HANDBOOK

Bio-T kit® BTV all genotypes

Cat. N° BIOTK028 - 100 reactions
Cat. N° BIOTK091 - 400 reactions

**Detection of all strains of Bluetongue Virus (BTV all genotypes)
by real-time RT-PCR (qRT-PCR)
with Endogenous internal positive control (IPC)**

RUMINANTS

Sample types

- Whole blood (on EDTA)
- Individual analysis or by pool up to 20 according to local regulations

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® BTV all genotypes has two technical handbooks:

- The extraction handbook for Bio-T kit® BTV all genotypes shared with the Bio-T kit® BTV-1, BTV-4 and BTV-8, displaying BioSellal's validated extraction protocols.
- The Bio-T kit® BTV all genotypes 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® BTV all genotypes.

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.

RUMINANTS Line

This kit belongs to the RUMINANTS line which gather a set of kits sharing common extraction and qRT-PCR protocols. It is compatible with BioSellal's other kits except with the ones belonging to the PIG and AVIAN lines (information available on www.biosellal.com).

Description of the Bio-T kit® BTV all genotypes

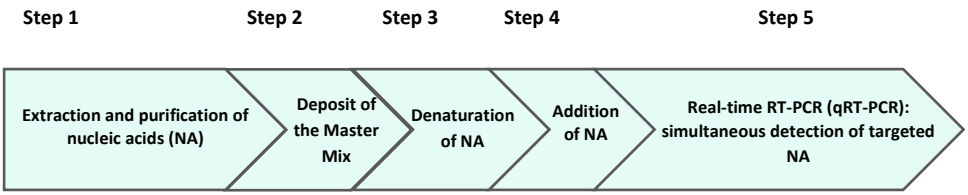
The **Bio-T kit® BTV all genotypes** (Cat. N° BIOTK028/BIOTK091) contains a ready to use **one-step RT-PCR Master Mix** allowing the detection **in the same reaction well of**:

- **all strains of Bluetongue Virus** (BTV serotype 1 to 27) with a 6-FAM labelling
- **An Endogenous internal positive control IPC** (gapdh), 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, based on qualitative dectection (detected or non-detected) from whole blood samples (Individual analysis or by pool up to 20 according to local regulation), 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 Bluetongue virus (ANSES Maison-Alfort, France).**

Extraction protocols validated by BioSellal are described in the extraction handbook shared between the Bio-T kit® BTV all genotypes, Bio-T kit® BTV-1, BTV-4 and BTV-8.

Description of the whole process



Extraction handbook shared between the Bio-T kit® BTV all genotypes, BTV-1, BTV-4 and BTV-8		qRT-PCR handbook of the Bio-T kit® BTV all genotypes			
Whole Blood	BioExtract® SuperBall® BioExtract® Column	Ready-to-use Master Mix MMBTV-B	Denaturation of BTV dsRNA	Samples NC/NCS Process positive control EPC (EPCBTV-B)	Dyes: FAM/Cy5 Passive reference: ROX Program: Classical program without RT Standard or Fast ramping

Kit contents and storage

Table 1. Description of the kit contents

Description	Reference	Volume /tube BIOTK028 100 reactions	Volume /tube BIOTK091 400 reactions	Presentation	Storage
Master Mix (MM) Ready to use	MMBTV-B	1000 µl	4x1000 µl	Transparent cap tube Bag A	≤-16°C Protected from light, « MIX » Area
External Positive Control (EPC) Positive PCR control of BTV all genotypes	EPCBTV-B	200 µl	200 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 » 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

Synthetic RNA of BTV all genotypes (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 this reagent under the following reference:

Table 3. Optional reagent*			
Reagent	**Description**	**Provider**	**Cat. N°**
BTV all genotypes RNA	Quantified RNA of BTV all genotypes (48×10^4 copies/qRT-PCR)	BioSellal	cARN-BTV -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 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.
- **Pathogen's genome detected by the RUMINANTS 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°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 BTV ALL GENOTYPES BY qRT-PCR WITH BIOTK028/BIOTK091 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 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 BTV all genotypes (EPC)**: Synthetic DNA (tube **EPCBTV-B**, **red** cap), containing specific target of BTV all genotypes.
  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 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) Denaturation of nucleic acids (NA)

Double-strand BTV RNA must be denaturated in single strand before to perform reverse-transcription and PCR.
To this purpose:

- Take 10 to 15 µl of nucleic acids extracts into a microplate – seal the plate
- Incubate during 3 minutes at 95°C ± 1.5.
- Place **immediately** the nucleic acids for at least 5 minutes at 5 ° C. ± 3 to limit the renaturation of the double-strands and to avoid the introduction into the Master Mix of a solution at high temperature which could degrade the Reverse Transcriptase enzyme.

It is strongly recommended to perform the dsRNA denaturation step just prior to the preparation of the qRT-PCR plate in order to prevent renaturation of the double stranded RNAs.

3) qRT-PCR plate preparation

In the "MIX" dedicated area

1. After thawing, vortex and rapid centrifugation, **transfer 10 µl Master Mix MMBTV-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 denaturated nucleic acids (or NCS, water, MRI or EPC**: **EPCBTV-B 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, Table 6, Table 7)

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.

4) 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 on ABI PRISM® 7500 Fast (Applied Biosystems) in Fast ramping. It is compatible with all thermal cyclers with at least 6-FAM and Cy5 channels. 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**	
BTV all genotypes	FAM	NFQ-MGB or None*	**15 µl**
Endogenous IPC	Cy5	NFQ-MGB or None*	= 10 µl Master Mix + 5 µl denaturated 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), EPC or extracted MRI.

Table 6. CLASSICAL 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
	30 sec* + data acquisition	60°C

* Set 31s for some thermal cyclers such as ABI PRISM® 7500.
NB: This amplification program is compatible with all Bio-T kits® except for ones belonging to the PIG and AVIAN LINES.

For thermal cycler such as LightCycler®480 and LightCycler®96 (Roche Life Science), it is recommended to use the following program:

Table 7. 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 kits® 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 8. PCR Controls results interpretation			
	Targets		Interpretation
	BTV all genotypes (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 PCR Control **OPTIONAL**	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 BTV all genotypes PCR external positive control **MANDATORY** *IN ABSENCE OF MRI*	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?
Sample 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 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.

Note:

Endogenous IPC targets a gene expressed by ruminants 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 9. Different types of results obtained for the samples		
Targets		
BTV all genotypes (FAM)	**Endogenous IPC (Cy5)**	**Interpretation**
Neg	**Pos***	**Negative or 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 : Risk of low positive sample non- detection** = 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 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.

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