HANDBOOK

Bio-T kit® PRRSV DIVA

Cat. N° BIOTK077 - 50 reactions
Cat. N° BIOTK086 - 100 reactions

Distinction between Suvaxyn® PRRS MLV vaccine strain (Suvaxyn® vaccine strain) and all other European strains (PRRSV EU other than Suvaxyn®) of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) by real-time RT-PCR (qRT-PCR) with Exogenous internal positive control (IPC)

SWINE

Sample types

- Whole blood (on EDTA), serum
- Organs
- Oral Fluids
- 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 DIVA has two technical handbooks:

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

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).

Description of the Bio-T kit® PRRSV DIVA

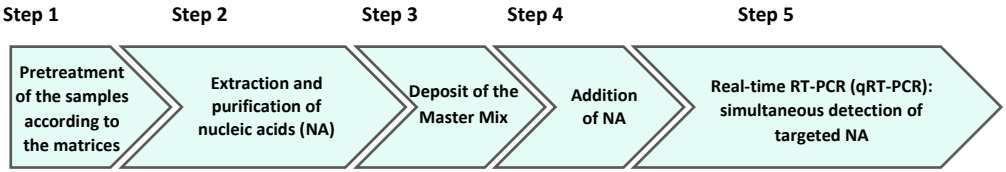
The **Bio-T kit® PRRSV DIVA** (Cat. N° BIOTK077/BIOTK086) contains a ready to use **one-step RT-PCR Master Mix** allowing the detection **in the same reaction well of**:

- **European field strains of PRRSV and European vaccine strains other than Suvaxyn® PRRS MLV (PRRSV EU other than Suvaxyn®)** with a 6-FAM labelling
- **Suvaxyn® PRRS MLV vaccine strain (Suvaxyn® vaccine strain)** 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 qualitative detection (detected or non-detected) from whole blood, serum, organs or oral fluids (individual analysis), was developed and validated according to the **French regulatory standard NF U47-600-2 edited by AFNOR**.

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

Description of the whole process



Extraction handbook of the Bio-T kit® PRRSV DIVA, PRRSV, PCV2 & PCV3 and PCV3		qRT-PCR handbook of the Bio-T kit® PRRSV DIVA		
whole blood, serum Organs* Oral Fluids*	BioExtract® SuperBall® BioExtract® Column	Ready-to-use Master Mix MMPRRSVDIVA-B	Samples NC/NCS Process positive control EPC (EPCPRRSVDIVA-B) Control of Artifact (CTLARTEFACT-A)	Dyes: FAM/VIC/Cy5 Passive reference: ROX Program: PIG/AVIAN program with RT Standard ramping

* pretreatment mandatory

Kit contents and storage

Table 1. Description of the kit contents					
Description	**Reference**	***Volume /tube***		**Presentation**	**Storage**
		BIOTK077 50 reactions	BIOTK086 100 reactions		
Master Mix (MM) Ready to use	MMPRRSVDIVA-B	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 Suvaxyn® vaccine strain and PRRSV EU other than Suvaxyn®	EPCPRRSVDIVA-B	110 µl		Red cap tube Bag C	≤-16°C « Addition of Nucleic acids » Area
Control of Artifact Positive PCR control of Suvaxyn® vaccine strain and Negative PCR control for other European PRRSV strains	CTLARTEFACT-A	110 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® 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 other than Suvaxyn® and of Suvaxyn® vaccine strain (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). An internal reference material (MRI) for PRRSV EU other than Suvaxyn® 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 other than Suvaxyn® RNA	Quantified RNA of PRRSV EU other than Suvaxyn® (1.2×10^5 copies/qRT-PCR)	BioSellal	cARNPRRSVEU-003
Suvaxyn® vaccine strain RNA	Quantified RNA of PRRSV EU other than Suvaxyn® (1.2×10^5 copies/qRT-PCR)	BioSellal	cARNPRRSVVAC001
MRI serum	PRRSV EU other than Suvaxyn®	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…).

DISCRIMINATION BETWEEN SUVAXYN® VACCINE STRAIN AND ALL OTHER PRRSV EU STRAINS BY qRT-PCR WITH BIOTK077/BIOTK086

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 Suvaxyn® vaccine strain and PRRSV EU other than Suvaxyn® (EPC)**: Synthetic DNA (tube **EPCPRRSVDIVA-B**, **red** cap), containing specific target of PRRSV EU other than Suvaxyn® and Suvaxyn® vaccine strain.
  This control is mandatory.

- **Control of Artifact:** Synthetic DNA (tube **CTLARTEFACT-A**, **orange** cap), containing specific target of Suvaxyn® vaccine strain. This control allows to evaluate the shape of the curve obtained in the "PRRSV EU other than Suvaxyn®" channel (artifact of the Suvaxyn® vaccine strain, strongly positive in VIC, which can induce a false positive signal in FAM) and thus to consider negative samples presenting later curves than this control in the FAM channel if these samples are positive for Suvaxyn® vaccine strain.
  This control is mandatory. It must be used pure and diluted 1/100 000 (5 serial dilutions by 1/10).

⚠ CAUTION: *EPC tube and control of artifact 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, serum or organs 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. BioSellal sells ready-to-use MRI for serum sample, positive for PRRSV EU other than Suvaxyn (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 MMPRRSVDIVA-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 Acids addition" dedicated area

2. **Add 5 µl of extracted nucleic acids (or NCS, water, MRI or Control of Artifact** (tube **CTLARTEFACT-A , Orange** cap) **and EPC** (tube **EPCPRRSVDIVA-B, Red** cap) 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: 90min.

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. 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	Ramping Fast by default
Passive Reference	**ROX**	**ROX**

Table 5. Thermal cycler Settings			
Target	**Detectors**		**Final Volume / well**
	Reporter	**Quencher**	
PRRSV EU other than Suvaxyn®	FAM	NFQ-MGB or None*	**25 µl** = **20 µl** Master Mix + 5 µl extracted nucleic acids or controls†
Suvaxyn® vaccine strain	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 and Control of Artifact.

Table 6. PIG/AVIAN Amplification program settings with RT		
	Standard 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.

The results obtained in the channel "PRRSV EU other than Suvaxyn®" in the presence of a positive signal for the Suvaxyn® vaccine strain should be compared with the result obtained for the artifact and EPC control.

Main Scenarios

Controls Reading

Table 7. PCR Controls results interpretation

	Targets			
	PRRSV EU other than Suvaxyn® (FAM)	Suvaxyn® vaccine strain (VIC)	Exogenous IPC (Cy5)	Interpretation
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 External positive control of PRRSV EU other than Suvaxyn® and Suvaxyn® vaccine strain **MANDATORY** *IN ABSENCE OF MRI*	Pos*	Pos*	Neg$	Valid
	Neg	Neg	Neg$	Problem during qRT-PCR plate preparation: Master Mix error? EPC omission?
	Pos*	Pos*	Pos	Contamination with a sample during qRT-PCR plate preparation?
Control of Artifact Negative signal control of PRRSV EU other than Suvaxyn® **MANDATORY**	Non-specific curve/Neg	Pos*	Neg$	Valid
Sample process positive Control MRI **RECOMMENDED** *IF AVAILABLE*	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 ?
	Neg	Neg	Pos¥	Process drift: extraction (in case of exogenous IPC addition directly into qRT-PCR 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 you to determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.
$ A slight signal can be observed for IPC, the Ct value of this signal must be higher than 35.

Samples Reading

Table 8. Different types of results obtained for the samples			
Targets			
PRRSV EU other than Suvaxyn® (FAM)	Suvaxyn® vaccine strain (VIC)	Exogenous IPC (Cy5)	Interpretation
Neg¥	Pos	Pos*	**Positive or Detected** Presence of Suvaxyn® vaccine strain
Pos	Neg¥		**Positive or Detected** Presence of PRRSV EU other than Suvaxyn®
Pos	Pos		**Positive or Detected** Presence of PRRSV EU other than Suvaxyn® and Suvaxyn® vaccine strain
Pos	Pos	Neg or Ct>35	**Positive or Detected** Problem during IPC addition? Presence of inhibitors ł? Competition with the main target?
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 Exogenous IPC omission during the extraction and/or qRT-PCR Presence of inhibitors ł? Nucleic acids degradation in the sample? Extraction problem? Competition with the main 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 ł? Nucleic acids degradation in the sample? Problem during the IPC addition? Extraction problem?

* Ct value obtained depends on the thermocycler, the analyzed matrix and the extraction methods used. It must be, at most, within the range specified on the certificate of analysis (CA). IPC values, obtained from the different matrices with the methods proposed by BioSellal are available on request. BioSellal recommends that the laboratory determines its own maximum permissible IPC value based on its extraction method and thermocycler.

ł In case of suspicion of inhibition, 1) Repeat qRT-PCR by pre-diluting NA extracted to 1/10 or even 1/100 in DNase / RNase free water or 2) Resume analysis from extraction.

¥ The data obtained by BioSellal during the validation of the Bio-T kit® PRRSV DIVA demonstrated a phenomenon of competition between targets. Indeed, when a sample is strongly positive for one of the two targets, the detection sensitivity of the other target is impacted. Also, in the case of a highly positive result for PRRSV EU strains other than Suvaxyn®, there is a risk of not detecting a small amount of Suvaxyn® vaccine strain. The negative result for the vaccine strain Suvaxyn® channel is therefore to be interpreted according to the vaccination status of the animals (date and vaccination protocol, date of sampling ...). Conversely, in the case of a highly positive result for the Suvaxyn® vaccine strain, there is a risk of non-detection of a PRRSV EU strains other than Suvaxyn® in a small quantity. The negative result for the PRRSV EU other than Suvaxyn® channel is therefore to be interpreted according to the epidemiological context (date of vaccination, viral circulation ...). It is possible to check the presence of the Suvaxyn® vaccine strain in combination with a PRRSV EU strain other than Suvaxyn® by Next-Generation Sequencing. Please contact BioSellal for more information. It may also be possible to renew the sampling later after the date of vaccination.

Features of BIOTK077 and BIOTK086

False positive FAM signal in the presence of Suvaxyn® vaccine strain	It should be noted that in the presence of the vaccine strain Suvaxyn®, a slight non-specific signal for valence PRRSV EU other than Suvaxyn®(FAM labelled) can be observed. To facilitate the analysis, an artifact control tube (**orange** cap) is provided in Bio-T kit® PRRSV DIVA. A dilution range of this positive control is recommended in order to verify the absence of artifact in FAM or, in case of a positive sample in the Suvaxyn® vaccine strain channel, to establish the shape of artifact signal and thus to consider negative, all curves obtained in FAM channel appearing later or at the same level as this control.
Evaluation of the competition effect between the two targets	During the validation of Bio-T kit® PRRSV DIVA, a phenomenon of competition between the targets PRRSV EU other than Suvaxyn® and Suvaxyn® vaccine strain has been identified. The evaluation of the impact of this inhibitory effect on the detectability of the kit has shown: - Equivalent detectability in case of equimolar mixing between the two types of strains (PRRSV EU strains other than Suvaxyn® and Suvaxyn® vaccine strain). - Similar detectability when mixing in a similar amount between the two types of strains (PRRSV EU strains other than Suvaxyn® and Suvaxyn® vaccine strain). More specifically, when the Ct value in PRRSV EU strains other than Suvaxyn® is in the range of 27 to 29, the presence of a large amount of vaccine strain (Ct ≈ 25) has a limited impact on detectability. When the Ct value in the Suvaxyn® vaccine strain is of the order of 28, the presence of a large amount of PRRSV EU strain other than Suvaxyn® (Ct ≈ 25) has a limited impact on the detectability. - A loss of detectability when one of the targets is in small quantity (Ct> 29) while the other target is in high quantity (Ct ≈ 25). This loss of detectability is on average 4 Ct and can lead to the non-detection of the target in small quantity.

Notes :

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