INSTRUCTION MANUAL

ADIAVET™ APP REAL TIME

TEST FOR THE DETECTION OF *ACTINOBACILLUS PLEUROPNEUMONIAE* BY REAL-TIME ENZYMATIC GENE AMPLIFICATION (PCR TEST)

Reference:
ADI033-100 (100 reactions)

VET

English version
NE033-02
2020/01

ADIAVET™ APP REAL TIME

I. Revision historic

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

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

Release Date	Part Number	Change type	Change summary
2016/12	NE033-01	N/A	First publication
2020/01	NE033-02	Technical change	Addition of a NF-Water tube in the kit Update of the ADIAMAG reference.

II. General informations

1. Purpose of the test

ADIAVET™ APP REAL TIME kit is intended to detect *Actinobacillus pleuropneumoniae*, **all serotypes from 1 to 15**, using real-time Polymerase Chain Reaction (PCR) technology from brush and biospies of tonsils or sample of lung from pig, as well as from bacterial culture.

2. *Actinobacillus pleuropneumoniae*

Actinobacillus pleuropneumoniae is the etiological agent of pig haemorrhagic pleuropneumonia, or actinobacillosis. This disease is the cause of economic losses in a large number of industrial farms (Gottschalk, 2012).

Actinobacillus pleuropneumoniae is a pleomorphic Gram-negative coccobacillus from the Pasteurellaceae family (Borr, 1991).

NAD requirements subdivide the *A. pleuropneumoniae* species into 2 distinct biovars: biovar 1 whose constituent strains are NAD-dependent and biovar 2, whose constituent strains are NAD-independent. Serotyping of *A. pleuropneumoniae* strains is based upon the organism's capsular polysaccharide antigens (Mittal *et al.*, 1983). In this manner, 15 serotypes have been distinguished, serotypes 1 and 5 being subdivided into 1a, 1b and 5a, 5b respectively (Jolie *et al.*, 1994 – Nielsen, 1986). There are 4 proteins cytotoxins (apxI, apxII, apxIII, apxIV). The combination of toxins makes the serotype more or less virulent. Serovar prevalence varies from country to country and with time (Gottschalk, 2015). *A. pleuropneumoniae* is frequently isolated from the nasal cavities, from tonsils and from lungs.

It is now known that other bacteria belonging to the Pasteurellaceae family are also present in the upper respiratory tract of pigs: *Haemophilus parasuis* (causative agent of Glasser's disease), *Actinobacillus minor, Actinobacillus porcinus, Actinobacillus indolicus* and *Actinobacillus taxon C* (Moller *et al.*, 1996).

All of these species share a certain number of biochemical properties with *A. pleuropneumoniae*, hence rendering their identification somewhat difficult.

3. Description and purpose of the test

This test is based on enzymatic gene amplification or PCR technology.

Amplified products are detected in real-time thanks to a specific labelled hydrolysis probe (5'-exonulease technology).

The ADIAVET™ APP REAL TIME kit enables the simultaneous detection of:

- *A. pleuropneumoniae*, **all serotypes from 1 to 15,** with probe labelled in FAM.
- The RNase P, an internal control of extraction and amplification steps specific from an endogenous DNA (probe labelled with a fluorochrome read in the same spectra as VIC and HEX).

ADIAGENE recommends using this test with DNA purification kits (Bio-X Diagnostics, Qiagen or 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
Tissue / Biospsie	☑
Brush / Swab	☑
Bacterial culture	☑

III. Material & reagents

1. Reagents provided with the kit

REF ADI033-100		
A5	amplification solution	2 x 500 µl tubes with green caps (a ready-to-use reagent)
APP CTL+	positive control *A. pleuropneumoniae*	1 tube with purple cap (to reconstitute)
NF-Water	Nuclease free Water	1 x 1000 µl tube with white cap (a ready-to-use reagent)

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 APP CTL+

APP CTL+ is a positive control of amplification.

Add **200 µl** of **NF-Water** to the **APP CTL+** tube and vortex at least 20 seconds.

Aliquot this solution by 6 or 12 µl and store them to <-15°C.

For each analysis, use **5 µl** of **APP CTL+** in one of the wells.

4. Equipment required but not supplied by Adiagene

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
- Etuve, heating baths or block heaters
- Vortex
- 1 - 10 µl pipette, 20 - 200 µl pipette and 200 - 1000 µl pipette
- Nuclease-free filter tips
- Nuclease-free microtubes: 1.5 ml and 2 ml
- Sterile tube of 5, 10 or 15 ml
- Latex or nitrile powder-free gloves
- 96-100% ethanol solution
- Sterile saline water (NaCl 8.5 g/l)
- Nuclease-free water

- DNA extraction kit (individual silica columns)

- QIAamp® DNA Mini Kit (Qiagen, 50 tests: ref. 51304 or 250 tests: ref. 51306)
- NucleoSpin® Tissue (Macherey-Nagel, 50 tests: ref.740952.50 or 250 tests: ref. 740952.250)

- Automated DNA/RNA extraction kit using magnetic beads

- ADIAMAG (Bio-X Diagnostics: réf. NADI003), 200 tests.

IV. Recommendations 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 Bio-X Diagnostics, Qiagen and Macherey-Nagel extraction kits. 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 handled with gloves and into a 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 saline water and to autoclave them 25 minutes at +120°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 DNAs are quite sensitive molecules. Crude extracts should be stored at the end of extraction on melting ice or at +2/8°C for 24 hours, then at <-15°C.

3. Controls preparation

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 Adiagene kit.

- The internal endogenous control (RNase P) naturally found in the samples verifies the extraction and amplification steps of each sample.
- The APP CTL+ valides 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. The control is a negative sample, for example 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 APP. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of APP. 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

1. Using QIAamp® DNA Mini kit

All the centrifugations are performed at room temperature.

	Biospsie/Tissue **Brush/Swab**
Preparation of the sample	Place one **biopsie or 0.1 g of tissue** or cut one **brush or swab** in the microtube. Add **1 ml of saline water.** Vortex**.** Transfer **200 µl** of surpernatant in a new microtube.
Lysis	Add **180 µl** of **ATL buffer**, **20 µl** of **proteinase K**. Vortex. Incubate **30 minutes** at **+70°C** (or a night at +56°C).
	Add **200 µl** of **AL buffer**. Vortex. Incubate **10 minutes** at **+70°C**.
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenise the mixture by pipeting (~10 times) or by vortex (~15 secondes).
Transfer to columns and binding to the membrane	Identify columns, apply the **whole** obtained solution to the corresponding column. Centrifuge 1 minute at 10 000 g. *If the whole sample has not been loaded once, apply the residual volume onto the column and centrifuge 1 minute at 10 000 g.*
1st wash	Change the collection tube and add **500 µl** of **AW1 buffer** to the column. Centrifuge 1 minute at 10 000 g.
2nd wash	Change the collection tube and add **500 µl** of **AW2 buffer** to the column. Centrifuge 1 minute 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 **200 µl** of **AE buffer**. Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.
Storage	Close the tubes, identify and store at +2/8°C for 24 hours, then at <-15°C.

2. Using NucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.

	Biospsie/Tissue **Brush/Swab**
Preparation of the sample	Place one **biopsie or 0.1 g of tissue** or cut one **brush or swab** in the microtube. Add **1 ml of saline water.** Vortex**.** Transfer **200 µl** of surpernatant in a new microtube.
Lysis	Add **180 µl** of **T1 buffer**, **25 µl** of **proteinase K**. Vortex. Incubate **30 minutes** at **+70°C** (or a night at +56°C).
	Add **200 µl** of **B3 buffer**. Vortex. Incubate **10 minutes** at **+70°C**.
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenise the mixture by pipeting (~10 times) or by vortexing (~15 secondes).
Transfer to columns and binding to the membrane	Identify columns, apply the **whole** obtained solution to the corresponding column. Centrifuge at 10 000 g/1 minute. *If the whole sample has not been loaded once, apply the residual volume onto the column and centrifuge 1 minute at 10 000 g.*
1st wash	Change the collection tube and add **500 µl** of **BW buffer** to the column. Centrifuge 1 minute at 10 000 g.
2nd wash	Change the collection tube and add **600 µl** of **B5 buffer** to the column. Centrifuge 1 minute 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 **200 µl** of **BE buffer**. Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.
Storage	Close the tubes, identify and store at +2/8°C for 24 hours, then at <-15°C.

3. Using ADIAMAG kit

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

4. Extraction from bacterial culture

	Bacterial culture
Preparation of the sample	Put **100 µl** of **Nuclease-free water** in a microtube. Take an isolated colony from an agar medium. Solubilise the colony.
	Incubate for 10 minutes at 95°C.
	Briefly centrifuge to remove condensation drops (optional).
DNA extraction - purification	N/A Directly perform the PCR analysis.

VI. Amplification

a - Determine the number of analysed samples including the controls (e.g. positive and negative extraction controls, positive control of amplification (CTL+) and No Template Control (NTC)).

b - Defrost the A5 solution at room temperature. Vortex. Dispense **10 µl** of the A5 solution in each PCR tubes or PCR plate wells with a micropipette with a Nuclease-free tip.

c- **Immediately replace the A5 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 **10 µl** of A5 solution.
For the CTL+, add **5 µl** of the solution obtained in § II-3 to the **10 µl** of A5 solution.
For the No Template Control (NTC), nothing is added to the A5 solution.
Take care to have no bubbles in the bottom of the wells.
Immediately replace purified DNA extracts at +2/8°C or at **<-15°C**.

e- Once all the tubes have been prepared, run the real-time PCR amplification.

The APP 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 60°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, for the Aria Mx from Agilent Technologies** and for the **Chromo 4** from **Biorad**:
2 minutes 50°C
10 minutes 95°C
15 seconds at 95°C and 1 minute at 60°C during 45 cycles

This program is concerning the **MX3000P** and **MX3005P from Agilent Technologies**:
2 minutes 50°C
10 minutes 95°C
30 seconds at 95°C and 1 minute at 60°C during 45 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.

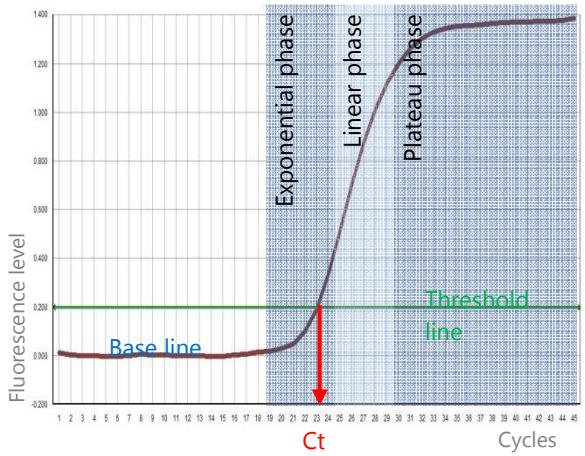
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

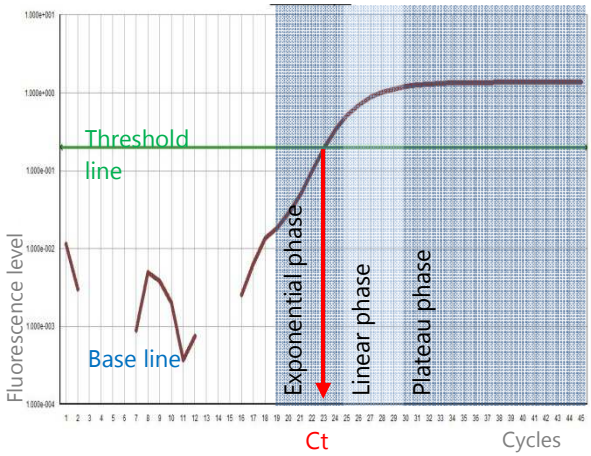
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present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

A. Validation of the run

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

Controls	No Template Control (NTC)	Amplification positive control (CTL+)	Extraction negative control	Extraction positive control *
FAM amplification	No	yes	no	yes
VIC/HEX amplification	No	no/yes	no	no/yes
Validation of	Absence of contamination for amplification	Amplification of the target	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM and VIC/HEX dyes) of the positive control (CTL+) are indicated in the certificate of analysis of the kit.

B. Result interpretation

DNA extraction and amplification for each sample are **valid** if at least a characteristic amplification curve is observed for APP (FAM) or for the internal control (VIC/HEX).

Example	A	B	C	D
FAM amplification	no	yes	yes	no
VIC/HEX amplification	yes	no	yes	no
Result	negative	positive	positive	undetermined

The sample is considered as **negative** if a characteristic amplification curve is observed in VIC or HEX without any amplification in FAM (example A).

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

A total absence of characteristic amplification curve for a sample (example D) shows a defective DNA extraction (lost or destruction of DNA) 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 DNA in Nuclease-free water. Then, if the test is still not valid, a new extraction is recommended.

VIII. References

Borr J. D., Ryan D. A. and MacInnes J. I. (1991) Analysis of *Actinobacillus pleuropneumoniae* and related organisms by DNA-DNA hybridization and restriction endonuclease fingerprinting. Int. J. Syst. Bacteriol. 41 (1): 121-129

Gottschalk, M. (2012) Actinobacillosis. In Diseases of Swine, 10th edn. Karriker, L., Ramirez, A., Schwartz, K., Stevenson, G., and Zimmerman, J. (eds). Hoboken, NJ: Wiley, pp. 653–669.

Gottschalk, M. (2015) The challenge of detecting herds sub-clinically infected with Actinobacillus pleuropneumoniae. The Veterinary Journal 206,30–38

Jolie R. A., Mulks M. H. and Thacker B. J. (1994) Antigenic differences within *Actinobacillus pleuropneumoniae* serotype 1. Vet. Microbiol. 38 (4): 329-349

Mittal K. R., Higgins R. and Larivière S. (1983) Detection of type-specific antigens in the lungs of *Haemophilus pleuropneumoniae*-infected pigs by coagglutination test. J. Clin. Microbiol. 18 (6): 1355-1357

Moller K., Fussing V., Grimont P., Paster B., Dewhirst F. and Killian M. (1996) *Actinobacillus minor* sp.nov, *Actinobacillus porcinus* sp.nov, and *Actinobacillus indolicus* sp.nov, three new V factor-dependent species from respiratory tract of pigs. Inter. J. System. Bacteriol. 37 (4): 463-464.

Nielsen R. (1986) Serological characterization of *Actinobacillus pleuropneumoniae* strains and proposal of a new serotype: serotype 12. Acta Vet. Scand. 27: 453-455

IX. 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

S.A.R.L. ADIAGENE
9, rue Gabriel Calloët-Kerbrat
22440 Ploufragan - France

RCS 417 876 299
Tel. +33 (0)2 96 68 40 20
www.biox.com