HANDBOOK

Bio-T kit® PRRSV

Cat. N° BIOTK001 - 50 reactions
Cat. N° BIOTK040 - 100 reactions

**Detection of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)
and genotyping of European (EU) and North American (NA) strains
by real-time RT-PCR (qRT-PCR)
with exogenous internal positive control (IPC)**

SWINE

Sample types

- Whole blood (on EDTA), serum
- Oral Fluids
- Cell culture supernatant
- Organs
- Individual analysis or by pool up to 10 according to the matrix

Recommended nucleic acids (NA) extractions

- Magnetic beads extraction (e.g.: BioSellal – BioExtract® SuperBall® Cat. N° BES384)
- Silica membrane columns extraction (e.g.: BioSellal – BioExtract® Column Cat. N° BEC050 or BEC250)

Veterinary use only

DOCUMENTS MANAGEMENT

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

- The extraction handbook shared between the Bio-T kit® PRRSV, PRRSV DIVA, PCV3 and PCV2 & PCV3, displaying BioSellal's recommended extraction protocols for each type of sample.
- The Bio-T kit® PRRSV 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® PRRSV.

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 mandatory to ship immediately after sampling or by default to store it at ≤ -16°C. Shipment has to be done within 24h 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 AVIAN lines. (information available on www.biosellal.com).

In addition to the kits belonging to the PIG line, BioSellal offers ELISA kit BioLisa® kit PRRSV. For more informations, contact BioSellal (contact@biosellal.com)

Description of the Bio-T kit® PRRSV

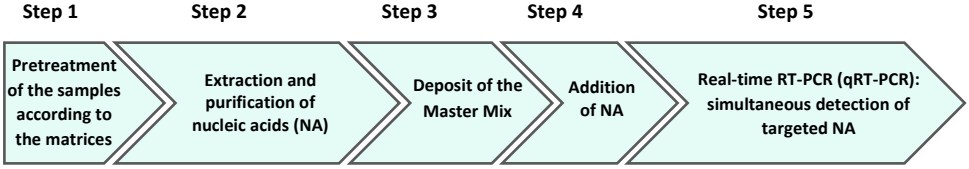
The **Bio-T kit® PRRSV** (Cat. N° BIOTK001/BIOTK040) contains a ready to use **one-step RT-PCR Master Mix** allowing the detection **in the same reaction well of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and the genotyping of**:

- **PRRSV European strains (PRRSV EU)** with a 6-FAM labelling
- **PRRSV North American strains (PRRSV NA**) with a VIC labelling
- **An exogenous internal positive control IPC RNA**, 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 detection of PRRSV and genotyping of Eurpean and North Amercian strains (detected or non-detected) from whole blood (on EDTA), serum, organs, oral fluids and cell culture supernatant samples (Individual analysis), was developed and validated according to the **French regulatory standard NF U47-600-2 edited by AFNOR**.

Extraction protocols validated by BioSellal are described in the extraction handbook shared between the Bio-T kit® PRRSV, PRRSV DIVA, PCV3 and PCV2 & PCV3.
The pool up to 10 is possible for whole blood (on EDTA) and serum with all Bio-T kit® mentioned before.

Description of the whole process



Extraction handbook shared between the Bio-T kit® PRRSV, PRRSV DIVA, PCV3 and PCV2 & PCV3		qRT-PCR handbook of the Bio-T kit® PRRSV		
Whole blood (on EDTA) Serum Oral Fluids* Cell culture supernatant* Organs*	BioExtract® SuperBall® BioExtract® Column	Ready-to-use Master Mix MMPRRSV-C	Samples NC/NCS Process positive control (MRI) EPC (EPCPRRSV-C)	Dyes: FAM/VIC/Cy5 Passive reference: ROX Program: PIG/AVIAN program with RT Standard or Fast ramping

* pretreatment mandatory

Kit contents and storage

Table 1. Description of the kit contents					
Description	**Reference**	**Volume/tube**		**Presentation**	**Storage**
		BIOTK001 50 reactions	**BIOTK040** 100 reactions		
Master Mix (MM) Ready to use	MMPRRSV-C	1000 µl	2x1000 µl	Transparent cap tube Bag A	≤-16°C Protected from light, « MIX » Area
Exogenous Internal Positive Control (IPC)	IPCRNA-A	250 µl	2x250 µl	Purple cap tube Bag B	≤-16°C « Extraction » Area
External Positive Control (EPC) Positive PCR control of PRRSV EU and PRRSV NA	EPCPRRSV-C	110 µl		Red 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® 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

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 PRRSV EU and of PRRSV NA (titrated in number of copies/qRT-PCR) used by BioSellal for the validation of the kit can be used to confirm the performance of your thermal cycler(s). To confirm the performance of your complete method, Viral suspension of PRRSV EU and of PRRSV NA (quantified in TCID50/ml) used by BioSellal in the validation file could be provided. An internal reference material (MRI) for serum samples is also available to confirm the performance of the complete method over the time (extraction + RT-PCR).

BioSellal sells these reagents under the following references:

Table 3. Optional reagents*

Reagent	Description	Provider	Cat. N°
PRRSV EU RNA	PRRSV EU quantified RNA (6×10^4 copies/qRT-PCR)	BioSellal	cARNPRRSVEU-002
PRRSV NA RNA	PRRSV NA quantified RNA (12×10^4 copies/qRT-PCR)	BioSellal	cARNPRRSVNA-002
PRRSV EU Viral suspension	PRRSV EU viral suspension quantified (10^3 TCID50/mL)	BioSellal	SV-PRRSV-EU-002
PRRSV NA Viral suspension	PRRSV NA viral suspension quantified ($10^{4,9}$ TCID50/mL)	BioSellal	SV-PRRSV-NA-002
MRI serum	PRRSV EU/NA serum (100 x LD_{METHOD})	BioSellal	MRI-PRRSV-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 Addition" (Nucleic Acids storage and addition of extracted nucleic acids 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 nucleic acids.
- Genomes of pathogens detected by the **PIG line** 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 PRRSV EU AND PRRSV NA BY qRT-PCR WITH BIOTK001/BIOTK040

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 PRRSV EU and PRRSV NA (EPC)**: Synthetic DNA (tube **EPCPRRSV-C**, **red** cap) containing specific target of PRRSV EU and PRRSV NA.
  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 for PRRSV EU and PRRSV NA (whole blood, serum, oral fluids, organs or cell culture supernatant) 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.
  BioSellal sells a ready-to-use MRI for serum sample (MRI-PRRSV-001).

2) qRT-PCR plate preparation

In the "MIX" dedicated area

1. After thawing, vortex and rapid centrifugation, **transfer 20 µl Master Mix MMPRRSV-C (**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 Acids addition" dedicated area

2. **Add 5 µl of extracted nucleic acids (or NCS, water, MRI or EPC**: **EPCPRRSV-C 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.

Note: if the exogenous IPC was not added during sample extraction, it can be added directly in the qRT-PCR plate:
- Add 1 µl of IPC (**purple** cap) with the extracted nucleic acids.
- Or add directly the IPC (1 µl per reaction) in an aliquot of Master Mix before the deposits of 21 µl of this mix into each well of interest. Then add 5 µl of extracted nucleic acids.
The reaction volume will be increased to 26 µl, without impacting the performances of the qRT-PCR.

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 qRT-PCR program. Approximate run time: 90 min

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) and ABI PRISM® 7500 Fast (Applied Biosystems) in Fast ramping. 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 or Fast Ramping	Fast Ramping by default
Passive Reference	**ROX**	**ROX**

Table 5. Thermal cycler Settings

Target	Detectors		Final Volume / well
	Reporter	Quencher	
PRRSV EU	FAM	NFQ-MGB or None*	**25 µl** = 20 µl Master Mix + 5 µl extracted nucleic acids or controls†
PRRSV NA	VIC	NFQ-MGB or None*	
exogenous IPC	Cy5	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 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 kit® of the PIG and AVIAN LINES.

RESULTS INTERPRETATION

To analyze and interpret the signals obtained by qRT-PCR, 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 qRT-PCR 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
	PRRSV EU (FAM)	PRRSV NA (VIC)	exogenous IPC (Cy5)	
NCS Negative Control Sample	Neg	Neg	Pos	Valid
	At least one of the two targets Pos		Pos	Contamination with a positive sample during extraction step or during qPCR plate preparation.
MANDATORY	Neg	Neg	Neg	Omission of exogenous IPC addition? Defective extraction?
NC Negative PCR Control **OPTIONAL**	Neg	Neg	Neg	Valid
	At least one of the three targets Pos			Contamination with a negative or a positive sample during PCR plate preparation? or Master Mix / Water contamination?
EPC PRRSV EU and PRRSV NA 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 qRT-PCR plate preparation?
Sample process positive Control MRI	Pos†	Pos†	Pos¥	Valid
	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 ?
RECOMMENDED *IF AVAILABLE*	Neg	Neg	Pos¥	Process drift: extraction (in case of exogenous IPC addition directly into qRT-PCR plate and not during extraction) Problem with MRSI 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 you to determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

Samples Reading

Table 8. Different types of results obtained for the samples			
Targets			
PRRSV EU (FAM)	PRRSV NA (VIC)	exogenous 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** Problem during the IPC addition? Presence of inhibitors ǂ? Competition with the mains targets?
One of the targets is **Neg**		**Neg or Ct>35**	**Positive or Detected** **for the positive target** **Uninterpretable for the negative target :** **Risk of low positive sample non- detection** = Repeat the analysis for the negative target IPC exogenous omission during the extraction and/or qRT-PCR? Presence of inhibitors ǂ? Extraction problem? Competition with the positive target?
Neg	**Neg**	**Neg or Ct>35**	**Uninterpretable** **Risk of low positive sample non- detection** = Repeat the analysis Nucleic acids extract omission or extract not in contact with Master Mix? Presence of inhibitors ǂ? Problem during the IPC addition? 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). IPC Ct values for recommended extraction protocols are available upon request. BioSellal recommends you to 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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