ml tubes
7. Centrifuge with rotor/adapter for 96-well microplates
8. PCR cooler, mini lab cooler or ice
9. Real-time PCR cycler

Procedure

Preparation of PCR reaction:

Component	Negative Control 1 reaction	DNA Sample 1 reaction	Positive Control 1 reaction	Final concentration
Triplex ASFV Fast Master Mix	20 µl	20 µl	20 µl	1 x
PC—positive control	-	-	5 µl	
NC—negative control	5 µl	-	-	
Purified DNA sample	-	5 µl	-	
Volume	25 µl	25 µl	25 µl	

Notes:

1. A reaction volume of 25 µl should be used with most real-time cyclers. Other reaction volumes may be used if recommended for a specific instrument.
2. Thaw, gently vortex and briefly centrifuge all solutions. Keep the kit components on ice. Avoid multiple thawing and freezing (> 2x) of Triplex ASFV Fast Master Mix, as this may reduce the sensitivity.
3. Set up PCR reactions at room temperature. Use Triplex ASFV Fast Master Mix allows room temperature reaction setup.
4. Dispense 20 µl into PCR tubes or plates.
5. Add 5 µl of NC- negative control, 5 µl of the sample DNA or 5 µl PC-positive control to the individual PCR tubes or wells containing the reaction mix.
6. For every PCR run prepare at least 2 additional reactions: negative and positive controls. Due to the possibility of contamination, prepare the Negative Control first and the Positive Control last.
7. Centrifuge briefly to settle down the reaction components and remove bubbles. Bubbles interfere with fluorescent detection.
8. Place the samples in the cycler and start the program prepared according to the table below.
9. Perform data analysis.

Real-time PCR Protocol:

Step	Standard mode	Fast mode	Number of cycles
Initial denaturation	95°C 2 min	95°C 2 min	1
Denaturation	95°C 15 s	95°C 10 s	40
Annealing/Extention	60°C 60 s	60°C 30 s	

Notes:

1. Run PCR reactions using standard ramp rate (standard mode) or fast mode depending on the user's preferences and the type of real-time PCR device used.
2. During the initial denaturation step Perpetual Taq DNA Polymerase is activated. Polymerase requires 2 min incubation at 95°C to restore activity.
3. Fluorescence analysis in the FAM, HEX and Cy5 channels should be performed by the device at the end of the extension step. ASF virus DNA is detected in the FAM channel, porcine beta-actin gene DNA is detected in the HEX channel and Ex-

Data Analysis and Interpretation:

Sample type	FAM channel	HEX channel	Cy5 channel	Sample result
Negative control	-	-	-	correct
Positive control	+	+	+	correct
Sample 1	-	+	+	negative ($ASFV^-$)
Sample 2	+	+	+	positive ($ASFV^+$)
Sample 3	+	-	+	positive ($ASFV^+$)
Sample 4	+	+	-	positive ($ASFV^+$)
Sample 5	+	-	-	positive ($ASFV^+$)
Sample 6	-	-	+	poor sample quality/low amount of DNA
Sample 7	-	+	-/high C_T	Partial inhibition of PCR
Sample 8	-	-	-	incorrect

1. Results should be analyzed in the FAM, HEX and Cy5 channels (if Ex-DNA was used) or only in the FAM and HEX channels (if Ex-DNA was not used during DNA isolation).
2. For the assay to be valid the positive control must give a signal in both the FAM and HEX channels (two-channel analysis) or in the all three channels FAM, HEX, Cy5 (three-channel analysis). The Negative Control must give no signal.
3. The sample is positive for ASFV and the assay is valid if:
   - the cryteria in the second point are met,
   - the sample yields a signal in the FAM channel with any configuration of signals in the HEX/Cy5 channels.

   Exceptionally very high concentrations of ASFV DNA in the sample may lead to a reduced HEX/Cy5 signal or no HEX/Cy5 signal at all due to the competition with the internal control.
3. The sample is negative for ASFV and the assay is valid if:
   - the cryteria in the second point are met,
   - the sample yields a signal in only the HEX channel (two-channel analysis) or in both HEX and Cy5 channels (if Ex-DNA was used).
5. The lack of a signal at all in the FAM, HEX and Cy5 channel simultaneously may indicate complete PCR reaction inhibition or that no DNA was added to the test sample. In this case it is recommended to dilute 10 times the sample DNA or repeat the DNA extraction and run the PCR reaction again.
6. The lack of fluorescence signal in the Cy5 channel may be caused by:
   - ineffective DNA isolation,
   - competition effect: a strong signal in the FAM channel in the case of a high concentration of ASF virus DNA in the sample,
   - inhibition of the PCR reaction,
   - not adding Ex-DNA in the DNA isolation process.
7. High C_T values in the Cy5 channel may indicate partial PCR reaction inhibition. In this case it is recommended to dilute 10 times the sample DNA or repeat the DNA extraction and run the PCR reaction again.
8. The absence of a signal in the HEX channel and the simultaneous presence of a signal in the Cy5 channel indicates poor sample quality/low amount of DNA in the sample.
9. Absence of a signal for the positive control indicates to an error, which could be due to incorrect setup of the PCR reaction.