HANDBOOK

Bio-T kit® CSFV

Cat. N° BIOTK058 - 50 reactions
Cat. N° BIOTK060 - 100 reactions

Detection of Classical Swine Fever Virus (CSFV) 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, lymph nodes)
- Swabs (blood or exudates)
- Individual analysis or by pool up to 10 according to the matrix

Recommended Nucleic Acids Extraction-Purification

- Silica membrane columns (e.g.: BioSellal – BioExtract® Column Cat. N° BEC050 ou BEC250 ; Qiagen – RNeasy® Mini Kit Cat N° 74104 ; Macherey-Nagel – NucleoSpin® RNA, Cat N° 740955, Macherey-Nagel – Nucleospin 8 virus, Cat N°740643)
- Qiagen – Cador® Pathogen 96 Qiacube® HT Kit Cat N°SP54161) on whole blood, serum, plasma and cell supernatant only
- Magnetic beads (e.g.: BioSellal – BioExtract® SuperBall® Cat. N° BES384 classical program 38 minutes and short program 19 minutes)

Veterinary use only

DOCUMENTS MANAGEMENT

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

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

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 of BioSellal which gather a set of kits sharing common extraction and RT-PCR protocols. It is also compatible with other kits of the AVIAN Line. (More information on www.biosellal.com).

Description of Bio-T kit® CSFV

The Bio-T kit® CSFV (Cat. N° BIOTK058/BIOTK060) 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,
- **An endogenous internal positive control IPC (beta actin)**, 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.

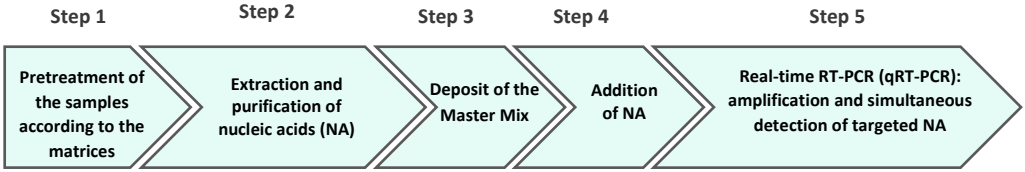
This kit can be used for the qualitative analysis of CSFV (detected or not detected) on samples such as whole blood, serum, plasma, cell culture supernatant, organs (spleen, tonsils, lymph nodes) and swabs (blood or exudates). It was developed and validated according to the **French regulatory standard NF U47-600-2 edited by AFNOR** and the specification of **the French National Laboratory (NRL) for CSF and ASF (Anses-Ploufragan-Plouzané, France).**

In order to improve endogenous IPC detection on swabs sample, the composition of the Master Mix has been changed. This leads to the change of the Master Mix's reference (MMCSFV-B).

Extraction protocols validated by BioSellal are described in the extraction handbook shared between the Bio-T kit® CSFV , 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 extraction and RT-PCR program for 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		qRT-PCR handbook of the Bio-T kit® CSFV		
Organs (spleen, tonsils, lymph nodes)[1] Whole blood, serum, plasma, cell culture supernatant[2] Swabs[1]	BioExtract® SuperBall® (38 and 19 minutes) BioExtract® Column RNeasy® Mini Kit NucleoSpin® RNA Cador® Pathogen 96 Qiacube® HT Kit[3] NucleoSpin® 8 virus	Ready to use Master Mix MMCSFV-B	Samples[4] NC/NCS MRI EPC (EPCCSFV-A)	Dyes : FAM/Cy5 Passive reference : ROX Program : PIG/AVIAN with RT Standard or Fast ramping

[1]: pretreatment mandatory, [2]: no pretreatment, [3]: only for whole blood, serum, plasma and cell supernatant, [4]: for organs extracted with BioExtract® short program 19 minutes, samples have to be diluted 1/10.

Kit contents and storage

Table 1. Description of the kit contents					
Description	**Reference**	**Volume/tube**		**Presentation**	**Storage**
		BIOTK058 50 reactions	BIOTK060 100 reactions		
Master Mix (MM) Ready to use	MMCSFV-B	750 µl	2x750 µl	Transparent cap tube Bag A	≤-16°C Protected from light, « MIX » Area
External Positive Control (EPC) Positive PCR control of CSFV	EPCCSFV-A	110 µl		Red 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 » Zone

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			
Consumable / Reagent	**Description**	**Provider**	**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
RNeasy® Mini Kit	RNA column extraction kit (50)	Qiagen	74104
NucleoSpin® RNA	RNA column extraction kit (50)	Macherey Nagel	740955
Cador® Pathogen 96 Qiacube® HT kit	DNA/RNA silica-membrane technology	Indical	SP54161
NucleoSpin® 8 Virus	RNA column extraction kit (12*8)	Macherey Nagel	740643

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), a CSFV synthetic RNA (titrated in number of copies/ qRT-PCR), used by BioSellal for the validation of the kit, is required. BioSellal sells this reagent under the following reference:

Table 3. Optional reagent*

Reagent	Description	Provider	Cat. N°
CSFV RNA	Quantified CSFV RNA (6×10^3 copies/qRT-PCR)	BioSellal	cARN-CSFV-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.
- **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.
- **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 CSFV BY qRT-PCR WITH BIOTK058/BIOTK060 KITS

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, **Blue** cap tube) replaces sample Nucleic Acid 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.

- **CSFV External Positive Control (EPC)**: synthetic DNA, containing the targeted sequence specific of CSFV (**EPCCSFV-A**, **red** cap tube)
  This control is mandatory.

⚠ CAUTION: *EPC tube handling represents a nucleic acid 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 blood, serum, plasma organs (spleens, tonsils, lymph nodes), swabs (blood or exudates) or cell culture supernatant is extracted in parallel with tested samples. After qRT-PCR, 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) Preparation of the qRT-PCR plate

In the «MIX » dedicated Area

1. After thawing, vortex and rapid centrifugation of the tube, **transfer 15µl of Master Mix MMCSFV-B** (transparent 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 5°C ± 3 during the deposit and to refreeze it immediately afterwards.*

In the «Nucleic Acid addition» dedicated Area

2. **Add 5 µl of Nucleic Acids extract (or NCS, MRI, water, EPC**: **EPCCSFV-A red** cap) in each well of interest. Make sure to pipet out the 5 µl 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 down the plate prior to place it into the thermal cycler**, in purpose to avoid the presence of drops on the walls of the wells and to eliminate the maximum of bubbles.

6. Start the qRT-PCR program. Approximate duration of the run: 90 minutes.

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 6-FAM 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 Ramping 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 or None*	**20 µl**
Endogenous IPC	CY5	NFQ-MGB or 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 RNA of CSFV).

Table 6. PIG/AVIAN Amplification program settings		
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: Amplification Program are compatible with all kits of PIG and AVIAN Lines from BioSellal.

RESULTS INTERPRETATION

To analyze and interpret the signals obtained by qRT-PCR, the Threshold line must be set up.
The Threshold must be assigned carefully 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 curves, at least 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 RT-PCR reaction when a calibrated extract is analyzed in the same qRT-PCR run.
The qRT-PCR run 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. Controls results interpretation			
	Targets		Interpretation
	CSFV (FAM)	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 qRT-PCR plate preparation.
NC Negative Amplification Control **OPTIONNAL**	Neg	Neg	Valid
	At least one of the two targets Pos		Contamination with a positive/negative sample during extraction step or during qRT-PCR plate preparation or Master Mix/water contamination
EPC CSFV PCR external Positive Control **MANDATORY** *IN ABSENCE OF PROCESS POSITIVE CONTROL*	Pos*	Neg	Valid
	Neg	Neg	Problem during qRT-PCR plate preparation: Master Mix error? EPC omission?
	Pos*	Pos	Contamination with a sample during qRT-PCR plate preparation?
Process positive Control MRI **RECOMMENDED** *IF AVAILABLE*	Pos†	Pos¥	Valid
	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, 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. However, 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 higher than 35.

Samples reading

Table 8. Different types of results for samples		
Targets		
CSFV (FAM)	Endogenous IPC (Cy5)	Interpretation
Neg	**Pos***	**Negative ou Undetected**
Pos		**Positive or Detected**
Pos	**Neg or Ct>35**	**Positive or 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 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. 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 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.

Notes :

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