HANDBOOK

Bio-T kit® Coronavirus & Rotavirus bovins

Cat. N° BIOTK075 - 50 reactions

Detection of bovine Coronavirus (bCor) and Type A and C Rotavirus (RotA&C) by real-time RT-PCR (qRT-PCR) with Exogenous Internal Positive control (IPC)

BOVINE

Sample types

- Faeces collected from calves between 5 to 21 days old and suffering from neonatal enteritis
- Individual analysis

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® Coronavirus & Rotavirus bovins has two technical handbooks:

- The extraction handbook shared between all the Bio-T kit® of the STOOL line, displaying BioSellal's recommended extraction protocols.
- The Bio-T kit® Coronavirus & Rotavirus bovins 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® Coronavirus & Rotavirus bovins.

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><td>Type of modification
Highlighting color</td><td>Minor modifications</td><td>Type 1 Major modifications</td><td>Type 2 Major modifications</td></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 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.

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.

STOOL Line

This kit belongs to the STOOL line which gather a set of kits dedicated to the detection of pathogens present in ruminant's faeces and 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 contact@biosellal.com).

Description of the

Bio-T kit® Coronavirus & Rotavirus bovins

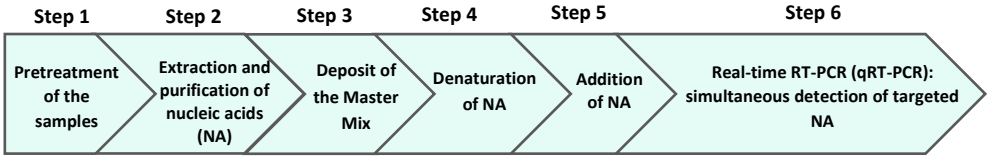
The **Bio-T kit® Coronavirus & Rotavirus bovins** (Cat. N° BIOTK075) contains a ready to use **one-step RT-PCR Master Mix** allowing the detection **in the same reaction well of**:

- **Coronavirus (bCor)** with a 6-FAM labelling
- **Type A and C Rotavirus (RotA&C)** 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 not detected) from faeces samples (Individual analysis), 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 extraction handbook of the STOOL line.

Description of the whole process



STOOL line Extraction handbook		qRT-PCR handbook of the Bio-T kit® Coronavirus & Rotavirus bovins			
Faeces*	BioExtract® SuperBall® BioExtract® Column	Ready-to-use Master Mix MMCorRotAC-A	Denaturation of Rotavirus dsRNA	Samples NC/NCS Process positive control EPC (EPCCorRotAC-A)	Dyes: FAM/VIC/Cy5 Passive reference: ROX Program: Classical program with RT Standard 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	MMCorRotAC-A	750 µl	Transparent cap tube Bag A	≤-16°C Protected from light, « MIX » Area
Exogenous Internal Positive Control (IPC)	IPCRNA-A	250 µl	**Purple** cap tube Bag B	≤-16°C « Extraction » Area
External Positive Control (EPC) Positive PCR control of RotA&C and bCor	EPCCorRotAC-A	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°**
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
BioPrep Coronavirus & Rotavirus bovins	Sample prep extraction kit for Coronavirus & Rotavirus detection (50) 15 ml conical bottom tube containing 2 glass beads	BioSellal	PREPbCR

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 bovine Coronavirus and Type A and C Rotavirus titrated in number of copies/qRT-PCR) used by BioSellal for the validation of the kit can be used to confirm the performances of your thermal cycler(s). BioSellal sells these reagents under the following references:

Table 3. Optional reagents*			
Reagent	**Description**	**Provider**	**Cat. N°**
Bovine Coronavirus RNA	Bovine Coronavirus quantified RNA (1.2×10^5 copies/qRT-PCR)	BioSellal	cARN-bCor-001
Bovine Type A and C Rotavirus RNA	Bovine type A Rotavirus quantified RNA (2.4×10^5 copies/qRT-PCR)	BioSellal	cARN-RotA -001

* These reagents are available only on demand, please contact BioSellal (contact@biosellal.com).
Note: The quantified Type C Bovine Rotavirus RNA can be provided on demand.

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.
- **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 BOVINE CORONAVIRUS AND ROTAVIRUS BY qRT-PCR WITH BIOTK075

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 bovine Coronavirus and Rotavirus (EPC)**: Synthetic DNA provided (tube **EPCCorRotAC-A**, **red** cap), containing specific target of bCor and RotA.
  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 Faeces 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.

2) Denaturation of nucleic acids (NA)

Double-strand bCor/RotA&C 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 15 µl Master Mix MMCorRotAC-A (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**: **EPCCorRotAC-A 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 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 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, 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: 90min.

4) 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. It is compatible with all thermal cyclers with at least 6-FAM, VIC and Cy5 channels. For more information, 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	Fast Ramping by default
Passive Reference	**ROX**	**ROX**

Table 5. Thermal cycler Settings

Target	Detectors		Final Volume / well
	Reporter	Quencher	
bCor	FAM	NFQ-MGB or None*	**20 µl** = 15 µl Master Mix + 5 µl extracted nucleic acids or controls†
RotA&C	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), extracted MRI and EPC.

Table 6. CLASSICAL Amplification program settings with RT		
Standard 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 bCor (FAM)	Targets RotA&C (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	Neg	Neg	Neg	Valid
OPTIONAL	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 bCor and RotA&C 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 MRSI*	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 determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

Samples Reading

Table 9. Different types of results obtained for the samples			
Targets			Interpretation
bCor (FAM)	RotA&C (VIC)	Exogenous IPC (Cy5)	
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 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 IPC exogenous omission during the extraction and/or qRT-PCR Presence of inhibitors ǂ? 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 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). 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.

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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