HANDBOOK

Bio-T kit® AIV genotypes H5 & H7

Cat. N° BIOTK082-50 - 50 reactions
Cat. N° BIOTK082-100 - 100 reactions

**Detection of Avian Influenza Virus Type A Subtype H5 (H5) and Avian Influenza Virus Type A Subtype H7 (H7)
by real-time RT-PCR (qRT-PCR)
with Endogenous internal positive control (IPC)**

AVIAN

Sample types

- Tracheal or oropharyngeal swabs
- Cloacal swabs
- Organs or supernatants of organs homogenates
- Individual analysis or by pool up to 5 according to the matrix and, unless otherwise indicated, according to the animal species, the geographical origin and the sampling date.

Recommended nucleic acids (NA) extractions

- 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® AIV genotypes H5 & H7 has two technical handbooks:

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

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

Sampling and preservation of samples must comply with the requirement indicated in the French standard NF U47-210.

Storage after reception

Recommended storage of samples at 5°C ± 3 for a maximum of 24h and ≤ -65°C beyond.

AVIAN Line

This kit belongs to the AVIAN line which gather a set of kits sharing common extraction and qRT-PCR protocols, unless exception (information available on contact@biosellal.com).

Description of the Bio-T kit® AIV genotypes H5 & H7

The Real-Time RT-PCR technique reveals the presence of target nucleic acids (NA) accurately and quickly.
The **Bio-T kit® AIV genotypes H5 & H7** (Cat. N° BIOTK082-50/BIOTK082-100) contains a ready to use **one-step RT-PCR Master Mix** allowing the detection **in the same reaction well of**:

- **Avian Influenza Virus Type A Subtype H5 targeting the HA gene** (segment 4), with a 6-FAM labelling
- **Avian Influenza Virus Type A Subtype H7 targeting the HA gene** (segment 4), with a VIC labelling
- An mRNA **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.

Scope of application of the Bio-T kit® AIV genotypes H5 & H7

This kit is based on qualitative detection of Avian Influenza Virus Type A Subtypes H5 and H7 (detected or not detected).

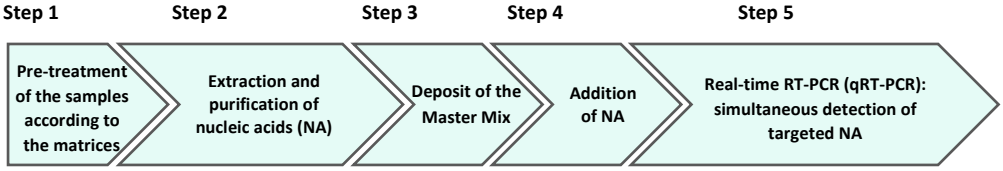
The Bio-T kit® AIV genotypes H5 & H7 was developed and validated according to the French regulatory standard NF U47-600-2 edited by AFNOR and the specification of the French National Laboratory (NRL) for the Avian Influenza (AI) from Anses-Ploufragan-Plouzané. It has been initially validated by the NRL of the AI for use :

- in a context of self-control on tracheal and cloacal swab matrices,

- in a context of official diagnosis (clinical suspicion) on tracheal and cloacal swab matrices, organs (intestines, brain, internal organs mixture such as trachea, spleen, heart, liver kidney, lung) or supernatant of organs homogenates (intestines, brain, internal organs mixture).

The used of any other matrices and in any other context (epidemiological monitoring,…) hasn't been validated by the NRL for AI.

Description of the whole process



Extraction handbook shared between the Bio-T kit® Avian & Swine Influenza Virus, Bio-T kit® AIV genotypes H5 & H7, Bio-T kit® AIV genotype H9 and Bio-T kit® NDV		**qRT-PCR handbook of the Bio-T kit® AIV genotypes H5 & H7**		
Tracheal or oropharyngeal swabs* Cloacal swabs* Organs or organs homogenates	BioExtract® SuperBall®¥ BioExtract® Column	Ready-to-use Master Mix MMH5H7-A	Samples NC/NCS Process positive control EPC (EPCASIV-B)§	Dyes: FAM/VIC/Cy5 Passive reference: ROX **Program: specific H5&H7** ramping Standard or Fast

* pre-treatment mandatory

¥ : Validated in association with standard 38 minutes program on all samples and in short 19 minutes program only on tracheal, oropharyngeal or cloacal swabs.

§ : EPC is common to the Bio-T kit® Avian & Swine Influenza Virus and Bio-T kit® AIV genotypes H5 & H7.

Kit contents and storage

Table 1. Description of the kit contents					
Description	**Reference**	***Volume /tube***		**Presentation**	**Storage**
		BIOTK082-50 50 reactions	BIOTK082-100 100 reactions		
Master Mix (MM) Ready to use	MMH5H7-A	750 µl	2x750 µl	tube grey cap Bag A	≤-16°C Protected from light, « MIX » Area
External Positive Control (EPC)[0] Positive PCR control of H7 and H5	EPCASIV-B	110 µl		tube red cap Bag B	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml		tube blue cap Bag B	5°C ±3 or ≤-16°C « Addition of Nucleic acids » Area

[0] : EPC is common to the Bio-T kit® Avian & Swine Influenza Virus and Bio-T kit® AIV genotypes H5 & H7.
Kit reagents are stable until the experimentation date on the label, subject to compliance with good storage conditions.

List of reagents to confirm laboratory performances

For adoption of RT-PCR and method, synthetic RNA of ASIV, H5 and H7, (titrated in number of copies/RT-PCR) and viral suspension of subtype H5 and H7 inactivated virus (titrated in equivalent LD_{METHOD} of NRL for AI) used by BioSellal in validation file are required. These materials are available from the NRL for AI. An internal reference material (MRI) of tracheal swab matrix (titrated in equivalent LDMETHOD of BioSellal), allowing to follow the good control of the analytical procedures, is proposed by BioSellal. This MRI is common to the Bio-T kit® Avian & Swine Influenza Virus and Bio-T kit® AIV genotypes H5 & H7.
BioSellal sells this reagent under the following references:

Table 2. Consumables and reagents not included in kit			
Reagent	**Description**	**Provider**	**Cat. N°**
Tracheal swab MRI	Tracheal swab positive for ASIV and double positive for H5 and H7 Titrated at 20 LD_{METHOD} BioSellal for ASIV, 1 LD_{METHOD} for H5 and 10 LD_{METHOD} for H7¥	BioSellal	MRI-AIV-001

¥ For H7 valence, MRI is titrated at 10 $LD_{METHODE}$ BioSellal for BioExtract® Column and BioExtract® SuperBall® extraction methods with classical program 38 minutes and at 1 $LD_{METHODE}$ BioSellal for BioExtract® SuperBall® extraction method with short program 19 minutes
For MRI of matrices other than tracheal swabs, please contact BioSellal (contact@biosellal.com).

List of consumables and reagents not included in kit

Table 3. Consumables and reagents not included in kit			
Consumables/ Reagents	**Description**	**Fournisseur**	**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.

Removal of Reagents Modalities

The implementation of Bio-T kit® AIV genotypes H5 & H7 doesn't generate any risk for the manipulator and the environment.

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 (NA) Addition" (Nucleic Acids storage and addition of extracted NA 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 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 SUBTYPE H5 AND H7 OF AVIAN INFLUENZA BY qRT-PCR WITH BIOTK082-50/BIOTK082-100

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 Subtypes H75 and H57 (EPC)** : Provided synthetic DNA (tube **EPCASIV-B**, **red** cap), containing specific target of H5 and H7. It also contains the specific sequences targeted by the Bio-T Kit® Avian & Swine Influenza Virus. The EPC is common for these two kits.
  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.*

- **Process Positive Control (MRI),** a weak positive sample of interest matrix 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) qPCR plate preparation

In the “MIX” dedicated area

1. After thawing, vortex and rapid centrifugation, **transfer 15 µl Master Mix MMH5H7-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 Acids addition” dedicated area

2. **Add 5 µl of extracted nucleic acids (or NCS, water, Process Control or EPC**: **EPCASIV-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)
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: 90 min.

3) 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 and Fast ramping. 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	Ramping Standard or Ramping Fast	Ramping Fast by default
Passive Reference	**ROX**	**ROX**

Table 5. Thermal cycler Settings			
Target	**Detectors**		**Final Volume / well**
	Reporter	**Quencher**	
H5	FAM	NFQ-MGB ou None*	**20 µl** = 15 µl Master Mix + 5 µl extracted nucleic acids or controls†
H7	VIC	NFQ-MGB ou None*	
Endogenous IPC	Cy5	NFQ-MGB ou 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), MRI and EPC.

Table 6. H5 & H7 Amplification program		
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
	60 sec¥ + data acquisition	**54°C¥**

¥The sensitivity analyzes carried out on the critical steps of the Bio-T kit® AIV genotypes H5 & H7 RT-PCR protocol showed that the variation of +/- 10% in nucleic acid and elongation time and +/- 1°C of the hybridization temperature, have no impact on the performance of the test. However, the use of the PIG/AVIAN program with RT, used for the Bio-T kit® Avian & Swine Influenza Virus and Bio-T kit® AIV genotype H9 doesn't maintain kit's performance. It is therefore very important to use the specific program H5 & H7.

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 7. PCR Controls results interpretation

	Targets			Interpretation
	H5 (FAM)	H7 (VIC)	Endogenous IPC (Cy5)	
NCS Negative Control Sample **MANDATORY**	Neg	Neg	Neg or Ct≥35 α	Valid
	At least one of the three targets Pos			Contamination with a positive/negative sample during extraction step or during qPCR plate preparation.α
NC Negative PCR Control **OPTIONAL**	Neg	Neg	Neg or Ct≥35 α	Valid
	At least one of the three targets Pos			Contamination with a positive/negative sample during extraction step or during qPCR plate preparation or Master Mix/water contamination.α
EPC H5 and H7 PCR external positive control **MANDATORY** *IN ABSENCE OF MRSI*	Pos*	Pos*	Neg or Ct≥35 α	Valid
	Neg	Neg	Neg or Ct≥35 α	Problem during qRT-PCR plate preparation: Master Mix error? EPC omission?
	Pos*	Pos*	Pos	Contamination with a sample during qPCR plate preparation?
Sample process positive Control MRSI **RECOMMENDED** *IF AVAILABLE*	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 ? 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. Ct values for IPC using the validated extraction protocols are presented in the validation file of the Bio-T kit® AIV genotypes H5 & H7. BioSellal recommends you determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

α: Endogenous IPC targets a gene expressed by avian cells, thus it cannot be detected in NCS, NC and EPC.

Due to cross-reaction between avian 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 obtained for the samples			
Targets			
H5 (FAM)	H7 (VIC)	IPC Endogenous (Cy5)	Interpretation
Neg	**Pos**	**Pos***	**Positive or Detected** **Presence of Avian Influenza Virus Type A and of Subtype H7**
Pos	**Neg**		**Positive or Detected** **Presence of Avian Influenza Virus Type A and of Subtype H5**
Pos	**Pos**		**Positive or Detected** **Presence of Avian Influenza Virus Type A and of Subtype H5 and H7**
Pos	**Pos**	**Neg or Ct>35**	**Positive or Detected** Competition with the main target? Lack of host cells? Presence of inhibitors ǂ? Sampling problem: lack of cells?
One of the targets is **Neg**		**Neg or Ct>35**	**Positive or Detected** **For the positive target** **Uninterpretable for the negative target** **Non- detection risk of law positive sample** = Repeat the analysis Competition with the main target? Extraction problem? Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Sampling problem: lack of cells?
Neg	**Neg**	**Neg ou Ct>35**	**Uninterpretable** **Non- detection risk of law positive sample** = 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? Sampling problem: lack of cells? Extraction problem?

* The obtained Ct value depends on the thermal cycler, the sample type and the used extraction protocol. Ct values for IPC using the validated extraction protocols are presented in the validation file of the Bio-T kit® AIV genotypes H5 & H7. 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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