HANDBOOK

Bio-T kit® ASFV Universal

Cat. N° BIOTK131 - 100 reactions
Cat. N° BIOTK132 - 400 reactions

Detection of African Swine Fever (ASFV) by real-time PCR (qPCR) with Endogenous (IPC) and Exogenous (IC) internal positive control

DOMESTIC SWINE AND WILD BOAR

Sample types

- Whole blood (on EDTA), serum, cell culture supernatant
- Organs (spleens, tonsils, lymph nodes)
- Swabs (blood or exudates)
- Individual analysis or by pool up to 10 or 20 according to the matrix and the extraction method

Recommended nucleic acids (NA) extractions

- Magnetic beads extraction (e.g.: BioSellal – BioExtract® SuperBall® Cat. N° BES384) classical program 38 min and short program 19 min according to the matrix

Veterinary use only

DOCUMENTS MANAGEMENT

The Bio-T kit® ASFV Universal has two technical handbooks:

- The extraction handbook shared between the Bio-T kit® ASFV Universal , Bio-T kit® CSFV, Bio-T kit® ASFV and Bio-T kit® CSFV & ASVF displaying BioSellal's validated extraction protocols for each type of sample.
- The Bio-T kit® ASFV Universal qPCR handbook, presenting the instruction information to perform the qPCR.

The last versions in use for each handbook are indicated on the certificate of analysis (CA) provided with the Bio-T kit® ASFV Universal.

Besides these two handbooks, a validation file is available on request, contact BioSellal (contact@biosellal.com).

MODIFICATIONS MANAGEMENT

BioSellal indicates modifications done to this document by highlighting them using the rules presented in the table below:

MODIFICATIONS MANAGEMENT			
Type of modification Highlighting color	**Minor modifications**	**Type 1 Major modifications**	**Type 2 Major modifications**
Impact on revision / version	**Change of revision date** **No change of version**	**Change of revision date** **+ change of version**	**Change of revision date** **+ change of version**
Examples of modifications	Corrections: typographical, grammatical or turns of phrase	EPC reference modification	Modification of Master Mix composition
	Addition of a new sample type for extraction	Exogenous IPC reference modification	Modification of validated extraction protocol
	Addition of information giving more details or alternative protocol		
	Addition/Suppression of optional information		

PRESENTATION

Recommendations for sampling, shipping and storage of samples

Real-time PCR is a powerful technique allowing the detection of few amounts of pathogen genome. Genome can be rapidly degraded depending on the pathogen nature (bacteria / parasites, enveloped viruses...), the genome nature (DNA / RNA) and the sample type (presence of DNase / RNase). Thus, BioSellal recommends the following instructions to guarantee an optimal diagnosis.

Sampling

To prevent cross-contamination between samples leading to false positive results, it is mandatory to use disposable materials for single use and to avoid direct contact between specimens.

Shipping

It is recommended to ship as soon as possible after sampling, under cover of positive cold.

Storage after reception

It is recommended to immediately analyze samples after receipt or freezing at ≤ -16 ° C for a few months and ≤ -65°C beyond 1 year.

PIG Line

This kit belongs to the PIG line which gathers a set of kits sharing common extraction and qPCR protocols. It is compatible with BioSellal's other kits of AVIAN Line. (information available on www.biosellal.com).

Description of the Bio-T kit® ASFV Universal

The **Bio-T kit® ASFV Universal** (Cat. N° BIOTK131/BIOTK132) contains a ready to use **PCR Master Mix** allowing the detection **in the same reaction well of**:

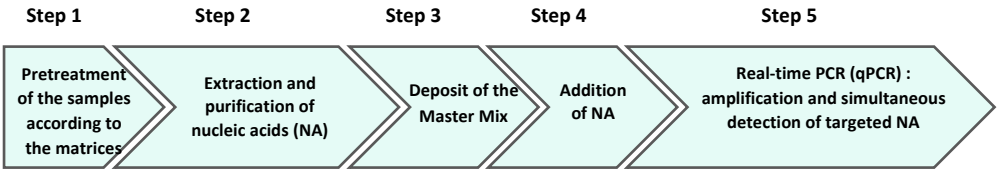
- **African Swine Fever (ASFV)** with a 6-FAM labelling
- An **Endogenous internal positive control IPC** (gapdh), with a Cy5 labelling, to assess the presence of sufficient amount of host cells, sample integrity, nucleic acids extraction quality and absence of RT- PCR inhibitors.
- An **Exogenous internal positive control IC** (DNA) with a VIC labelling

This kit, based on qualitative detection (detected or non-detected) from whole blood (on EDTA), serum, cell culture supernatant, organs (spleens, tonsils, lymph nodes) and swabs (blood or exudates) (Individual analysis or by pool up to 10 or 20 according to the matrix and the extraction method), was developed and validated according to the **French regulatory standard NF U47-600-2 edited by AFNOR**.

This kit has been evaluated by the European Union Reference Laboratory for African swine fever (EURL-ASF, INIA-CISA/CSIC, Valdeolmos, Spain). Data are available on demand, please contact BioSellal for more information.

Extraction protocols recommended and validated by BioSellal are described in the extraction handbook shared between the Bio-T kit® ASFV Universal, Bio-T kit® CSFV, Bio-T kit® ASFV and Bio-T kit® CSFV & ASVF.

Description of the whole process



Extraction handbook of the Bio-T kit® ASFV Universal		qPCR handbook of the Bio-T kit® ASFV Universal		
Whole blood, serum, cell culture supernatant Organs (spleens, tonsils, lymph nodes)* Swabs (blood or exudates)*	BioExtract® SuperBall® classical program 38 min and short program 19 min¥	Ready-to-use Master Mix MMASFVU-A	Samples NC/NCS MRI EPC (EPCASFVU-A)	Dyes: FAM/VIC/Cy5 Passive reference: ROX Program: PIG/AVIAN program ± RT FAST program Standard ramping

* Pretreatment mandatory

¥ according to the matrix

Kit contents and storage

Table 1. Description of the kit contents					
Description	**Reference**	***Volume / Tube***		**Presentation**	**Storage**
		BIOTK131 100 reactions	BIOTK132 400 reactions		
Master Mix (MM) Ready to use	MMASFVU-A	1500 µl	4x1500 µl	White cap tube Bag A	≤-16°C Protected from light, « MIX » Area
Exogenous Internal Positive Control (IC)	IPC-B	500 µl	4x500 µl	Pink cap tube Bag B	≤-16°C « Extraction » Area
External Positive Control (EPC) Positive PCR control of ASFV	EPCASFVU-A	200 µl	200 µl	Orange cap tube Bag C	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml		Blue cap tube Bag C	5°C ± 3 or ≤-16°C « Addition of Nucleic acids » Area

Kit reagents are stable until the expiration date stated on the label, subject to compliance with good storage conditions.

List of consumables and reagents not included in kit

Table 2. Consumables and reagents not included in kit			
Consumables/ Reagents	**Description**	**Provider**	**Cat. N°**
ATL Buffer	Lysis Buffer	BioSellal	ATL19076
BioExtract® SuperBall®	DNA/RNA Magnetic beads extraction kit (4 x 96)	BioSellal	BES384
Other equivalent extraction kit/reagents			

For consumables related to the thermal cycler, refer to the user manual of the device.

List of reagents to confirm laboratory performances

To confirm the performance of your thermal cycler(s), Synthetic DNA of ASFV (titrated in number of copies/qPCR) provided with the qPCR kit (**orange** cap tube) could be used. Please, contact BioSellal for more information (contact@biosellal.com).

Main critical points

- Wear appropriate personal protective equipment (lab coat, disposable gloves frequently changed).
- Work in dedicated and separate areas to avoid contamination: "Extraction" (unextracted samples storage, extraction equipment area), "Mix" (ready to use MM storage, qPCR plates preparation), "Nucleic acids Addition" (Nucleic Acids storage and addition of extracted nucleic acids and controls in the qPCR plate), "PCR" (final area containing the thermal cycler(s)).
- Use dedicated equipment for each working area (gloves, lab coat, pipettes, vortex, ...).
- Use filter tips.
- Before use, thaw all components at room temperature.
- Vortex and spin briefly (mini centrifuge) all reagents before use.
- Avoid the repetition of freezing-thawing cycles for samples, lysates, extracted nucleic acids.
- **Pathogen's genome detected by the PIG line's kits can be DNA or RNA. Working with RNA is more demanding than working with DNA** (RNA instability and omnipresence of the RNases). For these reasons, special precautions must be taken:
    - Always wear gloves, change them frequently, especially after contact with skin or work surfaces.
    - Treat all surfaces and equipment with RNases inactivation agents (available commercially).
    - When wearing gloves and after material decontamination, minimize the contact with surfaces and equipment in order to avoid the reintroduction of RNases.
    - Use "RNase free" consumable.
    - It is recommended to store the RNA at ≤ 5°C ± 3 during the manipulation and then freeze it as soon as possible, preferably at ≤ -65°C or by default at ≤ -16°C.
    - Open and close tubes one by one in order to limit the opening times and avoid any contact with RNases present in the environment (skin, dust, working surfaces…).

DETECTION OF ASFV BY qPCR WITH BIOTK131/BIOTK132

Global Procedure

1) Establish qPCR plate setup defining each sample position and **including the following controls**:

- **Negative Control Sample (NCS)**: water (or PBS) replaces the sample from the first step of sample preparation.
  This control is mandatory for each extraction series.

- **Negative Amplification Control (NC)**: 5 µl of water RNase/DNase free (Aqua-A tube, **blue** cap) replaces sample Nucleic Acids extract on qPCR plate.
  This control is recommended when using the kit for the first time or to verify the absence of Master Mix contamination.

- **External Positive Control of ASFV (EPC)** : Synthetic DNA provided (tube **EPCASFVU-A**, **orange** cap), containing specific target of ASFV .
  This control is mandatory.

⚠ CAUTION: *EPC and standards tube handling represents nucleic acids contamination hazard, it is thus recommended to open and handle it in a restricted area, away from other PCR components and to take precautions to avoid cross-contamination with nucleic acids extracts during deposit on the qPCR plate.*

- If available, **a Process Positive Control (MRI),** a weak positive sample is extracted in parallel with tested samples. After qPCR, MRI Ct value will be monitored on a Shewhart control card. Obtaining conform Ct value validates the whole process. In this case, the use of the EPC, provided with the kit, is not mandatory.

2) qPCR plate preparation

In the "MIX" dedicated area

1. After thawing, vortex and rapid centrifugation, **transfer 15 µl Master Mix MMASFVU-A (white** cap) in each well of interest (samples and controls).

In the "Nucleic Acids addition" dedicated area

2. **Add 5 µl of extracted nucleic acids (or NCS, water, MRI, standards or EPC**: **EPCASFVU-A orange** cap tube) in each well of interest. Make sure to pipet out in the bottom of the well, in the Master Mix, and to avoid the formation of bubbles.

Note: if the exogenous IC was not added during sample extraction, it can be added directly in the qPCR plate:

- Add 1 µl of IC (**pink** cap) with the extracted nucleic acids
- Or directly add the IC (1 µl per reaction) in an aliquot of Master Mix before the deposits of 16 µl of this mix into each well of interest. Then add 5 µl of extracted nucleic acids.

The reaction volume will be increased to 21 µl, without impacting the performances of the qPCR.

3. **Seal the plate with an optically clear sealer** or **close the strip caps**.

In the "PCR" amplification dedicated area

4. **Define the thermal cycler parameters** (see Table 3, Table 4, Table 5)

5. It is recommended to **spin the plate down prior to place it in the thermal cycler**, to remove drops from the well pit walls.

6. Start the qPCR program. Approximate run time: 70 minutes (55 minutes for the fast program).

3) Thermal cycler settings

This kit was developed and validated on ABI PRISM® 7500 Fast (Applied Biosystems) in standard ramping and confirmed on AriaMx™ (Agilent Technologies, fast ramping by default), on CFX96 (Bio-Rad, fast ramping by default) and QuantStudio5® (Applied Biosystems) in standard ramping . It is compatible with all thermal cyclers with at least 6-FAM, VIC and Cy5 channels. For more information, contact our technical support.

Table 3. Thermal cycler configuration

	ABI PRISM® 7500 Fast	AriaMx™	CFX96	QuantStudio® 5
Mode	Quantitation – Standard curve	Quantitative PCR, Fluorescence Probe	All Channels	Quantitation – Standard curve
Ramping	Standard Ramping	Fast Ramping by default	Fast Ramping by default	Standard Ramping
Passive Reference	**ROX**	**ROX**	-	**ROX**

Table 4. Thermal cycler Settings

Target	Detectors		Final Volume / well
	Reporter	Quencher	
ASFV	FAM	NFQ-MGB or None*	**20 µl** = 15 µl Master Mix + 5 µl extracted nucleic acids or controls†
Endogenous IPC	Cy5	NFQ-MGB or None*	
Exogenous IC	VIC	NFQ-MGB or None*	
To assign to samples and controls†			

* Depends on the thermal cycler model. Do not hesitate to contact the BioSellal Technical Support (tech@biosellal.com)

† Controls are NC (water), NCS (extracted water), EPC and or extracted MRI.

Table 5. PIG/AVIAN Amplification program settings without RT†		
Standard ramping		
Cycles	**Time**	**Temperature**
1 cycle	5 min	95°C
40 cycles	10 sec	95°C
	45 sec + data acquisition	60°C

† Optional step, in case of simultaneous detection of RNA genomes. Achieving a reverse-transcription (RT) step prior to PCR for the amplification of RNA genomes has no impact on the performances of the Bio-T kit® ASFV Universal (see the summary of the validation file).

NB: This amplification program is compatible with all Bio-T kits® of the PIG and AVIAN LINES.

A fast program has also been validated:

Table 1. FAST program without RT settings		
Standard Ramping		
Cycles	**Time**	**Temperature**
1 cycle	5 min	95°C
40 cycles	5 sec	95°C
	25 sec + data acquisition	60°C

NB: This amplification program is not compatible with others BioSellal kits.

RESULTS INTERPRETATION

To analyze and interpret the signals obtained by qPCR, the threshold must be set up.
The threshold must be assigned carefully in order to obtain the most reproducible result between different manipulations according to the requirements defined in Annex C of the French Standard **NF U47-600 (part 1)**. A consistent set of positive controls, usually an In-house Reference Material (MRI) or the EPC, is used to set the threshold value above the baseline and in the exponential amplification phase of the plot.
The Threshold Cycle, named « Ct » or « Cq » (depending on thermal cyclers), corresponds to the intersection between the amplification curves and the threshold line. It allows the relative measurement of the concentration of the target in the PCR reaction when a calibrated extract is analyzed in the same series.
The qPCR series is validated if the controls (EPC, MRI, NCS and NC) present valid results, then the result of each sample can be interpreted.

Main Scenarios

Controls Reading

Table 6. PCR Controls results interpretation

	Targets			Interpretation
	ASFV (FAM)	Exogenous IC (VIC)	Endogenous IPC (Cy5)	
NCS Negative Control Sample **MANDATORY**	Neg	Pos	Neg	Valid
	Pos / Neg	Pos	Pos/ Neg	If at least the target ASFV or endogenous IPC is positive: Contamination with a positive sample during extraction step or during qPCR plate preparation.
NC Negative PCR Control **OPTIONAL**	Neg	Neg$^{\Delta}$	Neg	Valid
	At least one of three targets Pos			Contamination with a negative or a positive sample during PCR plate preparation? or Master Mix / Water contamination?
EPC ASFV PCR external positive control **MANDATORY** *IN ABSENCE OF MRI*	Pos*	Neg$^{\Delta}$	Neg	Valid
	Neg	Neg$^{\Delta}$	Neg	Problem during qPCR plate preparation: Master Mix error? EPC omission?
	Pos *	At least one of two targets Pos$^{\Delta}$		Contamination with a sample during qPCR plate preparation?
Sample process positive Control MRI RECOMMENDED *IF AVAILABLE*	Pos†	Pos†	Pos$^{¥}$	Valid
	Neg	Neg	Neg/ Pos$^{¥}$	Problem during qPCR: Master Mix error? Nucleic acids extract omission or extract not in contact with Master Mix? Process drift: extraction (in case of exogenous IPC addition directly into qPCR plate and not during extraction) Problem with MRI preparation? Degradation of the sample process positive control?

* The Ct value obtained must be conform with the value indicated on the Certificate of Analysis (CA). † The Ct value must be included within control card limits. ¥ The obtained Ct value depends on the thermal cycler, the sample type and the used extraction protocol. IPC Ct values for recommended extraction protocols are available upon request. BioSellal recommends determining your own maximal IPC Ct value depending on your own extraction method and thermal cycler. Δ: If the exogenous IC is added directly in the Master Mix, a positive signal is expected for NC and EPC.

Note: Endogenous IPC targets a gene expressed by pig cells, thus it cannot be detected in NCS, NC and EPC. Due to cross-reaction between ruminant GAPDH and human GAPDH, a slight signal can be observed for IPC in the controls, the Ct value of this signal must be lower than 35.

Samples Reading

Table 7. Different types of results obtained for the samples			
Targets			
ASFV (FAM)	Exogenous IC (VIC)	Endogenous IPC (Cy5)	Interpretation
Neg	Pos*	Pos*	**Negative or Undetected**
Pos			**Positive or Detected**
Pos	Pos*	Neg or Ct>35	**Positive or Detected** Quantity of cells insufficient? (Only if endogenous IPC is negative or Ct >35) Omission of IC addition? (Only if exogenous IC is negative). Presence of inhibitors ǂ? Competition between targets? Extraction problem?
	Neg or Ct>35	Pos*	
		Neg or Ct>35	
Neg	Neg or Ct>35	Pos*	**Uninterpretable = Repeat the analysis** Problem during qPCR plate preparation: Master Mix error? Nucleic acids extract omission or extract not in contact with Master Mix? (Only if both controls are negative). Presence of inhibitors ǂ? Competition between targets? Nucleic acids degradation in the sample? Sampling problem: lack of cells? (Only if endogenous IPC is negative or Ct >35 and exogenous IC is pos). Extraction problem?
		Neg or Ct>35	
	Pos*	Neg or Ct>35	

* Obtained Ct values depends on the thermal cycler, the analysed matrix and the extraction methods used. For IC, it must be, at most, within the range specified on the certificate of analysis (CA). IPC and IC values, obtained from the different matrices with the methods recommended by BioSellal, are available upon request. BioSellal recommends that the laboratory determines its own maximum tolerated IC/IPC value based on its extraction method and thermal cycler.

ǂ In case of suspicion of inhibition, 1) Repeat qPCR by pre-diluting nucleic acids to 1/10 or even 1/100 in DNase / RNase free water or 2) Resume analysis from extraction.

biosellal

www.biosellal.com

Technical Support

tech@biosellal.com +33 (0) 4 26 78 47 62

Information and orders

contact@biosellal.com +33 (0) 4 26 78 47 60