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HANDBOOK

Bio-T kit® BTV-1

Cat. N° BIOTK031 - 25 reactions

Genotyping of Blue Tongue Virus serotype 1 (BTV-1)
by real-time RT-PCR (qRT-PCR)
with endogenous internal positive control (IPC)

RUMINANTS

Sample types

- Whole Blood (on EDTA)
- Individual test

Recommended Nucleic Acids Extraction-Purification

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

Veterinary use only

DOCUMENTS MANAGEMENT

The Bio-T kit® BTV-1 has two technical handbooks:

- The extraction handbook for Bio-T kit® BTV-1 shared with the Bio-T kit® BTV all genotypes, BTV-4 and BTV-8, displaying BioSellal's validated extraction protocols.
- The Bio-T kit® BTV-1 qRT-PCR handbook, presenting the instruction information to perform the qRT-PCR.

The latest versions in use for each manual are indicated on the certificate of analysis (CA) provided with Bio-T kit® BTV-1.

Besides these two handbooks, a summary report of the validation file is available on request, contact BioSellal (contact@biosellal.com).

REVISION 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		

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Samples reading

Table 8. Different types of results for samples		
Targets		
BTV-1 (VIC)	**Endogenous IPC (Cy5)**	**Interpretation**
Neg	**Pos***	**Negative or Un-detected**
Pos		**Positive or Detected**
Pos	**Neg or Ct>35**	**Positive or Detected** RT or PCR inhibitors‡? Competition with BTV target?
Neg	**Neg or Ct>35**	**Uninterpretable** = analysis to be renewed Omission of extracted RNA during plate setup or addition not in contact with the MM during plate preparation? RT or PCR inhibitors‡? NA degradation? Trouble during NA extraction?

* The value of Ct obtained depends on the thermocycler and the extraction methods used. It must be, at most, within the range specified on the certificate of analysis (CA). BioSellal recommends that the laboratory determines its own maximum tolerated IPC value based on its extraction method and thermocycler.

‡ 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.

Main scenarios

Controls reading

Table 7. Controls results interpretation

	Targets		Interpretation
	BTV-1 (VIC)	Endogenous IPC (Cy5)	
NCS Negative Control Sample **MANDATORY**	Neg	Neg	Valid
	At least one valence Pos		Contamination with negative / positive sample during extraction or preparation of plate.
NC Negative Amplification Control **OPTIONNAL**	Neg	Neg	Valid
	At least one valence Pos		Contamination with negative / positive sample during extraction or preparation of plate or Master Mix /water contamination.
EPC BTV-1 External Positive Control **MANDATORY**	Pos*	Neg	Valid
	Neg	Neg	Master Mix error? EPC omission?
	Pos*	Pos	Contamination during the preparation of the plate by a sample.

* Ct value obtained must comply with the value given on the certificate of analysis (CA).

Note:
Endogenous IPC targets a gene expressed by ruminant's cells, thus it cannot be detected in NCS, NC and EPC.

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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 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 7 days and ≤ -16°C beyond.

RUMINANTS Line

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

Description of Bio-T kit® BTV-1

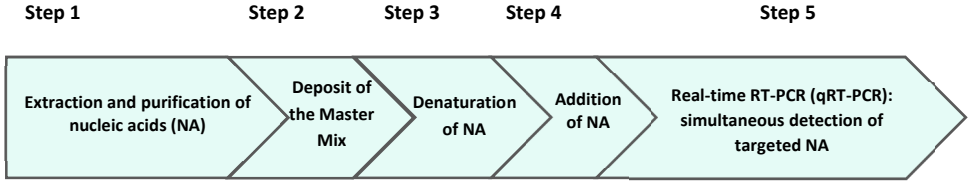
The Bio-T kit® BTV-1 (Cat. N° BIOTK031) contains a ready to use **one-step RT-PCR Master Mix** allowing the detection **in the same reaction well of**:

- **Serotype 1 of BTV** with VIC labelling,
- **A mRNA 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 can be used for the qualitative analysis of BTV-1 (detected or not detected) on samples such as whole blood. It was developed and validated according to the **French standard NF U47-600 part -2 published by AFNOR and the specifications of the French National Reference Laboratory (NRL) for Bluetongue virus from ANSES Maisons-Alfort, France.**

This kit is used for genotyping purposes on sample found positive for BTV with the Bio-T kit® BTV all genotypes. In order to guarantee the traceability of the samples, BioSellal recommends performing on the same qRT-PCR analysis, a confirmation of the BTV positivity with the Bio-T kit® BTV all genotypes, and the genotyping of the strain with the Bio-T kit® BTV-1. Thus, amplification programs are common to all BTV kits.
In order to differentiate genotyping and identification, two different dyes were chosen: 6-FAM for BTV all genotypes and VIC for genotyping kits.
If further extraction is required, validated methods are described in the Bio-T kit® all genotypes, BTV-1, BTV-4 and BTV-8 extraction handbook.

Description of the whole process



Extraction handbook shared between the Bio-T kit® BTV all genotypes, BTV-1, BTV-4 and BTV-8		Bio-T kit® BTV-1 handbook			
Whole Blood	BioExtract® SuperBall® BioExtract® Column	Ready to use Master Mix MMBTV1-A	Denaturation of BTV dsRNA	Samples NC/NCS EPC (EPCBTV1-A)	Dyes: VIC/Cy5 Passive reference: ROX Program: Classical program with RT ramping Standard or Fast

Table 5. Thermocycler settings			
Target	Dyes		Final Volume / well
	Reporter	Quencher	
BTV-1	VIC	NFQ-MGB or None*	**15 µl**
Endogenous IPC	CY5	NFQ-MGB or None*	= 10 µl MM + 5 µl NA denatured or controls†
To assign to samples and controls†			

* Depending on thermocycler, contact our technical support (tech@biosellal.com)
† Controls are NC (water), NCS and EPC (BTV-1).

Table 6. Amplification CLASSICAL PROGRAM with RT		
Ramping Standard or Fast		
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

NB: This amplification program is compatible with all Bio-T kits® except the PIG and AVIAN Lines.
* Set 31 sec for some thermocyclers as ABI PRISM® 7500.

RESULTS INTERPRETATION

To analyse 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 thermocyclers), 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 analysed in the same qRT-PCR run.
The qRT-PCR run is validated if the controls (EPC, NCS and NC) present valid results, then the result of each sample can be interpreted.

3) Preparation of the qRT-PCR plate

In the «MIX » dedicated Area

1. After thawing, vortex and rapid centrifugation of the tube, **transfer 10µl of Master Mix MMBTV1-A** (grey cap) in each well of interest (samples and controls).

⚠ *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 4 ° C 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, water, EPC**: **EPCBTV1-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 thermocycler parameters** (see Table 4, Table 5, Table 6)
5. It is recommended **to spin down the plate prior to place it into the thermocycler**, 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.

4) Thermocycler settings

This kit was developed and validated on AriaMx™ (ramping Fast) and ABI PRISM® 7500 Fast (ramping Standard or Fast. For other thermocyclers, contact our technical support.

Table 4. Thermocycler Configuration

	ABI PRISM® 7500 Fast	AriaMx™
Mode	Quantitation – Standard curve	Quantitative PCR, Fluorescence Probe
Ramping	Ramping Standard or Ramping Fast	Ramping Fast
Passive Reference	**ROX**	**ROX**

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Kit contents and Storage conditions

Table 1. Description of the kit contents

Description	*Reference*	*Volume /tube*	Presentation	Conservation
Master Mix (MM) Ready to use	MMBTV1-A	250 µl	Tube cap grey Bag A	≤-16°C Protected from light, « MIX » Zone
External Positive Control (EPC) BTV-1 positive PCR control	EPCBTV1-A	100 µl	Tube cap red Bag B	≤-16°C « Addition of Nucleic acids » Zone
Water RNase/DNase free	Aqua-A	1 ml	Tube cap blue 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°
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 thermocycler, refer to the user manual of the device.

List of reagents to confirm laboratory performance

A BTV-1 synthetic RNA (titrated in number of copies/RT-PCR), used by BioSellal in its validation file, can be used to confirm the performance of your thermocycler(s). BioSellal sells this reagent under the following reference:

Table 3. Optional reagent*

Reagent	Description	Provider	Cat. N°
RNA BTV-1	BTV-1 quantified RNA (5×10^6 copies/qRT-PCR)	BioSellal	cARN-BTV1-001

*This reagent is available only on demand, please contact BioSellal (contact@biosellal.com).

General precautions

- 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 Acid storage and addition of extracted NA and controls in the qRT-PCR plate), "PCR" (final area containing the thermocycler(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 4 ° C 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.
- **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 BTV-1 BY qRT-PCR WITH BIOTK031

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 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.

- **BTV-1 External Positive Control (EPC)**: synthetic DNA, containing the targeted sequence specific of BTV-1 (tube **EPCBTV1-A**, **red** cap)
  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.*

2) Denaturation of 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.