

African swine fever virus qPCR Kit

Catalogue number	Presentation
MD05121	100 reactions
MD05122	8 reactions

Description

African swine fever virus (ASFV) is a large, double-stranded DNA virus that belongs to the Asfarviridae family and is able to replicate in the cytoplasm of infected cells. It is the causative agent of African swine fever (ASF) causing a haemorrhagic fever with high mortality rates in domestic pigs. African swine fever virus qPCR Kit is designed for the *in vitro* detection of ASFV genomes. The kit is built to have the broadest possible detection profile whilst remaining specific to ASFV. Thus, this kit has been designed for the specific (inclusivity) and exclusive (exclusivity) *in vitro* detection of this species. The primers and probe sequences have very high (>95%) homology with a broad range of ASFV genomes based on a comprehensive bioinformatic analysis with all reference data within the NCBI database. However, due to the inherent instability of viral genomes, it is not possible to guarantee detection of all clinical isolates. Other closely related virus are not detected. Real-time PCR detection methods have been used several studies to identify ASFV genomes (Tignon M. et al 2011). This kit was meticulously designed and validated to meet the rigorous criteria of a quantitative assay. However, it is important to note that the provided Positive Control is not intended for quantification purposes. We recommend checking NZYtech website for the availability of a suitable Quantitative Standard for an accurate quantification. In alternative, commercially genomic DNA standards can also be used. If required, a complementary kit for the detection of an endogenous gene of the species from which samples are being extracted is available at NZYtech (see <https://nzytech.com/en/molecular-diagnostics/>). The complementary usage of an Endogenous Detection reaction provides a solid confirmation that nucleic acids were properly extracted from the selected biological matrix. If you require further information or have a specific question about the detection profile of this kit, please send an e-mail to info@nzytech.com and our scientific team will answer your question. This kit is designed to be used by trained users in a suitable molecular biology laboratory environment.

Shipping & Storage Conditions

This product can be shipped at a range of temperatures from dry ice to room temperature (RT). Although kit components are stable at room temperature, they should be immediately stored at -85°C to -15°C upon arrival. Also proceed with the following recommendations:

- Once the lyophilized components have been resuspended, they should not be exposed to temperatures above -15°C for longer than 30 minutes at a time.
- Minimise the number of freeze-thaw cycles by storing kit components in working aliquots. The kit is stable for six months from the date of resuspension.
- The PPMix must be stored protected from light. Particularly, do not expose the Lyo NZYSupreme qPCR master mix (2x) to direct sunlight after combining it with the PPMix.
- If the package that protects the kit arrived damaged, please contact NZYtech.
- Beware of the expiry date indicated on the packaging. NZYtech does not recommend using the kit after the expiry date. On this date, the kit must be discarded.

Components

For kit reference MD05121

The kit provides a comprehensive set of reagents sufficient to perform 100 *in vitro* Real-time PCR reactions.

COMPONENT	# TUBES	VOLUME	CAP COLOR
Lyo NZYSupreme qPCR master mix (2x)	2	-	Yellow
qPCR master mix reconstitution buffer ^Δ	1	1100 µL	Yellow
Lyo ASFV/IEC PPMix (10x) *	1	-	Brown
NTC [†]	1	760 µL	Neutral
ASFV Positive control	1	100 µL	Red
Internal Extraction Control (IEC) DNA	1	525 µL	Neutral

^Δ for resuspension of Lyo NZYSupreme qPCR master mix (2x).

* It includes ASFV and Internal Extraction Control (IEC) Primers and Probes Mix (PPMix), FAM™ and HEX™ labelled, respectively.

[†] for usage as No Template Control (NTC) and for Primers and Probe Mix (PPMix) resuspension.

For kit reference MD05122

Alternatively, this kit is available with a lyophilized solution that combines a master mix with a primer-probe mix (Lyo PMix), allowing for the performance of 8 real-time PCR reactions.

COMPONENT	# TUBES	VOLUME	CAP COLOR
Lyo PMix ASFV/IEC 2x *	1	-	Brown
PMix reconstitution buffer ^Δ	1	100 µL	Yellow
NTC [†]	1	760 µL	Neutral
ASFV Positive Control	1	8 µL	Red
Internal Extraction Control (IEC) DNA	1	50 µL	Neutral

* It includes ASFV and Internal Extraction Control (IEC) Primers and Probes Mix (PPMix), FAM™ and HEX™ labelled, respectively.

^Δ for resuspension of Lyo PMix.

[†] for usage as No Template Control (NTC).

Reagents, Materials and Equipment Required but Not Provided

- Real-time PCR Instrument that detects FAM™ and HEX™/JOE™/VIC™ fluorescent dyes (emission wavelengths of 520 and 556/555/554 nm, respectively).
- DNA extraction kit: we recommend using NZYtech's DNA extraction kits which are constantly fine-tuned to optimize Molecular Diagnostic applications.
- Quantitative Standard for quantification of African swine fever virus-containing samples - - MD07001 (African Swine Fever virus Quantitative qPCR Standard).
- RNase/DNase-free qPCR plasticware: PCR tubes, strips, caps, 96-well plates, and adhesive films (also available at NZYtech).
- Pipettes and filter tips (RNase/DNase-free).
- Disposable gloves.
- Vortex and centrifuge.

Sample Material

All nucleic acid samples that are suitable for PCR amplification can be used with this kit. However, procedures for the collection of biological sample material, shipping conditions, storage and processing times influence the quality of nucleic acids and may need to be optimized. Please ensure the samples are suitable in terms of purity, concentration, and DNA integrity.

This kit provides a DNA Internal Extraction Control (termed IEC) that can be co-purified and then co-amplified with the target nucleic acid. This is useful for checking the efficiency of DNA extraction and/or the presence of PCR inhibitors contaminating the sample nucleic acids. In addition, we recommend running at least one negative control with the samples (see below). To prepare a negative control, replace the template DNA sample with the No Template Control (NTC).

The Dynamic Range of the Test

Under optimal PCR conditions this NZYtech Molecular Diagnostic Real-time PCR Kit displays high priming efficiencies (>95%) and can detect at least 10 copies of the target template per reaction using different sample matrices.

Rational for the test

Real-time PCR

NZYtech African swine fever virus qPCR Kit includes all reagents to qualitatively identify the presence of African swine fever virus. The isolated and purified DNA is amplified in a single reaction using a highly ASFV-specific primers and probe set, exploiting the so-called TaqMan® principle. During this process, both primers and probe specifically anneal to a selected target region of the ASFV genome. The fluorogenic probe, which consists of a DNA sequence labelled with a 5'-dye and a 3'-quencher, is degraded during PCR amplification and the reporter dye and quencher are separated, increasing fluorescence. This can be detected on a wide range of real-time PCR platforms.

Negative Control

To validate any positive findings, a negative control reaction should be included every time the kit is used. To perform this, a reaction should be performed using the No Template Control (NTC) solution provided, instead of the DNA template. A negative result for the two channels (FAM and HEX/JOE/VIC) indicates that the reagents have not become contaminated while setting up the run.

Positive control (PC)

The kit includes a positive control template (PC) that allows the confirmation of a correct PCR setup. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that primers and probe used to detect the ASFV specific target are working properly. In contrast, if a negative result is obtained, the overall test results are invalid, and the test must be repeated. Care should be taken to ensure that the PC does not contaminate any other kit component which would lead to false-positive results. This can be achieved by handling this component in a post-PCR environment. In addition, care should also be taken to avoid cross-contaminating experimental samples when adding the PC to the run. This can be avoided by correctly sealing negative controls and all other samples before pipetting the PC into the positive control well. If such sealing is not possible, we suggest pipetting the PC into a well located the furthest possible from the negative control and biological sample wells. Please note that the PC in the kit is a representative sequence associated with the designed target region and does not contain the organism's entire genome.

Internal Extraction Control (IEC) DNA

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This Internal Extraction Control (IEC) DNA is then co-purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the IEC DNA also indicate that PCR inhibitors are not present at a compromising concentration. The set of primers and probe that detect IEC DNA is supplied in combination with ASFV-specific PPMix. Amplification of IEC DNA does not interfere with the detection of the ASFV target gene even when it is present at low-copy numbers. IEC DNA is detected through the HEX/JOE/VIC channel and should result in a Cq value <28.

Standard Protocol

Procedures before starting

To help preventing any carry-over DNA contamination, we recommend assigning independent areas for reaction set-up, the addition of samples and PC, PCR amplification and any post-PCR gel analysis. Also keep the kit components containing nucleic acids, specifically the ASFV Positive control (PC) and the Internal Extraction Control (IEC) DNA, separated from the remaining components of the kit and in the positive control setup area. It is essential that any tubes containing amplified PCR product are not opened in the PCR setup area. We also recommend the use of RNase/DNase-free plasticware/reagents, filter tips (preferably of low retention) and a clean area to work. Prepare the kit contents as described below:

A) For kit reference MD05121 – 100 reactions

Prepare the kit contents as described below:

1. Pulse-spin all Lyo tube components in a centrifuge before opening. This will ensure that the lyophilized qPCR master mix (2x) and PPMix remain at the base of the tube, avoiding spilling upon opening the tubes.

Note: *never pulse tubes containing PC and IEC in the same centrifuge used for non-DNA kit components.*

2. In the clean reaction set-up area, reconstitute the **Lyo NZYSupreme qPCR master mix (2x)** with 525 µL of **qPCR master mix reconstitution buffer**, as stated below. Flick gently until complete resuspension and spin. Do not replace the reconstitution buffer with water or any other buffer. The master mix is then ready to use as a 2x qPCR master mix.

COMPONENT	VOLUME (µL/per tube)
Lyo NZYSupreme qPCR master mix (2x) (Yellow)	525

3. Reconstitute the **PPMix** with 210 µL of the supplied **NTC**. To ensure complete resuspension, vortex the tube thoroughly until complete resuspension and spin. Do not replace the NTC as the reconstitution agent with water or any other buffer. The PPMix is then ready to use as 10x qPCR PP mix.

COMPONENT	VOLUME (µL)
Lyo ASFV/IEC PPMix (10x) (Brown)	210

B) For kit reference MD05122 – 8 reactions

Prepare the kit components according to the following instructions:

1. Pulse-spin Lyo PMix in a centrifuge before opening to ensure that the content remain at the base of the tube, avoiding spilling upon opening the tube.

Note: *never pulse tubes containing PC and IEC in the same centrifuge used for non-nucleic acid kit components and ensure that these tubes are opened and processed away from other kit components to avoid cross-contamination.*

2. In the clean reaction set-up area, reconstitute the **Lyo PMix ASFV/IEC 2x** with 82,5 µL of **PMix reconstitution buffer**, as stated below. Flick gently until complete resuspension and spin. Do not replace the reconstitution buffer with water or any other buffer. The PMix is then ready to use as a 2x PMix. **Please be aware that after reconstitution, the Lyo PMix should be kept on ice.**

COMPONENT	VOLUME (µL/per tube)
Lyo PMix ASFV/IEC 2x (Brown)	82,5

Nucleic Acids Extraction

The **Internal extraction control (IEC) DNA** can be added either to the DNA lysis/extraction buffer or to the biological sample once it has been resuspended in the lysis buffer.

Note: *Do not add the Internal Extraction Control DNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.*

1. Add 4 µL of the **IEC DNA** to the DNA lysis/extraction buffer that will be added to each sample (**4 µL of IEC DNA/sample**).
2. Proceed to DNA extraction according to the manufacturer's protocols.

Procedure

1. qPCR reaction mixture preparation

A) For kit reference MD05121 – 100 reactions

Prepare the qPCR reaction mixture according to the table below that specifies the volumes for 1 and n reactions (n , number of reactions). NZYtech highly recommends performing qPCR analyses in duplicates, which increases the probability of detection of the pathogen and facilitates the interpretation of results.

COMPONENT	1 REACTION VOLUME (μL)	n REACTIONS * VOLUME (μL)
Lyo NZYSupreme qPCR master mix (2x) (Yellow) **	10	$n \times 10$
Lyo ASFV/IEC PPMix (10x) (Brown)	2	$n \times 2$
NTC (Neutral) ***	3	$n \times 3$
Final Volume	15	$n \times 15$

* Include sufficient reactions for the negative and positive controls. For negative control use NTC. Positive controls include the ASFV Positive control (**mandatory**) and the Internal Extraction Control (IEC) DNA (**optional**: include this positive control in case IEC was added during sample extraction to confirm the correct function of the IEC detection reaction). We strongly recommend performing replicates of all reactions.

** Please note that a precipitate in the bottom of the master mix tube may be observed after resuspension, in particular after multiple freeze/thaw cycles. To ensure optimal performance, please make sure all components are thawed and resuspended prior to use. In this case do not spin the master mix before pipetting.

*** In case you prefer to use the IEC DNA as an amplification control (instead of adding it during the extraction protocol), add 2 μL of NTC and 1 μL of IEC DNA per reaction and adjust the volumes needed depending on the number of reactions (n).

B) For kit reference MD05122 – 8 reactions

Prepare the qPCR reaction mixture according to the table below that specifies the volumes for 1 and n reactions (n , number of reactions). NZYtech highly recommends performing qPCR analyses in duplicates, which increases the probability of detection of the pathogen and facilitates the interpretation of results.

COMPONENT	1 REACTION VOLUME (μL)	n REACTIONS * VOLUME (μL)
Lyo PMix ASFV/IEC 2x (Brown)	10	$n \times 10$
NTC (Neutral) **	5	$n \times 5$
Final Volume	15	$n \times 15$

* Include sufficient reactions for the negative and positive controls. For negative control use NTC. Positive controls include the ASFV Positive Control (**mandatory**) and the Internal Extraction Control (IEC) DNA (**optional**: include this positive control in case IEC was added during sample extraction to confirm the correct function of the IEC detection reaction). We strongly recommend performing replicates of all reactions.

** In case you prefer to use the IEC DNA as an amplification control (instead of adding it during the extraction protocol), add 4 μL of NTC and 1 μL of IEC DNA per reaction and adjust the volumes needed depending on the number of reactions (n).

2. Reaction setup

- 2.1. Pipette 15 μL of each qPCR mix into individual wells according to your real-time PCR experimental plate setup.
- 2.2. For the negative control reaction (**mandatory**), add 5 μL of NTC instead of the DNA template into the negative control well. The final volume in each well is 20 μL.
Note 1: Negative controls should be prepared and properly sealed before the addition of the biological samples and positive controls. If this is not possible, avoid pipetting the negative control in adjacent wells to the positive control and biological samples.
- 2.3. For the biological sample(s) reaction(s), pipette 5 μL of each extracted DNA sample into the corresponding wells, according to your experimental plate setup. The final volume in each well should be 20 μL.
Note 2: Seal all biological samples and negative controls before pipetting the PC into the positive control well. If not possible, avoid pipetting the positive and negative controls and the biological samples in adjacent wells.
Note 3: Up to 8 μL of biological sample can be used. When using an amount other than 5 μL of the sample, the amount of NTC in the reaction mixture must be changed accordingly. Please be aware that a higher volume of sample material may result in partial reaction inhibition.
- 2.4. For the ASFV positive control reaction (**mandatory**):
 - A) For kit reference MD05121 (100 reactions), add 5 μL of ASFV Positive control template into the corresponding well. The final volume in each well should be 20 μL.
 - B) For kit reference MD05122 (8 reactions), add 3 μL of ASFV Positive Control template plus 2 μL of NTC into the corresponding well. The final volume in each well should be 20 μL.
- 2.5. For the IEC positive control reaction (**optional**; just in case IEC DNA was added to the sample during DNA extraction), add 5 μL of Internal Extraction Control (IEC) DNA into the corresponding well. The final volume in each well should be 20 μL.

Suggested thermal cycling conditions

NZYtech's Lyo Master Mixes are optimized and highly efficient reaction mixtures developed for real-time qPCR. The table below displays a standard protocol optimized on several platforms. However, these conditions may be adapted to suit different machine-specific protocols.

CYCLES	TEMPERATURE	TIME	NOTES
1	95 °C	2 min	Polymerase activation
40	95 °C	5 s	Denaturation
	60 °C	30 s	Annealing/Extension*

* Fluorogenic data should be collected during this step through the FAM channel. HEX/JOE/VIC channel should be also used in case IEC DNA was added during sample extraction or if it was used as an amplification control.

Quality Control

Genomic DNA contamination

The product must comply with internal standards of DNA contamination as evaluated through real-time qPCR

Nucleases assay

To test for DNase contamination, 0.2-0.3 µg of pNZY28 plasmid DNA are incubated with the kit component in test for 14-16 h at 37 °C. To test for RNase contamination, 1 µg of RNA is incubated with the kit component in test for 1 h at 37 °C. Following incubation, the nucleic acids are visualized on a GreenSafe-stained agarose gel. There must be no visible nicking or cutting of the nucleic acids. To test DNases or RNases contamination of the nucleic acid controls, dilutions of the controls are incubated for 14-16 h at 37 °C and at -20 °C. After incubation, a qPCR/RT-qPCR reaction is performed comparing Ct values of the samples incubated at 37 °C and at -20 °C. There must be a deviation of less than 2 Cts between the two samples.

Functional assay

The qPCR/RT-qPCR reactions must ensure the consistent amplification of target DNA/RNA and internal extraction control across serial dilutions, meeting specified acceptance criteria for assay performance.

Data analysis

Before analysing sample results, we recommend verifying if the real-time PCR test is valid. Thus, for each plate, please confirm if the results for Positive and Negative controls performed as expected, according to the following criteria:

- **ASFV Positive control (PC):** the amplification curve of FAM (for ASFV target gene) is positive. The positive control is expected to amplify with a Cq <32. *Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised. Repeat the test.*
- **Positive IEC control (Optional:** in case the IEC DNA was added during sample extraction): the amplification curve of HEX/JOE/VIC, which relates to IEC DNA, is positive. Positive IEC control is expected to amplify with a Cq <28. *Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised. Repeat the test.*
- **Negative control (No Template Control, NTC reaction):** no amplification is detected. If the negative control has an amplification curve with a sigmoidal shape, sample contamination may have occurred. *Repeat the test following good qPCR practices.*

After verification of the validity of the test, use the following table for the interpretation of principal results (evaluate the overall shape of the amplification curves; only sigmoidal amplification curves are indicative of true amplification).

Sample Target Cq < 40 (FAM)	Internal Extraction Control Cq < 28 (HEX/JOE/VIC)*	Negative Control Cq > 40	Positive Control Cq < 32	Result
+	+/-	-	+	POSITIVE result
-	+	-	+	NEGATIVE result

* **Internal Extraction Control** (in case IEC DNA was added to the sample during DNA extraction): The Cq value obtained with the IEC in each biological sample will vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. Cq values < 28 are within the normal range. When amplifying a ASFV sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.