HANDBOOK

Bio-T kit® *Toxin A* of *Pasteurella multocida*

Cat. N° BIOTK056 - 50 reactions

Detection of *Toxin A* gene of *Pasteurella multocida* (ToxA of PMT)
by real-time PCR (qPCR)
with Exogenous internal positive control (IPC)

SWINE

Sample types

- Swabs of the 2 nasal or tonsillar cavities
- Organ: tonsil biopsy
- Bacterial colonies
- Individual analysis or by pool up to 5 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® *Toxin A* of *Pasteurella* multocida has two technical handbooks:

- The extraction handbook for Bio-T kit® *Toxin A* of *Pasteurella multocida*, displaying BioSellal's recommended extraction protocols for each type of sample.
- The Bio-T kit® *Toxin A* of *Pasteurella multocida* 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® *Toxin A* of *Pasteurella multocida*.

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><td>Impact on revision / version</td><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><td rowspan="4">Examples of modifications</td><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

Recommended storage of samples at 5°C ± 3 for a maximum of 24h (Swabs) or 8 days (organs) and ≤ -16°C for a few months and ≤ -65°C beyond 1 year. In case of culture analysis, do not freeze the sample.

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 belonging to the AVIAN lines. (information available on www.biosellal.com).

Description of the Bio-T kit® *Toxin A* of *Pasteurella multocida*

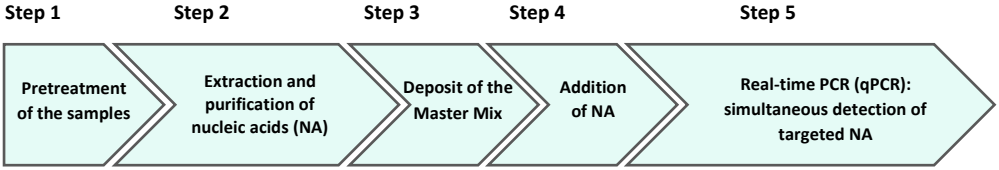
The **Bio-T kit® *Toxin A* of *Pasteurella multocida*** (Cat. N° BIOTK056) contains a ready to use **PCR Master Mix** allowing the detection **in the same reaction well of**:

- ***Toxin A* gene of *Pasteurella multocida*** (ToxA of PMT) with a 6-FAM labelling
- An **Exogenous internal positive control IPC DNA**, 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 swabs of the 2 nasal or tonsillar cavities or tonsil biopsy samples (Individual analysis or by pool up to 5 according to the matrix), was developed and validated according to the **French regulatory standard NF U47-600-2 edited by AFNOR** for the PCR part.

Extraction protocols recommended by BioSellal are described in the Bio-T kit® *Toxin A* of *Pasteurella multocida* extraction handbook.

Description of the whole process



Extraction handbook of the Bio-T kit® *Toxin A* of *Pasteurella multocida*		**qPCR handbook of the Bio-T kit® *Toxin A* of *Pasteurella multocida***		
Swabs of the 2 nasal or tonsillar cavities* Organ: tonsil biopsy* Bacterial colonies*	BioExtract® SuperBall® BioExtract® Column	Ready-to-use Master Mix MMPMT-A	Samples NC/NCS Process positive control EPC (EPCPMT-A)	Dyes: FAM/Cy5 Passive reference: ROX Program: PIG/AVIAN program ± RT Standard or Fast ramping

* pretreatment mandatory

Kit contents and storage

Table 1. Description of the kit contents				
Description	**Reference**	**Volume/tube**	**Presentation**	**Storage**
Master Mix (MM) Ready to use	MMPMT-A	750 µl	White cap tube Bag A	≤-16°C Protected from light, « MIX » Area
Exogenous Internal Positive Control (IPC)	IPC-A	250 µl	Pink cap tube Bag B	≤-16°C « Extraction » Area
External Positive Control (EPC) Positive PCR control of ToxA of PMT	EPCPMT-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°**
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

To confirm performances of your thermal cycler(s), ToxA of PMT DNA (quantified in copy number/qPCR) provided with the qPCR kit (**orange** cap tube) could be used. Please, contact BioSellal for more information (tech@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, qPCR plates preparation), "Nucleic acids Addition" (Nucleic Acids storage and addition of extracted nucleic acids and controls in the qPCR 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 nucleic acids.
- **Pathogens of PIG LINE could 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 ToxA OF PMT BY qPCR WITH BIOTK056 KIT

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 Toxina A of Pasteurella mutlocida** (**EPC ToxA of PMT**) : Synthetic DNA provided (tube **EPCPMT-A**, **orange** cap), containing specific target of ToxA of PMT.
  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 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 MMPMT-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, water, MRI or EPC**: **EPCPMT-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.

Note: if the exogenous IPC was not added during sample extraction, it can be added directly in the qPCR plate:
- Add 1 µl of IPC (**pink** 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 16 µl of this mix into each well of interest. Then add 5 µl of extracted nucleic acids.
The reaction volume will be increased to 21 µl, without impacting the performances of the qPCR.

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 3, 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. Approximate run time: 70 min.

3) Thermal cycler settings

This kit was developed and validated on ABI PRISM® 7500 Fast (Applied Biosystems) in standard ramping and confirmed on ABI PRISM® 7500 Fast (Applied Biosystems) in Fast ramping and AriaMx™ (Agilent Technologies, Fast ramping by default) . For other thermal cyclers, contact our technical support.

Table 3. 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 4. Thermal cycler Settings			
Target	**Detectors**		**Final Volume / well**
	Reporter	**Quencher**	
ToxA of PMT	FAM	NFQ-MGB or None*	**20 µl** = 15 µl Master Mix + 5 µl extracted nucleic acids or controls†
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 5. 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. Achieving a reverse-transcription (RT) step prior to PCR for the amplification of RNA genomes has no impact on the performances of the Bio-T kit® *Toxin A* of *Pasteurella multocida* (see the summary of the validation file).
NB: This amplification program is compatible with all Bio-T kits® 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 6. PCR Controls results interpretation			
	Targets		
	ToxA of PMT (FAM)	Exogenous IPC (Cy5)	Interpretation
NCS Negative Control Sample **MANDATORY**	Neg	Pos	Valid
	Pos	Pos	Contamination with a positive/negative sample during extraction step or during qPCR plate preparation.
	Neg	Neg	Omission of exogenous IPC addition? Defective extraction?
NC Negative PCR Control **OPTIONAL**	Neg	Neg	Valid
	At least one of the two targets Pos		Contamination with a negative or a positive sample during PCR plate preparation? or Master Mix / Water contamination?
EPC ToxA of PMT PCR external positive control **MANDATORY** *IN ABSENCE OF MRI*	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?
Sample 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 ?
	Neg	Pos¥	Process drift: extraction (in case of exogenous IPC addition directly into qPCR 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 determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

Samples Reading

Table 7. Different types of results obtained for the samples		
Targets		Interpretation
ToxA of PMT (FAM)	Exogenous IPC (Cy5)	
Neg	Pos*	Negative or Undetected
Pos		Positive or Detected
Pos	Neg or Ct>35	**Positive or Detected** Problem during the IPC addition? Presence of inhibitors ǂ? Competition with the main target?
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? Nucleic acids degradation in the sample? 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). Ct values for IPC using the recommended 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 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.

biosellal

www.biosellal.com

Technical Support

tech@biosellal.com +33 (0) 4 26 78 47 62

Information and orders

contact@biosellal.com +33 (0) 4 26 78 47 60