HANDBOOK

Bio-T kit® Avian & Swine Influenza Virus

Cat. N° BIOTK076 - 100 reactions

Detection of Avian and Swine Influenza Virus Type A (ASIV) by real-time RT-PCR (qRT-PCR) with endogenous (IPC) and exogenous (IC) internal positive control

AVIAN & SWINE

Sample type in birds

- Tracheal or oropharyngeal swabs
- Cloacal swabs
- Organs or organs homogenates
- Individual analysis or by pool up to 10, according to local regulations and according to the type of matrix and, unless otherwise indicated, according to the animal species, the geographical origin and the sampling date.

Sample type in swine*

- Nasal swabs;
- Tracheobronchial lavages;
- Organs or organs homogenates
- Allantoic fluid or cell supernatant.
- Individual analysis or by pool up to 10, according to local regulations and according to the type of matrix and, unless otherwise indicated, according to the animal species, the geographical origin and the sampling date.

* Only Avian influenza virus detection has been evaluated by the NRL for Avian Influenza (ANSES Ploufragan-Plouzané, VIPAC Unit). Also, the Avian Bio-T kit® & Swine Influenza Virus can only be used on suids for research purposes.

Recommended Nucleic Acids Extraction-Purification

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

Veterinary use only

DOCUMENTS MANAGEMENT

The Bio-T kit® Avian & Swine Influenza Virus has two technical handbooks:

- The extraction handbook shared between the Bio-T kit® Avian & Swine Influenza Virus, Bio-T kit® AIV genotypes H5 & H7 and Bio-T kit® AIV genotype H9, displaying BioSellal's validated extraction protocols for each type of sample.
- The Bio-T kit® Avian & Swine Influenza Virus qPCR 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® Avian & Swine Influenza Virus.

Besides these two handbooks, a summary report of the validation file is 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

Sampling must comply with the requirement of the relevant legislation of your country and by default, with the specifications of OIE manual.

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.

AVIAN and PIG Line

This kit belongs to the AVIAN and PIG Lines of BioSellal which group together a set of kits that share common extraction and RT-PCR protocols (More information on www.biosellal.com).

Description of Bio-T kit® Avian & Swine Influenza Virus

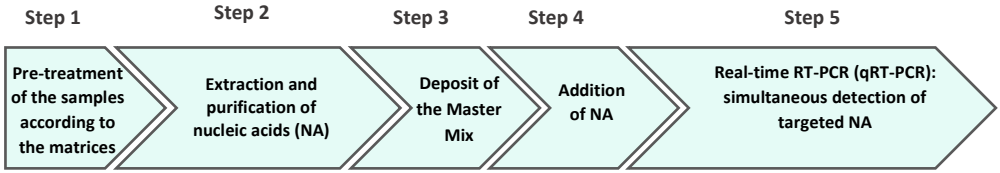
The Bio-T kit® Avian & Swine Influenza Virus (Cat. N° BIOTK076) contains a ready to use **one-step RT-PCR Master Mix** allowing the detection **in the same reaction well of**:

- **Avian and Swine Influenza Virus Type A (ASIV)** with a 6-FAM labelling
- **An exogenous internal positive control IC** (VIC labelled) to assess the absence of RT-PCR inhibitors
- **An endogenous internal positive control IPC** (beta actin), 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) on samples such as swabs and organs (Individual analysis or by pool up to 10 according to the matrix), was developed and validated according to the **French standard NF U47-600-2 published by AFNOR and specifications of the French National Reference Laboratory (NRL) for Avian Influenza (AI) from Anses-Ploufragan-Plouzané.**

Extraction protocols validated by BioSellal are described in the extraction handbook shared between the Bio-T kit® Avian & Swine Influenza Virus, Bio-T kit® AIV genotypes H5 & H7 and Bio-T kit® AIV genotype H9.

Description of the whole process



Extraction handbook shared between the Bio-T kit® Avian & Swine Influenza Virus, Bio-T kit® AIV genotypes H5 & H7 and Bio-T kit® AIV genotype H9		**qRT-PCR handbook of the Bio-T kit® Avian & Swine Influenza Virus**		
For avian species - Tracheal or oropharyngeal swabs* - Cloacal swabs* - Organs or organs homogenates * For suidae species - Nasal swabs* - Tracheobronchial lavages - Organs or organs homogenates * - Allantoic fluid or cell supernatant	BioExtract® SuperBall®¥ BioExtract® Column	Ready to use Master Mix MMASIV-A	Samples NC/NCS EPC (EPCASIV-B)	Dyes: FAM/VIC/Cy5 Passive reference: ROX Program: PIG & AVIAN with RT ramping Standard or Fast

* pre-treatment mandatory

¥ Validated in combination with the standard 38-minute program on all samples and in short 19-minute programs only on tracheal, oropharyngeal or cloacal swabs.

Kit contents and storage

Table 1. Description of the kit contents

Description	Reference	Volume/tube	Presentation	Conservation
Master Mix (MM) Ready to use	MMASIV-A	2x750 µl	Tube cap grey Bag A	≤-16°C Protected from light, « MIX » Area
External Positive Control (EPC) ASIV PCR Positive control	EPCASIV-B	110 µl	Tube cap red Bag B	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml	Tube cap blue Bag B	5°C ±3 or ≤-16°C « Addition of Nucleic acids » Area
Exogenous Internal Control (IC)	IPCRNA-A	500 µl	Tube cap purple Bag C	≤-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 the kit

Table 2. Consumables and reagents not included in the kit

Consumable / Reagent	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 thermocycler, refer to the user manual of the device.

List of reagents to confirm laboratory performance

A ASIV synthetic RNA (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 thermocycler(s). BioSellal sells this reagent under the following references:

Table 3. Optional reagent*

Reagent	Description	Provider	Cat. N°
ASIV RNA	quantified ASIV RNA (number of copies/qRT-PCR)	BioSellal	cARN-ASIV-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 Master Mix 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 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 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 AVIAN & SWINE INFLUENZA VIRUS BY qRT-PCR WITH BIOTK076

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.

- **ASIV External Positive Control (EPC)**: synthetic DNA, containing the targeted sequence specific of ASIV (tube EPCASIV-B, **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.*

- **If available, Positive Control Sample, MRI :** For each of the extraction methods it is recommended to include a positive sentinel process control (MRI), consisting of a low positive sample (Tracheal or oropharyngeal swabs, Cloacal swabs, Organs or organs homogenates, Nasal swabs, Broncho alveolar liquid) is extracted at the same time as the samples in one or more specimens (depending on the number of samples analysed). After qRT-PCR, Ct values of this extraction indicator will be transferred and tracked over time on a control card. The fact of obtaining, after extraction and qRT-PCR expected Ct values with this positive control validates the entire method.

Note: Exogenous IC (IPCRNA-A, tube with **purple caps**) supplied in Bio-T kit® Avian & Swine Influenza Virus should be used during extraction for each sample and extraction controls (NCS, MRI).

2) Preparation of the qRT-PCR plate

In the «MIX » dedicated Area

1. After thawing, vortex and rapid centrifugation of the tube, **transfer 15µl of Master Mix MMASIV-A** (grey 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 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: EPCASIV-B**, **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.

Note: if the exogenous IC was not added during sample extraction, it can be added directly in the qRT-PCR plate:
- Add 1 µl of IC (**purple** cap) with the extracted nucleic acids
- Or add directly the IC (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 effectiveness 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 thermocycler parameters** (see **Table 4**, **Table 5**, **Table 6**)

5. It is recommended to **spin the plate down prior to place it in the thermocycler**, to prevent drops in the well pit walls.

6. Start the qPCR program. Approximate run time: 90 min.

3) Thermocycler settings

This kit was developed and validated on AriaMx™ (ramping Fast) and ABI PRISM® 7500 Fast (ramping Standard or Fast). 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. Thermocycler configuration

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

Table 5. Thermocycler Settings

Target	Detectors		Final Volume / well
	Reporter	Quencher	
ASIV	FAM	NFQ-MGB ou None*	**20 µl** = 15 µl Master Mix + 5 µl extracted nucleic acids or controls†
Exogenous IC	VIC	NFQ-MGB ou None*	
endogenous IPC	Cy5	NFQ-MGB ou None*	
To assign to samples and controls†			

* Depends on the thermocycler model. Do not hesitate to contact the BioSellal Technical Support (tech@biosellal.com)
† Controls are NC (water), NCS (extracted water) and/or EPC.

Table 6. PIG/AVIAN Amplification program settings with RT

Ramping Standard ou Fast		
Cycles	**Temps**	**Température**
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.
*Please note that elongation time of 45 seconds is a critical parameter for the sensitivity of the Bio-T kit® Avian & Swine Influenza Virus.

RESULTS INTERPRETATION

To analyze 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 analyzed 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.

Main scenarios

Controls reading

Table 7. PCR Controls results interpretation

	Targets			Interpretation
	ASIV (FAM)	Exogenous IC (VIC)	Endogenous IPC (Cy5)	
NCS Negative Control Sample **MANDATORY**	Neg	Pos	Neg	Valid
	Pos / Neg	Pos	Pos/ Neg	If at least the target ASIV or endogenous IPC is positive: Contamination with a positive sample during extraction step or during qPCR plate preparation.[α]
NC Negative PCR Control **OPTIONAL**	Neg	Neg	Neg	Valid
	At least one of three targets Pos			Contamination with a negative or a positive sample during PCR plate preparation? or Master Mix / Water contamination?[α]
EPC ASIV PCR external positive control **MANDATORY**	Pos*	Neg	Neg	Valid
	Neg	Neg	Neg	Problem during qRT-PCR plate preparation: Master Mix error? EPC omission?
	Pos *	At least one of two targets Pos		Contamination with a sample during qPCR plate preparation?
Sample process positive Control MRI **RECOMMENDED** *(IF AVAILABLE)*	Pos†	Pos†	Pos¥	Valid
	Neg	Neg	Neg/ Pos¥	Problem during qRT-PCR: Master Mix error? Nucleic acids extract omission or extract not in contact with Master Mix? Process drift: extraction (in case of exogenous IPC addition directly into qPCR 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 thermocycler, the sample type and the used extraction protocol. Ct values for IC and IPC using the validated extraction protocols are available upon request. BioSellal recommends you determine your own maximal IC/ IPC Ct value depending on your own extraction method and thermocycler.

α: Endogenous IPC targets a gene expressed by swine and avian cells, thus it cannot be detected in NCS, NC and EPC. Due to cross-reaction between avian & swine β-actin and human β-actin, a slight signal can be observed for IPC in the negative controls, the Ct value of this signal must be lower than 35.

Samples reading

Table 8. Different types of results for samples			
	Targets		
ASIV (FAM)	Exogenous IC (VIC)	Endogenous IPC (Cy5)	Interpretation
Neg	Pos*	Pos*	**Negative or Un-detected**
Pos			**Positive or Detected**
Pos	Pos*	Neg or Ct>35	**Positive or Detected** Quantity of cells insufficient? Competition with ASIV target?
	Neg or Ct>35	Pos*	Omission of IC addition? Trouble during nucleic acids extraction?
		Neg or Ct>35	RT or PCR inhibitors[ł]?
Neg	At least one valence Neg or Ct>35		**Uninterpretable** = repeat the analysis Omission of nucleic acids during plate setup or addition not in contact with the Master Mix during plate preparation? Omission of IC addition? RT or PCR inhibitors[ł]? nucleic acids degradation? Trouble during nucleic acids extraction?

* The value of Ct obtained depends on the thermocycler, the matrix analysed 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 validated by Biosellal, are presented in the validation file of the Bio-T kit®ASIV. BioSellal recommends that the laboratory determines its own maximum tolerated IPC value based on its extraction method and thermocycler.

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

Sample Reading: In case of IC exogenous addition

Table 9. Different types of results obtained for the samples if the exogenous IC was added during the extraction or the qRT-PCR			
	Targets		
ASIV (FAM)	Exogenous IC (VIC)	Endogenous IPC (Cy5)	Interpretation
Neg	Pos*	Pos†	**Negative or Undetected**
Pos			**Positive or Detected**
Pos	Pos*	Neg or Ct>35	**Positive or Detected** Quantity of cells insufficient? (only if endogenous IPC is negative or Ct >35) Omission of IC addition? (only if exogenous IC is negative) Presence of inhibitors ǂ? Competition with ASIV target? Extraction problem?
	Neg or Ct>35	Pos†	
		Neg or Ct>35	
Neg	Neg or Ct>35	Pos†	**Uninterpretable** **Risk of low positive sample non- detection** = repeat the analysis Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Sampling problem: Quantity of cells insufficient? (only if endogenous IPC is negative or Ct >35 and exogenous IC is pos) Extraction problem? Competition between targets? Nucleic acids extract omission or extract not in contact with Master Mix? (only if both controls are negative)
		Neg or Ct>35	
	Pos*	Neg or Ct>35	

* Obtained Ct values depends on the thermocycler, the analysed matrix and the extraction methods used. For IC, it must be, at most, within the range specified on the certificate of analysis (CA). IPC and IC values, obtained from the different matrices with the methods validated by Biosellal, are available upon request. BioSellal recommends that the laboratory determines its own maximum tolerated IC/IPC value based on its extraction method and thermocycler.

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

Samples Reading: in the absence of IC exogenous

Table 10. Different types of results obtained for the samples if the IC exogenous was not added during the extraction or the qRT-PCR		
Targets		
ASIV (FAM)	IPC endogenous (Cy5)	Interpretation
Neg	Pos†	**Negatif or Undetected**
Pos		**Positive or Detected**
Pos	Neg or Ct>35	**Positive or Detected** Lack of hostcells? Presence of inhibitors ǂ? Competition with the main target? Extraction problem?
Neg	Neg or Ct>35	**Ininterprétable** **Non- detection risk of law positive samples** = repeat the analyse Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Sampling problem: lack of cells? Nucleic acids extract omission or extract not in contact with Master Mix?

† The obtained Ct value depends on the thermocycler, the analysed matrix and the extraction methods used. IPC values, obtained from the different matrices with the methods validated by Biosellal, are available upon request. BioSellal recommends you determine your own maximal IPC Ct value depending on your own extraction method and thermocycler.

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.

Note:

biosellal

www.biosellal.com

Technical Support

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

Information and order

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