HANDBOOK

Bio-T kit® AIV Genotyping H9

Cat. N° BIOTK083 - 50 reactions

Detection of Avian Influenza Virus Type A Subtype H9 (H9)
by real-time RT-PCR (qRT-PCR)
with Exogenous internal positive control (IC)

AVIAN

Sample types

- 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.

Recommended nucleic acids (NA) extractions

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

Veterinary use only

DOCUMENTS MANAGEMENT

The Bio-T kit® AIV Genotyping H9 has two technical handbooks:

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

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 IC 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 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 Genotyping H9

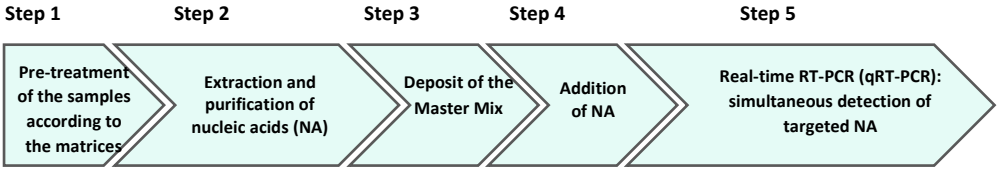
The **Bio-T kit® AIV** Genotyping H9 (Cat. N° BIOTK083) contains a ready to use **one-step RT-PCR Master Mix** allowing the detection **in the same reaction well of**:

- **Avian Influenza Virus Type A Subtype H9 (H9)** with a 6-FAM labelling
- An **Exogenous internal positive control IC** 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 non-detected) from tracheal or oropharyngeal swabs, cloacal swabs, organs or organ homogenates samples (Individual analysis or by pool up to 10 according to the matrix), 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 for Avian Influenza (AI) from Anses-Ploufragan-Plouzané.**

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

Description of the whole process



Extraction handbook shared between the Bio-T kit® Avian & Swine Influenza Virus, Bio-T kit® AIV genotyping H5 & H7 and Bio-T kit® AIV Genotyping H9		**qRT-PCR handbook of the Bio-T kit® AIV Genotyping H9**		
Tracheal or oropharyngeal swabs * Cloacal swabs* Organs or organ homogenates	BioExtract® SuperBall® BioExtract® Column	Ready-to-use Master Mix MMH9-A	Samples NC/NCS EPC (EPCH9-A)	Dyes: FAM/Cy5 Passive reference: ROX Program: PIG/AVIAN program with RT ramping Standard or Fast

* pre-treatment mandatory

Kit contents and storage

Table 1. Description of the kit contents				
Description	**Reference**	**Volume/tube**	**Presentation**	**Storage**
Master Mix (MM) Ready to use	MMH9-A	750 µl	tube grey cap Bag A	≤-16°C Protected from light, « MIX » Area
Exogenous Internal Positive Control (IC)	IPCRNA-A	250 µl	tube purple cap Bag B	≤-16°C « Extraction » Area
External Positive Control (EPC) Positive PCR control of H9	EPCH9-A	110 µl	tube red cap Bag C	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml	tube blue cap 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			
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 thermal cycler, refer to the user manual of the device.

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 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 to limit the opening times and avoid any contact with RNases present in the environment (skin, dust, working surfaces…).

DETECTION OF SUBTYPE H9 OF AVIAN INFLUENZA BY qRT-PCR WITH BIOTK083

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 H9 (EPC)**: Synthetic DNA provided (**EPCH9-A** tube, **red** cap), containing specific target of H9.
  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.*

2) qPCR plate preparation

In the "MIX" dedicated area

1. After thawing, vortex and rapid centrifugation, **transfer 15 µl Master Mix MMH9-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, EPC**: **EPCH9-A** tube**, red** cap) 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 IC was not added during sample extraction, it can be added directly in the qPCR 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 qPCR.

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 3, Table 4, Table 5)

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 3. 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 4. Thermal cycler Settings

Target	Detectors		Final Volume / well
	Reporter	Quencher	
H9	FAM	NFQ-MGB or None*	**20 µl**
Exogenous IC	Cy5	NFQ-MGB or None*	= 15 µl Master Mix + 5 µl extracted 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) and/or EPC.

Table 5. PIG/AVIAN Amplification program settings with RT

Ramping Standard or Fast		
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 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, NCS and NC) present valid results, then the result of each sample can be interpreted.

Main Scenarios

Controls Reading

Table 6. PCR Controls results interpretation			
	Targets		Interpretation
	H9 (FAM)	Exogenous IC (Cy5)	
NCS Negative Control Sample **MANDATORY**	Neg	Pos	Valid
	Pos	Pos	Contamination with a positive/negative sample during extraction step or during qPCR plate preparation.
	Neg	Neg	Omission of exogenous IC addition? Defective extraction?
NC Negative PCR Control **OPTIONAL**	Neg	Neg	Valid
	At least one of the two targets Pos		Contamination with a negative or a positive sample during PCR plate preparation? or Master Mix / Water contamination?
EPC H9 PCR external positive control **MANDATORY** *IN ABSENCE OF MRSI*	Pos*	Neg	Valid
	Neg	Neg	Problem during qRT-PCR plate preparation: Master Mix error? EPC omission?
	Pos*	Pos	Contamination with a sample during qPCR plate preparation?

* 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.

Samples Reading

Table 7. Different types of results obtained for the samples		
Targets		Interpretation
H9 (FAM)	Exogenous IC (Cy5)	
Neg	Pos*	**Negative or Undetected**
Pos		**Positive or Detected**
Pos	Neg or Ct>35	**Positive or Detected** Problem during the IC addition? Presence of inhibitors ǂ? Competition with the main target?
Neg	Neg or Ct>35	**Uninterpretable** = 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 IC 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). Ct values for IC using the validated extraction protocols are available upon request. BioSellal recommends you determine your own maximal IC 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 :

biosellal

www.biosellal.com

Technical Support

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

Information and orders

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