ADIAVET™ AIV H5-H7 REAL TIME

TEST FOR THE DETECTION OF AVIAN INFLUENZA Type A Subtypes H5 and H7 VIRUS BY REAL-TIME ENZYMATIC AMPLIFICATION (RT-PCR TEST)

References:
ADI531-50 (50 reactions)
ADI531-100 (100 reactions)

VET

In accordance with EU legislation, all Member States have AI contingency plan. Please refer to your authority for Avain flu diagnosis.

English version
NE531-01
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ADIAVET™ AIV H5-H7 REAL TIME

I. Revision history

N/A	Not Applicable (first publication)
Correction	Correction of document anomalies
Technical change	Addition, revision and/or removal of information related to the product
Administrative	Implementation of non-technical changes noticeable to the user

Note: minor typographical, grammar and formatting changes are not included in the revision history.

Release Date	Part Number	Change type	Change summary
2017/11	NE531-01		Creation

II. General information

1. Purpose of the test

ADIAVET™ AIV H5-H7 REAL TIME kit is intended to detect Avian Influenza Viruses (AIV), subtypes H5 and H7 using real-time Polymerase Chain Reaction (PCR) technology from swab, tissue, faeces, feather, FTA cards specimens of poultry and viral strain culture. **Depending of the country AI detection are subject to official control**.

2. Pathogen

All Avian influenza (AI) viruses belong to the Influenzavirus A genus of the orthomyxoviridae family. They are negative single stranded RNA viruses divided into subtypes based on two surface proteins: hemagglutinin and neuraminidase. Today, there are 16 subtypes of hemagglutinin (H1-H16) and 9 subtypes of neuraminidase (N1-N9) described.

AIV can cause severe diseases in domestic poultry, including chickens and turkeys but can also infect pheasants, quails, ducks, geese... The mammals, human including, may contract occasionnaly the influenza virus.

It exists many strains of avian influenza virus, classed into 2 levels, low and highly pathogenic. A influenza virus is classed as highly pathogenic if one of the following criteria is verified:

- Determination of pathogenicity index by intravenous (IVPI) greater than 1.2
- Presence of an amino acid sequence of the cleavage site of haemagglutinin similar to a sequence already observed for highly pathogenic IA isolates (presence of several basic amino acids)

All H5 and H7 subtypes (low and highly pathogenic) must be declared to the OIE.

Real-time PCR could be a method to obtain result within one day, with a high specificity and sensitivity.

3. Description and purpose of the test

This test is based first on the reverse transcription (RT) of RNA into complementary DNA (cDNA). Then, cDNA is amplified (PCR) by a DNA polymerase using specific primers. Both enzymatic reactions occur in the same tube (One-step RT-PCR).
Amplified products are detected in real-time thanks to a specific labelled hydrolysis probe (5'-exonulease technology).

The ADIAVET™ AIV H5-H7 REAL TIME kit enables the simultaneous detection of:

- Subtype H5 Avian influenza virus (probe labelled in FAM),
- Subtype H7 Avian influenza virus (probe labelled in Cy5),
- GAPDH, an internal control of extraction and amplification steps specific from an endogenous RNA (probe labelled with a fluorochrome read in the same spectra as VIC and HEX).

ADIAGENE validated the test using RNA purification kits (Qiagen, Macherey-Nagel). Other purification kits can be used if they have been validated by the user.

Analysis options according to the specimen:

Specimen	Individual analysis	Pool of sample is possible*, up to
Swab (tracheal, cloacal...)	☑	10
Tissue (lung)	☑	☒
Feather	☑	☒
FTA cards	☑	☒
Faeces	☑	☒

* It depends on the epidemiological case and on the quality of the specimen.
Depending of the country AI detection are subject to specific directives

III. Material and reagents

1. Reagents provided with the kit

	Kits
REF **ADI531-50**	Pack of 50 tests
REF **ADI531-100**	Pack of 100 tests

REF **ADI531-50**		
A5	amplification solution	1 x 1000 µl tube with green cap (a ready-to-use reagent)
H5-H7 CTL+	positive control H5, H7 and IPC	1 tube with purple cap (to reconstitute)
Package insert downloadable from www.biox.com		
REF **ADI531-100**		
A5	amplification solution	2 x 1000 µl tubes with green cap (a ready-to-use reagent)
H5-H7 CTL+	positive control H5, H7 and IPC	1 tube with purple cap (to reconstitute)
Package insert downloadable from www.biox.com		

2. Validity and storage

On receipt, the kit should be stored at <-15°C.
It is recommended to make fractions of A5 solution if it should be defrosted more than 3 times.
Do not defrost reagents more than 3 times.
Realtime reagents are susceptible to light: store them in the darkness.
The A5 reagent is ready to use for PCR reaction.
Do not mix reagents of two different batches.

3. Use of controls

Add 200 µl of Nuclease-free water to the "H5-H7 CTL+" tube included in the kit. Homogenize tube contents using a mixer such as vortex, at least 20 seconds. Aliquot this solution by 6 or 12 µl and store to <-15°C. For each analysis, we recommend to use 5 µl of "H5-H7 CTL+" in a well.

4. Equipment required but not supplied

Material should be Nuclease-free (e.g. autoclaved 25 minutes twice at +120°C or once 60 minutes at +121°C)

- Thermal cycler with consumables for real-time PCR: 0.2 ml PCR tubes or closed 96-wells PCR plates with optical quality.
- Class II Microbiological Safety Cabinet
- Centrifuge for microtubes, 96-wells plate
- Universal laboratory Grinder Mixer Mill or Fast Prep
- Etuve, heating baths or block heaters
- Instrument for homogenous mixing of tubes
- 96 wells plates' agitator
- 1 - 10 µl pipette, 20 - 200 µl pipette and 200 - 1000 µl pipette
- Multichannels pipette 1000 µl
- Nuclease-free filter tips
- Nuclease-free microtubes: 1.5 ml and 2 ml
- 96-well plates (ELISA-like)
- Powder-free Latex gloves
- Metal beads 3 mm (tungsten carbide or stainless-steel bead)
- 96-100% ethanol solution
- Nuclease-free water
- Sterile saline water (NaCl 8.5 g/l)
- ß-mercaptoethanol 14.5 M
- MEM medium + antibiotic (penicillin 100 IU/ml and streptomycin 100 µg/ml)

- RNA extraction kit (individual columns)

- RNeasy® Mini Kit (Qiagen, 50 extractions: ref. 74104 or 250 extractions: ref. 74106)
- QIAamp® Viral RNA (Qiagen, 50 extractions: ref. 52904 or 250 extractions: ref. 52906)

or

- NucleoSpin® RNA (Macherey-Nagel, 50 extractions: ref. 740955.50 or 250 extractions: ref. 740955.250)

-Automated DNA/RNA extraction kit using magnetic beads

See the NEKF user manual available on the web site mentioned on the certificate of analysis included in the used ADIAVET™ kit.

IV. Recommendation before the analysis of samples

Before starting the test, read the entire protocol and scrupulously respect it.

1. Precautions

Adiagene has elaborated this PCR test with the use of extraction kits from Qiagen, Macherey-Nagel. Other extraction kits can be used with a previous validation.

Follow the supplier's instructions for the storage, the preparation and the use of the extraction reagents.

Some kits include and/or need the use of toxic reagents. These reagents should be use with gloves and into chemical cabinet.

We strongly recommend that only appropriately trained personnel perform this test. Ensure the accuracy and precision of the micropipettes used. The quality of the obtained results depends upon rigorous respect of good laboratory practices.

The PCR generates large amount of amplified DNA. A few molecules of amplified products are sufficient to generate a positive result. It is important to reserve 2 rooms, one for manipulation of samples to be tested, and another one for amplified products analysis. Do not open the PCR tubes after amplification. Samples for analysis should be handled and disposed of as biological waste. **Take all measures of security and confinement required for the manipulation of the concerned biological agents.**

We recommend using fractions of demineralised and saline water and to autoclave them twice 25 minutes at +120°C or once 60 minutes at +121°C. *Take a new fraction for each new manipulation to avoid contamination.*

2. Storage of samples and DNA extracts

Samples can be stored a couple of days at +2/8°C. After 2 days, we recommend to store them at <-15°C. Extracted RNAs are quite sensitive molecules. Extraction is made at room temperature and should be performed as fast as possible to avoid degradations. We then recommend to read the entire protocol before starting the test and to rigorously respect it. Crude extracts should be stored at the end of extraction on melting ice or at +2/8°C for few hours, then at <-15°C.

3. Samples preparation

A. Swab

Add **1 swab** into **1 ml** of MEM medium + antibiotic (in order to allow an ulterior viral culture) or of sterile saline water in the tube.
Homogenize the swab.
In case of pools until 10, mix 1 volume of each individual mixture to make the pool and keep the individual mixture *<-65°C*
Pools of 10 swabs can be performed but some weak samples can be non-detected.
Take **200 µl of sample**.

Or

Add **1 to 5 swabs** into **2 ml** of MEM medium + antibiotic (in order to allow an ulterior viral culture) or of sterile saline water in the tube.
Homogenize the swab.
In case of pool of 10, mix 1 volume of each mixture to make the pool.
Take **200 µl of sample**.

See § V for the extraction and purification of RNA following the protocol specific to liquid biological samples.

B. Tissue

Put **0.1 g** of organ in a 2 ml-microtube with **1 ml** of sterile saline water
Add **1 tungsten carbide or stainless-steel bead**.
Grind twice at 30 hertz for 2 minutes.
Centrifuge 2 minutes at 6 000g.
Take **200 µl of sample**.

NB: Store the rest of the liquid at <-15°C *for a new analysis or for a viral culture.*
See § V for the extraction and purification of RNA following the protocol specific to liquid biological samples.

C. Faeces

Place 1 g of faeces
Add 5 ml of sterile saline water.
Homogenize.
Allow to settle.
Take **200 µl of sample**.
NB: Store the rest of the liquid at <-15°C *for a new analysis.*
See § V for the extraction and purification of RNA following the protocol specific to liquid biological samples.

D. Feather

Cut the calamus of 1 to 5 feathers precociously to avoid any projections and place them in 2 ml of physiological saline.
Homogenize and take **200 µl of sample.**

NB: Store the rest of the liquid at <-15°C for a new analysis.
See § V for the extraction and purification of RNA following the protocol specific to liquid biological samples.

E. FTA card

Cut the FTA card and transfer them in a 2 ml microtube
Add 1 ml of sterile saline water
Homogenize and take **200 µl of sample.**

NB: Store the rest of the liquid at <-15°C for a new analysis.
See § V for the extraction and purification of RNA following the protocol specific to liquid biological samples.

F. Viral strain culture, supernatant of cellular culture

Briefly centrifuge if necessary to clear bronchial fluids.
Take **200 µl of sample**.

NB: Store the rest of the fluid at <-15°C for a new analysis or for a viral culture.
See § V for the extraction and purification of RNA following the protocol specific to liquid biological samples.

4. Controls to include

The use of controls allows verifying the reliability of the results.
The controls are included per trial of analysis. A trial is defined as all the samples treated in the same conditions.

All the steps of the analysis procedure (extraction+amplification), for all the types of samples, are validated with the association of the controls included in the kit.

- The internal endogenous control (GAPDH) naturally found in the samples allows verifying the extraction and amplification steps of each sample.
- The H5-H7 CTL+ allows validating the amplification of the target.

Other controls must or could be added:

- **Negative control of extraction (required)**

To verify the absence of cross-contamination, at least one negative control must be included per trial (e.g. the normative requirement and recommendation for the development and the validation of veterinary PCR NF U47-600-1 suggests the use of 1 negative control for 24 samples or 4 negative samples for a 96 wells-plate). This control could be a negative matrix, or a buffer used for dilutions.

- **Positive control of extraction (recommended)**

A positive control could be added in each trial. The control is a sample including AIV H5 and H7. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of AIV H5 and/or H7. This positive control will be closed to the limit of detection of the method. It will inform about the fidelity of the obtained results between different trials.

V. Extraction and purification

All the centrifugations are performed at room temperature.

1. Using RNeasy® kit

	Liquid biological samples
Lysis	Add **350 µl** of **buffer RLT + β-mercaptethanol (10µl/ml)** to the **200 µl** of **samples** prepared as previously described. Homogenize.
Binding preparation	Add **350 µl** of **ethanol 70%**. Homogenize.
Transfer to columns and binding to the membrane	Identify columns, apply **700 µl** of the **obtained solution** to the corresponding column. Centrifuge 1 minute at 8 000 g.
1st wash	Change the collection tube and add **700 µl** of **buffer RW1**. Centrifuge 1 minute at 8 000 g.
2nd wash	Change the collection tube and add **500 µl** of **buffer RPE**. Centrifuge 1 minutes at 8 000 g.
3rd wash	Change the collection tube and add **500 µl** of **buffer RPE**. Centrifuge 3 minutes at 10 000 g.
Elution	Transfer the column to a microtube. Add **50 µl** of **Nuclease-free water**. Incubate ~2 minutes at room temperature and centrifuge 1 minute at 8 000 g.
Storage	Close the tubes, identify and store on ice if using immediately, or store to <-15°C.

2. Using NucleoSpin® RNA kit

	Liquid biological samples
Lysis	Add **350 µl** of **RA1 buffer + β-mercaptethanol (10µl/ml)** to the **200 µl** of **samples** prepared as previously described. Homogenize.
Binding preparation	Add **350 µl** of **ethanol 70%**. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).
Transfer to columns and binding to the membrane	Identify columns, apply **700 µl** of the **obtained solution** to the corresponding column. Centrifuge 30 seconds at 11 000 g.
1st wash	Change the collection tube and add **350 µl** of **buffer MDB**. Centrifuge 1 minute at 11 000 g.
2nd wash	Add **200 µl** of **buffer RAW2**. Centrifuge 30 seconds at 11 000 g.
3rd wash	Change the collection tube and add **600 µl** of **buffer RA3**. Centrifuge 30 seconds at 11 000 g.
4th wash	Change the collection tube and add **250 µl** of **buffer RA3**. Centrifuge 2minutes at 11 000 g.
Elution	Transfer the column to a microtube. Add **60 µl** of **Nuclease-free water**. Incubate ~2 minutes at room temperature and centrifuge 1 minute at 11 000 g.
Storage	Close the tubes, identify and store on ice if using immediately, or at < -15°C.

3. Using QIAamp® Viral RNA kit

	Liquid biological samples
Lysis	Add **560 µl** of **buffer AVL + RNA carrier** to the **200 µl** of **samples** prepared as previously described. Homogenize.
	Incubate at room temperature during 10 minutes. Briefly centrifuge the lysate.
Binding preparation	Add **560 µl** of **ethanol 100%**. Homogenize.
Transfer to columns and binding to the membrane	Identify columns, apply **630 µl** of the **obtained solution** to the corresponding column. Centrifuge 1 minute at 10 000 g. Change the collection tube, put the rest of the mix on the column and centrifuge 1 minute at 10 000 g.
1st wash	Change the collection tube and add **500 µl** of **buffer AW1**. Centrifuge 1 minute at 10 000 g.
2nd wash	Change the collection tube and add **500 µl** of **buffer AW2**. Centrifuge 1 minutes at 10 000 g.
Column dry step	Change the collection tube. Centrifuge 3 minutes at 10 000 g.
Elution	Transfer the column to a microtube. Add **60 µl** of **buffer AVE**. Incubate ~1 minutes at room temperature and centrifuge 1 minute at 10 000 g.
Storage	Close the tubes, identify and store on ice if using immediately, or at -< -15°C.

4. Using DNA/RNA extraction magnetic beads kit

See the NEKF user manual available on the web site mentioned on the certificate of analysis included in the used ADIAVET™ kit.

VI. Amplification

a- Determine the number samples analysed including the controls (e.g. positive and negative extraction controls, control of amplification (CTL+ and CTL-) and PCR reagent control (NTC)).

b- Thaw the A5 solution at room temperature. Homogenize. Dispense **20 µl** of A5 solution in each PCR tubes or PCR plate wells with a micropipette with a Nuclease-free tip.

c- **Immediately replace the A5 solution tube at <-15°C and in darkness.**

d- For each sample, the extraction negative control (obligatory) and the extraction positive control (recommended) add **5 µl** of purified extract to the 20 µl of A5 solution.
For the controls, add **5 µl**, per well, of each solution obtained in § III-3 to the 20 µl of A5 solution.
For the PCR reagent control (NTC), nothing is added to the A5 solution.
Immediately replace purified RNA extracts on melting ice or at **<-15°C**. Take care to have no bubbles in the bottom of the wells.

e- Store the plate or the tubes on melting ice or at +2/8°C until the cycler is programmed and start quickly the run after you have placed the plate or the tubes in the cycler.

The AIV target is read in FAM. The Internal Control is read in VIC or HEX. The Quencher is non fluorescent. The solution contains a passive reference ROX for the ABI machines. Fluorescence is read during the elongation step (1 minute at 54°C).

The following program is defined for **ABI Prism** thermalcyclers (like 7500, StepOne...) from **Applied Biosystems** (check the "emulation 9600" option if available), for the **Rotorgene** from **Qiagen** and for the **Chromo 4** from **Biorad**:
10 minutes 45°C
10 minutes 95°C
15 seconds at 95°C and 1 minute at **54°C** during 40 cycles

This program is concerning the **MX3000P** and **MX3005P** of **Stratagene**:
10 minutes 45°C
10 minutes 95°C
30 seconds at 95°C and 1 minute at **54°C** during 40 cycles

Roche diagnostic: LightCycler 2*, LightCycler 480*
**** NOTE:*** *The use of LightCycler thermalcyclers requires a calibration manipulation. Adiagene will furnish process chart and reagents required for this calibration.*

Contact us if you wish to use other thermalcyclers.

VII. Interpretation of results

1. Definitions

The **« base line »** corresponds to the background of fluorescence and qualifies the non characteristic part of the curve observed during the first cycles.

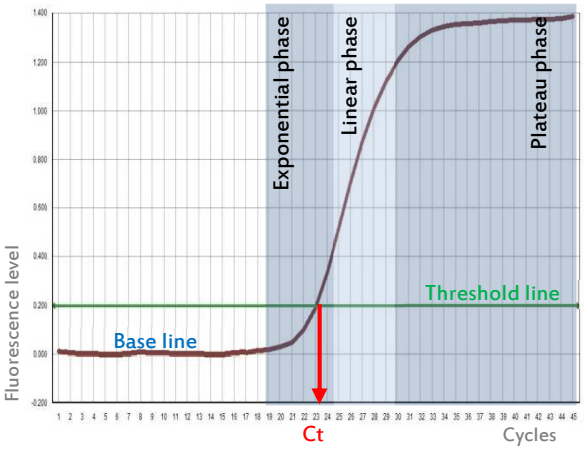
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

The **« threshold line »** has to be placed over the background in the exponential phase of a characteristic amplification curve or a group of characteristic amplification curves.

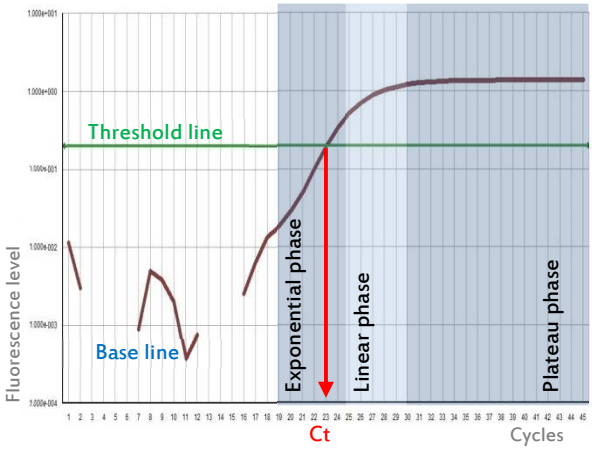
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present in the PCR well.

Example of characteristic amplification curve

Arithmetic scale of Y axis



Logarithmic scale of Y axis



2. Validation and interpretation of results

Display the FAM curves from the plate and set the threshold value as indicated above.
Proceed in the same mean for the VIC or HEX curves.

A. Validation of the run

Amplification is considered to be **valid** if the following results are obtained for the controls:

Controls	Reagent control (NTC)	H5-H7 CTL+	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	yes
Cy5 amplification	no	yes	no	yes
VIC/HEX amplification	no	yes	no	no/yes
Validation of	Absence of contamination for amplification	Amplification of the targets	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM, Cy5 and VIC/HEX dyes) of the CTL+ were indicated in the certificate of analysis of the kit.

B. Result interpretation

RNA extraction and amplification for each sample are considered to be **valid** if at least a characteristic amplification curve is observed for H5 (FAM), H7 (Cy5) or for the internal control (VIC or HEX).

Exemple	A	B	C	D	E
FAM amplification	No	Yes	No	Yes	No
Cy5 amplification	No	No	Yes	Yes	No
VIC/HEX amplification	Yes	No/Yes	No/Yes	No/Yes	No
Résultat	H5 et H7 Not detected	H5 detected H7 not detected	H7 detected H5 no detected	H5 and H7 detected	undetermined

The sample is considered as **not detected** if a characteristic amplification curve is observed in VIC or HEX without any amplification in FAM and Cy5 (A example).

The sample is considered as **H5 detected** if a characteristic amplification curve is observed in FAM (B example). Internal control can be co-amplified.

The sample is considered as **H7 detected** if a characteristic amplification curve is observed in Cy5 (C example). Internal control can be co-amplified.

The sample is considered as **H5-H7 detected** if a characteristic amplification curve is observed in FAM and Cy5 (D example). Internal control can be co-amplified.

A total absence of characteristic amplification curve for a sample (E example) shows a defective RNA extraction (lost or destruction of RNA) or a deficient real-time PCR (inhibitors in the sample, program error or no template added). In this case, we recommend first to repeat the test with pure and tenfold diluted RNA in Nuclease-free water. Then, if the test is still not valid, a new extraction is recommended.

VIII. Index of symbols

Symbol	Meaning
	Catalogue number
	Manufacturer
	Upper temperature limit
	Use by date
	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests
	Keep away from sunlight
	For veterinary in vitro use only – For animal use only

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