HANDBOOK

Bio-T kit® CSFV & ASFV

Cat. N° BIOTK074 - 100 reactions

Detection of Classical Swine Fever Virus (CSFV) and African Swine Fever Virus (ASFV) by real-time RT-PCR (qRT-PCR) with Endogenous internal positive control (IPC)

DOMESTIC SWINE AND WILD BOAR

Sample types

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

Recommended nucleic acids (NA) extractions

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

Veterinary use only

DOCUMENTS MANAGEMENT

The Bio-T kit® CSFV & ASFVhas two technical handbooks:

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

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

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 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 RT-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 qRT-PCR protocols. It is compatible with BioSellal's other kits belonging to the PIG and AVIAN lines. (information available on www.biosellal.com).

Description of the Bio-T kit® CSFV & ASFV

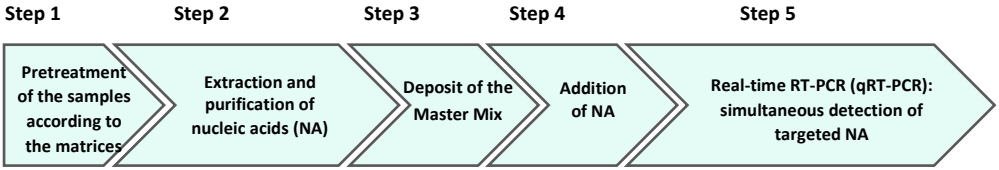
The **Bio-T kit® CSFV & ASFV** (Cat. N° BIOTK074) contains a ready to use **one-step RT-PCR Master Mix** allowing the detection **in the same reaction well of**:

- **Classical Swine Fever Virus (CSFV)** with a 6-FAM labelling.
- **African Swine Fever Virus (ASFV)** with a VIC labelling,
- An **Endogenous internal positive control IPC (beta actin),** with a Cy5 labelling, to add at the extraction step to assess nucleic acids extraction quality and absence of RT-PCR inhibitors.

This kit, based on qualitative detection (detected or non-detected) from whole blood (on EDTA), serum organs (spleen, tonsils and lymph nodes) and cell culture supernatant (Individual analysis), 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 the Classical and African Swine Fever (ANSES Ploufragan-Plouzané).**

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

Description of the whole process



Extraction handbook of the Bio-T kit® CSFV & ASFV		qRT-PCR handbook of the Bio-T kit® CSFV & ASFV		
whole blood (on EDTA), serum cell culture supernatant swabs*	BioExtract® SuperBall® 38 and 19 minutes BioExtract® Column Cador Pathogen 96 Qiacube HT kit[1]	Ready-to-use Master Mix MMCSFV&ASFV-A	Samples NC/NCS Process positive control EPC (EPCCSFV&ASFV-A)	Dyes: FAM/VIC/Cy5 Passive reference: ROX Program: PIG/AVIAN program with RT ramping Standard or Fast
organs (spleen, tonsils and lymph nodes)*	BioExtract® SuperBall® 38 minutes BioExtract® Column	Ready-to-use Master Mix MMCSFV&ASFV-A	Samples NC/NCS Process positive control EPC (EPCCSFV&ASFV-A)	Dyes: FAM/VIC/Cy5 Passive reference: ROX Program: PIG/AVIAN program with RT ramping Standard or Fast
	BioExtract® SuperBall® 19minutes		**1/10 diluted samples** NC/NCS Process positive control EPC (EPCCSFV&ASFV-A)	Dyes: FAM/VIC/Cy5 Passive reference: ROX Program: TLF program with RT ramping Standard or Fast

* pretreatment mandatory, 1: only for blood, serum, plasma and cell supernatant

Kit contents and storage

Table 1. Description of the kit contents				
Description	**Reference**	**Volume/tube**	**Presentation**	**Storage**
Master Mix (MM) Ready to use	MMCSFV&ASFV-A	2x750 µl	tube transparent cap Bag A	≤-16°C Protected from light, « MIX » Area
External Positive Control (EPC) Positive PCR control of ASFV and CSFV	EPCCSFV&ASFV-A	200µl	tube red cap Bag B	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml	tube blue cap 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			
Consumables/ Reagents	**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
Cador Pathogen 96 Qiacube HT kit	DNA/RNA extraction kit (x5)	Qiagen	5461

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

List of reagents to confirm laboratory performances

Synthetic RNA of CSFV and Synthetic DNA of ASFV (titrated in number of copies/RT-PCR) used by BioSellal for the validation of the kit can be used to confirm the performance of your thermal cycler(s). BioSellal sells these reagents under the following references:

Table 3. Optional reagents*

Reagent	Description	Provider	Cat. N°
CSFV RNA	Quantified RNA of CSFV (6×10^4 copies/qRT-PCR)	BioSellal	cARN-CSFV-001
ASFV DNA	Quantified DNA of ASFV (1.5×10^4 copies/qRT-PCR	BioSellal	cADN-ASFV-001

* These reagents are 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.
- **One-step RT-PCR Master-Mix is less stable than PCR Master-Mix. To guarantee its optimal performance, it is mandatory to extemporaneously defrost the tubes just before the use, to vortex it, to keep it at 5°C ± 3 during the deposit and to refreeze it immediately afterwards.**
- Vortex and spin briefly (mini-centrifuge) all reagents before use.
- Avoid the repetition of freezing-thawing cycles for samples, lysates, extracted NA.
- **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 CSFV AND ASFV BY qRT-PCR WITH BIOTK074

Global Procedure

1) Establish qRT-PCR 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 qRT-PCR 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 and CSFV (EPC)**: Synthetic DNA provided (tube **EPCCSFV&ASFV-A, red** cap), containing specific target of CSFV and 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 qRT-PCR plate.*

- If available, **a Process Positive Control (MRI),** a weak positive sample of whole blood (on EDTA), serum is extracted in parallel with tested samples. After qRT-PCR, MRI Ct values will be monitored on a Shewhart control card. Obtaining conform Ct values 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 MMCSFV&ASFV-A** (grey cap) in each well of interest (samples and controls).

⚠ *NOTE: One-step RT-PCR Master-Mix is less stable than PCR Master-Mix. To guarantee its optimal performance, it is mandatory to extemporaneously defrost the tubes just before the use, to vortex it, to keep it at 4 ° C during the deposit and to refreeze it immediately afterwards.*

In the "Nucleic Acids addition" dedicated area

2. **Add 5 µl of extracted nucleic acids (or NCS, water, MRSI or EPC**: **EPCCSFV&ASFV-A red** 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)

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.
   a. TLF with RT: for organs extracted with BioExtract® SuperBall® short program 19 minute. Approximate run time: 120 min.
   b. PIG/AVIAN WITH RT: for all other matrices and extractions protocols. Approximate run time: 90 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 ABI PRISM® 7500 Fast (Applied Biosystems) in Fast ramping, and Rotor-Gene Q (QIAGEN). It is compatible with all thermal cyclers with at least 6-FAM, VIC and Cy5 channels. For more information, contact our technical support.

Table 4. Thermal cycler configuration

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

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

Table 6. PIG/AVIAN Amplification program settings with RT		
Standard or Fast ramping		
Cycles	Time	Temperature
1 cycle	20 min	50°C
1 cycle	5 min	95°C
40 cycles	10 sec	95°C
	45 sec + data acquisition	60°C

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

For nucleic acids extracted with the BioExtract® SuperBall® short program 19 minutes from organs, a 1/10 dilution after extraction and a specific amplification program are needed.

Table 7. TLF Amplification program settings with RT		
Standard or Fast ramping		
Cycles	Time	Temperature
1 cycle	20 min	50°C
1 cycle	5 min	95°C
40 cycles	15 sec	95°C
	1 min + data acquisition	60°C

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, NCS and NC) present valid results, then the result of each sample can be interpreted.

Main Scenarios

Controls Reading

Table 8. PCR Controls results interpretation

	Targets			Interpretation
	CSFV (FAM)	ASFV (VIC)	Endogenous IPC (Cy5)	
NCS Negative Control Sample **MANDATORY**	Neg	Neg	Neg	Valid
	At least one of the three targets **Pos**			Contamination with a positive/negative sample during extraction step or during qPCR plate preparation.
NC Negative PCR Control **OPTIONAL**	Neg	Neg	Neg	Valid
	At least one of the three targets **Pos**			Contamination with a positive/negative sample during extraction step or during qPCR plate preparation or Master Mix/water contamination.
EPC CSFV and ASFV PCR external positive control	Pos*	Pos*	Neg	Valid
	Neg	Neg	Neg	Problem during qRT-PCR plate preparation: Master Mix error? EPC omission?
MANDATORY *IN ABSENCE OF MRI*	Pos*	Pos*	Pos	Contamination with a sample during qPCR plate preparation?
Sample process positive Control MRI	Pos†	Pos†	Pos¥	Valid
RECOMMENDED *IF AVAILABLE*	Neg	Neg	Neg	Problem during qRT-PCR plate preparation: Master Mix error? Nucleic acids extract omission or extract not in contact with Master Mix? Process drift: extraction and/or qRT-PCR ? 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 using the validated extraction protocols are available upon 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 pig cells, thus it cannot be detected in NCS, NC and EPC. However, due to cross-reaction between swine β-actine and human β-actine, 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 9. Different types of results obtained for the samples			
Targets			
CSFV (FAM)	ASFV (VIC)	Endogenous IPC (Cy5)	Interpretation
Neg	**Neg**	**Pos***	**Negative or Undetected**
Pos	**Pos**		**Positive or Detected**
At least one of two targets **Pos**			**Positive or Detected for the positive target** **Negative or Undetected for the negative target**
Pos	**Pos**	**Neg or Ct>35**	**Positive or Detected** Lack of host cells? Presence of inhibitors ǂ? Competition with the main target?
At least one of the target is **Neg**		**Neg or Ct>35**	**Positive or Detected for the positive target** **Uninterpretable for the negative target= Repeat the analysis.** Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Sampling problem: lack of cells? Extraction problem?
Neg	**Neg**	**Neg or Ct>35**	**Uninterpretable = Repeat the analysis** Problem during qRT-PCR 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. IPC Ct values for validated extraction protocols are available upon 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 qRT-PCR 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.

Table 10. Different types of results obtained for the samples			
Targets			
CSFV (FAM)	ASFV (VIC)	IPC Endogenous (Cy5)	Interpretation
Neg	**Neg**	**Pos***	**Negative or Undetected**
Pos	**Pos**		**Positive or Detected**
Pos	**Pos**	**Neg or Ct>35**	**Positive or Detected** Lack of host cells? Presence of inhibitors ǂ? Competition with the main target?
At least one of the target is **Neg**		**Neg or Ct>35**	**Positive or Detected for the positive target** **Uninterpretable** = Repeat the analyse for the negative target. Extraction problem? Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Sampling problem: lack of cells?
Neg	**Neg**	**Neg or Ct>35**	**Uninterpretable** = Repeat the analyse 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 using the validated extraction protocols are available upon 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 qRT-PCR 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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