HANDBOOK

Bio-T kit® ASFV

Cat. N° BIOTK084 - 50 reactions
Cat. N° BIOTK085 - 100 reactions

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

DOMESTIC SWINE AND WILD BOAR

Sample types

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

Recommended nucleic acids (NA) extractions

- Silica membrane columns extraction (e.g.: BioSellal – BioExtract® Column Cat. N° BEC050 or BEC250, Macherey-Nagel – NucleoSpin® 8 Virus, Cat N° 740643)
- Qiagen – Cador® Pathogen 96 Qiacube®HT Kit Cat N°54161) on whole blood, serum, plasma and cell supernatant only
- Magnetic beads extraction (eg: BioSellal – BioExtract® SuperBall® Cat. N° BES384 classical program 38 minutes and short program 19 minutes)

Veterinary use only

DOCUMENTS MANAGEMENT

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

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

Besides these two handbooks, a summary report of the validation file and a performances confirmation handbook are 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:

<table>
<tr><th colspan="4">MODIFICATIONS MANAGEMENT</th></tr>
<tr><th>Type of modification
Highlighting color</th><th>Minor modifications</th><th>Type 1 Major modifications</th><th>Type 2 Major modifications</th></tr>
<tr><th>Impact on revision / version</th><td>Change of revision date
No change of version</td><td>Change of revision date
+ change of version</td><td>Change of revision date
+ change of version</td></tr>
<tr><th rowspan="4">Examples of modifications</th><td>Corrections: typographical, grammatical or turns of phrase</td><td>EPC reference modification</td><td>Modification of Master Mix composition</td></tr>
<tr><td>Addition of new sample type for extraction</td><td>Exogenous IPC reference modification</td><td>Modification of validated extraction protocol</td></tr>
<tr><td>Addition of information giving more details or alternative protocol</td><td></td><td></td></tr>
<tr><td>Addition/Suppression of optional information</td><td></td><td></td></tr>
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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 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 gather 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

The **Bio-T kit® ASFV** (Cat. N° BIOTK084/BIOTK085) contains a ready to use **PCR Master Mix** allowing the detection **in the same reaction well of**:

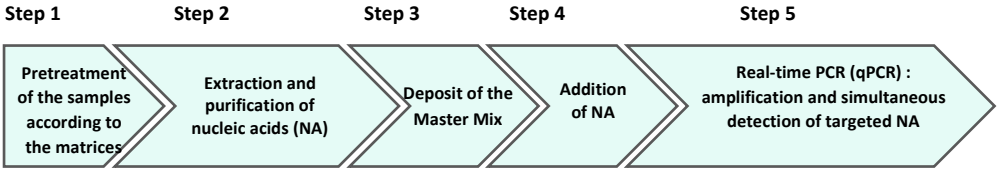
- **African Swine Fever Virus (ASFV)** with a VIC labelling,
- **An Endogenous internal positive control IPC** (gapdh), with a Cy5 labelling, to assess the presence of sufficient amount of host cells, sample integrity, nucleic acids extraction quality and absence of PCR inhibitors.

This kit, based on qualitative detection of ASVF (detected or not detected) from whole blood, serum, plasma cell culture supernatant, organs (spleens, tonsils, lymph nodes), swabs (blood or exudates), was developed and validated according to the **French regulatory standard NF U47-600-2 edited by AFNOR** and the specification of **the French National Laboratory (NRL) for CSF and ASF (Anses-Ploufragan-Plouzané, France).**

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

In order to facilitate the differential diagnosis of swine fever, BioSellal has validated a unique program of extraction and RT-PCR with Bio-T kit® CSFV, Bio-T kit® ASFV and Bio-T kit® CSFV & ASFV.

Description of the whole process



Extraction handbook shared between the Bio-T kit® ASVF, Bio-T kit® CSFV, Bio-T kit® CSFV & ASVF		qPCR handbook of the Bio-T kit® ASFV		
Whole blood, serum, plasma Cell culture supernatant[2] Organs (spleens, tonsils, lymph nodes)[1] Swabs (blood or exudates)[1]	BioExtract® SuperBall® 38 and 19 minutes BioExtract® Column NucleoSpin® 8 Virus Cador® Pathogen 96 Qiacube® HT Kit[3]	Ready-to-use Master Mix MMASFV-A	Samples NC/NCS MRI EPC (EPCASFV-A)	Dyes: VIC/Cy5 Passive reference: ROX Program: PIG/AVIAN program ± RT Fast or Standard ramping

[1]: pretreatment mandatory, [2]: no pretreatment, [3] : only for whole blood, serum, plasma and cell supernatant

Kit contents and storage

Table 1. Description of the kit contents					
Description	**Reference**	**Volume/tube**		**Presentation**	**Storage**
		BIOTK084 50 reactions	BIOTK085 100 reactions		
Master Mix (MM) Ready to use	MMASFV-A	750 µl	2x750 µl	white cap tube Bag A	≤-16°C Protected from light, « MIX » Area
External Positive Control (EPC) Positive PCR control of ASFV	EPCASFV-A	110 µl		orange cap tube Bag B	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml		blue cap tube Bag B	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			
Consommable / Réactif	**Description**	**Fournisseur**	**Cat. N°**
ATL Buffer	Lysis Buffer	BioSellal	ATL19076
BioExtract® Column	DNA/RNA column extraction kit (50)	BioSellal	BEC050
BioExtract® Column	DNA/RNA column extraction kit (250)	BioSellal	BEC250
BioExtract® SuperBall®	DNA/RNA Magnetic beads extraction kit (4 x 96)	BioSellal	BES384
NucleoSpin® 8 Virus	RNA column extraction kit (12*8)	Macherey Nagel	740643
Cador® Pathogen 96 Qiacube® HT Kit	DNA/RNA silica-membrane technology	Indical	SP54161

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

List of reagents to confirm laboratory performance

To confirm the performance of your thermal cycler(s), Synthetic DNA of ASFV (titrated in number of copies/qPCR), used by BioSellal for the validation of the kit, is required. The EPC provided with the qPCR kit (**orange** cap tube) could also be used. However, to avoid the repetition of freezing-thawing cycles, BioSellal recommends the use of this companion product. BioSellal sells this reagent under the following reference:

Table 3. Optional reagent*

Reagent	Description	Provider	Cat. N°
ASFV DNA	Quantified DNA of ASFV (1.5×10^4 copies/qPCR)	BioSellal	cADN-ASFV-001
Serum MRI	ASFV positive serum sample	BioSellal	MRI-ASFV-001

* This reagent is available only on demand, please contact BioSellal (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, qRT-PCR plates preparation), "Nucleic acids (NA) Addition" (Nucleic Acids storage and addition of extracted NA and controls in the qRT-PCR 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 NA.
- **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 ±3°C 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 BIOTK084/BIOTK085 KITS

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, **blue** cap tube) 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 (**EPCASFV-A**, **orange** cap tube), containing specific target of ASFV.
  This control is mandatory.

⚠ CAUTION: *EPC 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 of blood, serum, organs (spleens, tonsils, lymph nodes), swabs (blood or exudates) or cell culture supernatant 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 MMASFV-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, MRI, water or EPC**: **EPCASFV-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.

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 4, Table 5, Table 6).

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

6. Start the qPCR program. Approximate run time: 70 min.

3) Thermal cycler settings

This kit was developed and validated on AriaMx™ (Agilent Technologies, Fast ramping by default) and confirmed on ABI PRISM® 7500 Fast (Applied Biosystems) in standard ramping and fast ramping, and Rotor-Gene Q (QIAGEN). It is compatible with all thermal cyclers with at least VIC and Cy5 channels. For other thermal cyclers, contact our technical support.

Table 4. Thermal cycler configuration

	ABI PRISM® 7500 Fast	AriaMx™
Mode	Quantitation – Standard curve	Quantitative PCR, Fluorescence Probe
Ramping	Standard Ramping or Fast Ramping	Fast Ramping by default
Passive Reference	**ROX**	**ROX**

Table 5. Thermal cycler Settings			
Target	**Detectors**		**Final Volume / well**
	Reporter	**Quencher**	
ASFV	VIC	NFQ-MGB ou None*	**20 µl**
Endogenous IPC	Cy5	NFQ-MGB ou None*	= 15 µl Master Mix + 5 µl extracted nucleic acids or controls†
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) MRI (Process Positive Control) and EPC (Target DNA of ASVF).

Table 6. PIG/AVIAN Amplification program settings (without RT)†		
Standard or Fast 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 such as CSFV. Achieving a reverse-transcription (RT) step prior to PCR has no impact on the performances of the Bio-T kit® ASFV (see the summary of the validation file).
NB: This amplification program is compatible with all Bio-T kit® of the PIG and AVIAN LINES.

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 positives 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 7. PCR Controls results interpretation

	Targets		Interpretation
	ASFV (VIC)	Endogenous IPC (Cy5)	
NCS Negative Control Sample **MANDATORY**	Neg	Neg	Valid
	At least one of the two targets **Pos**		Contamination with a positive/negative sample during extraction step or during qPCR plate preparation.
NC Negative PCR Control **OPTIONAL**	Neg	Neg	Valid
	At least one of the two targets **Pos**		Contamination with a positive/negative sample during extraction step or during qPCR plate preparation or Master Mix/water contamination
EPC ASFV PCR external positive control **MANDATORY** *IN ABSENCE OF PROCESS POSITIVE CONTROL*	Pos*	Neg	Valid
	Neg	Neg	Problem during qPCR plate preparation: Master Mix error? EPC omission?
	Pos*	Pos	Contamination with a sample during qPCR plate preparation?
Process positive Control MRI **RECOMMENDED** *IF AVAILABLE*	Pos†	Pos¥	Valid
	Neg	Neg	Problem during qPCR plate preparation: Master Mix error? Nucleic acids extract omission or extract not in contact with Master Mix? Process drift: extraction and/or qPCR ? 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. Ct values for IPC, obtained from different sample types with methods validated by BioSellal, are available on request. BioSellal recommends you determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

Note:

Endogenous IPC targets a gene expressed by swine cells, thus it cannot be detected in NCS, NC and EPC.

Samples Reading

Table 8. Different types of results obtained for the samples		
Targets		
ASFV (VIC)	IPC Endogenous (Cy5)	Interpretation
Neg	Pos*	Negative ou Undetected
Pos		Positive ou Detected
Pos	Neg or Ct>35	Positive ou Detected Lack of host cells? Presence of inhibitors ǂ? Competition with the main target?
Neg	Neg or Ct>35	Uninterpretable = Repeat the analyse Problem during qPCR plate preparation: Master Mix error? Nucleic acids extract omission or extract not in contact with Master Mix? Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Sampling problem: lack of cells? Extraction problem?

*The obtained Ct value depends on the thermal cycler, the sample type and the used extraction protocol. This value must be, at least, included within the specified range in the certificate of analysis (CA). Ct values for IPC, obtained from different sample types with methods validated by BioSellal, are available on request. BioSellal recommends you determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

ǂ In case of inhibition suspicion, 1) Repeat the qPCR with the dilution of extracted nucleic acids at 1/10 or 1/100 in the DNase/RNase free water. 2)Restart the analysis from the extraction step.

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