INSTRUCTION MANUAL

ADIAVET™ APP

TEST FOR THE DETECTION AND TYPING OF *ACTINOBACILLUS PLEUROPNEUMONIAE* BY ENZYMATIC GENE AMPLIFICATION (PCR TEST)

References:
ADI032-50 (50 reactions)
ADI032-100 (100 reactions)

NF032-02
2020/01

ADIAVET™ APP

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Revision historic

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 historic

Release Date	Part Number	Change type	Change summary
2017/01	NE032-01	N/A	Not Applicable (first publication)
2020/01	NE032-02	Technical change	Addition of a NF-Water tube in the kit

Preamble

The ADIAVET™ APP kit (References ADI032-50 and ADI032-100) contains strictly the same Amplification solutions A1 and A2 than those in the previous reference ADI031-0. Only the storage conditions of these 2 buffers change in order to increase the stability to 18 months. The performances are the same.

The ADIAVET™ APP kit (References ADI032-50 and ADI032-100) can be used to typing of positive samples identified with the ADIAVET™ APP REAL TIME kit Reference ADI033-100 (real time PCR kit for the detection of all APP serotypes) from the same DNA extracts.

I. General information

1. Purpose of the test

ADIAVET™ APP kit is intended to detect *Actinobacillus pleuropneumoniae* using Polymerase Chain Reaction (conventional PCR) technology and the typing of its constituent strains into 4 groups 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.

4. Description and purpose of the test

This test is based on enzymatic gene amplification or PCR technology.
Amplified products are visualised on a 2% agarose gel.

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

- *A. pleuropneumoniae* into 4 groups:

PCR typing	Serotyping
Groupe I	Serotypes 1-9-11-12*-14
Groupe II	Serotypes 5a-5b-10
Groupe III	Serotypes 2-4-7-8-12
Groupe IV**	Serotypes 3-4*-6-7-13-15

* only serotypes 4 and 12 reference strains are included in these groups
** Some *Actinobacillus lignieresii* strains can also give false positive result.

- An internal control of amplification step specific from an exogenous DNA

ADIAGENE recommends using this test with DNA purification kits (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	☑

II. Material and reagents

1. Reagents included in the kit

REF ADI032-50

A1	Amplification Solution	2 x 1250 µl tubes red caps (a ready-to-use reagent)
A2	Amplification Solution	1 x 60 µl tube red caps (a ready-to-use reagent)
E1	Size marker	1 x 100 µl tube colorless caps (a ready-to-use reagent)
E2	Loading buffer	1 x 500 µl tube blue caps (a ready-to-use reagent)
APP I CTL+	Positive control *A. pleuropneumoniae group I*	1 tube purple caps (to reconstitute)
APP II CTL+	Positive control *A. pleuropneumoniae group II*	1 tube purple caps (to reconstitute)
APP III CTL+	Positive control *A. pleuropneumoniae group III*	1 tube purple caps (to reconstitute)
APP IV CTL+	Positive control *A. pleuropneumoniae group IV*	1 tube purple caps (to reconstitute)
NF-Water	Nuclease free Water	1 x 1000 µl tube with white cap (a ready-to-use reagent)

REF ADI032-100

A1	Amplification Solution	4 x 1250 µl tubes red caps (a ready-to-use reagent)
A2	Amplification Solution	2 x 60 µl tubes red caps (a ready-to-use reagent)
E1	Size marker	1 x 100 µl tube colorless caps (a ready-to-use reagent)
E2	Loading buffer	1 x 1000 µl tube blue caps (a ready-to-use reagent)
APP I CTL+	Positive control *A. pleuropneumoniae group I*	1 tube purple caps (to reconstitute)
APP II CTL+	Positive control *A. pleuropneumoniae group II*	1 tube purple caps (to reconstitute)
APP III CTL+	Positive control *A. pleuropneumoniae group III*	1 tube purple caps (to reconstitute)
APP IV CTL+	Positive control *A. pleuropneumoniae group IV*	1 tube purple caps (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 A1 and A2 solutions if it should be defrosted more than 3 times.
Do not defrost reagents more than 3 times.
Do not mix reagents of two different batches.

On opening the kit, E1 and E2 solutions should be stored at room temperature (+18/25°C) and in the room where the amplified products are being analysed.

3. Use of APP CTL+

"APP CTL+" tubes are 4 positive controls of amplification.
Add **200 µl** of **NF-Water** to each **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 **2 µl** of each **APP CTL+** in an independent well.
Amplify each positive control separately for a better visibility on gel.

4. Equipment required but not supplied

Disposable material should be Nuclease-free or autoclaved 25 minutes at +120°C.

- Thermal cycler with PCR consumables: 0.2 ml PCR tubes or closed 96-wells PCR plates.
- A centrifuge for microtubes
- Sterile microtubes: 1.5 ml and 2 ml
- Vortex
- Latex gloves
- 2 heating baths or block heaters for microtubes
- 1-10 µl pipette, 20-200 µl pipette and 200-1000 µl pipette
- Nuclease-free filter tips
- Power supply
- Horizontal gel electrophoresis apparatus and comb stand
- Transilluminators (λ = 254 nm for ethidium bromide stain)
- Polaroid camera system
- Agarose (molecular biology grade)

- Ethidium bromide (Et Br) solution
- TBE 1X (Tris Borate EDTA) or TAE 1X (Tris Acetate EDTA) buffer
- 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)

III. DNA extraction

1. Precautions – safety instructions

Adiagene has elaborated this PCR test with the use of 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 outside the room devoted to electrophoresis.
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.**
***NOTE: Ethidium bromide is hazardous. Please review the Material Safety Data Sheet before handling. Always handle with gloves.**

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.

We recommend to include at least one negative extraction control (= extraction without sample) by run of extraction.
A positive sample in *Actinobacillus pleuropneumoniae* (culture or field sample) can be included and extracted in each run, it will be considered as an extraction positive control.

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

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.

IV. Extraction and Purification

1. Extraction using QIAamp® DNA Mini kit

All the centrifugations are performed at room temperature.

	biopsy/Tissue **Brush/Swab**
Preparation of the sample	Place one **biopsy or 0.1 g of tissue** or cut one **brush or swab** in the microtube. Add **1 ml of saline water.** Vortex. Transfer the totality of the supernatant in the new microtube. Centrifuge 10 minutes at 3 300 g. Remove the supernatant.
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. Extraction using NucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.

	biopsy/Tissue **Brush/Swab**
Preparation of the sample	Place one **biopsy or 0.1 g of tissue** or cut one **brush or swab** in the microtube. Add **1 ml of saline water.** Vortex**.** Transfer the totality of the supernatant in the new microtube. Centrifuge 10 minutes at 3300g. Remove the supernatant.
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. Extraction from bacterial culture

	Bacterial culture
Preparation of the sample	Put 100 µl of Nuclease-free water in a microtube. Or take an isolated colony from an agar medium. Solubilize 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.

IV. Amplification

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

b - Defrost the A1 and A2 solutions at room temperature. Vortex. Transfer **48** x (n+1) **µl** of **Amplification solution A1** to a sterile microtube, then add **1.2** x (n+1) **µl** of **Amplification solution A2** using a micropipette fitted with a sterile tip. Vortex the resulting solution.

c- **Immediately replace the A1 and A2 tubes at <-15°C.**

d- Dispense **48 µl** of the A1+A2 solution in each PCR tubes or PCR plate wells with a micropipette with a Nuclease-free tip

e- For each sample, the extraction negative control (obligatory) and the extraction positive control (recommended) add **2 µl** of purified extract to the 48 µl of solution.
For the APP Positives Controls, add **2 µl** of the solution obtained in § II-3 to the 48 µl of solution in independent wells.
For the No Template Control (NTC), nothing is added to the solution.
Immediately replace purified DNA extracts at +2/8°C or at **<-15°C**. Take care to have no bubbles in the bottom of the wells.

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

The following programme is defined for the GeneAmp PCR system 9700 (mode 9600), 9600, 2400 or 2700 produced by Applied Biosystems. For the Perkin-Elmer 480 system, or for any other thermal cycler, contact us.

Cycle :

Once	94°C for 5 minutes
45 times	(94°C for 15 seconds, 55°C for 40 seconds, 72°C for 1 minute)
Once	72°C for 10 minutes
Once	10°C maintained until analysis of the amplified product

V. Reading and interpretation of results

1. Detection of amplified products

Detection of amplified products is performed on 2% agarose gel in a TBE 1X or TAE 1X buffer.

a- Prepare a 2% agarose gel in a buffer solution of TBE 1X or TAE 1X.
For each gel, weigh out 2 grams of agarose and add to 100 ml of buffer. Heat the mixture to a boil. Allow to cool for several minutes, homogenise and pour the mixture onto the electrophoresis rack. Use a comb with 10 µl wells. Allow the gel to solidify for at least 45 minutes prior to use.

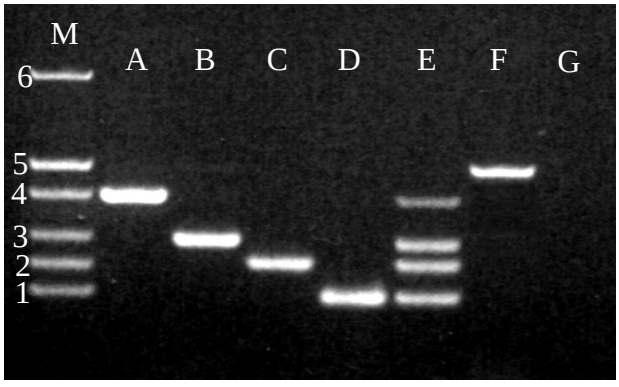
b- Place the electrophoresis rack into the tank and flood the gel with TBE 1X or TAE 1X buffer (the same as to make the gel). Add 2 drops (100 µl) of an ethidium bromide solution (1 mg/ml) to the tank buffer per litter of electrophoresis buffer.
Mix 2 µl of Loading buffer E2 with 10 µl of amplified product and dispense 10 µl of this solution to one of the wells. Place 5 µl of Size Marker E1 ALONE in one of the wells.

c- Allow the samples to migrate under an electric field of 3 volts per cm for 2 hours.

d- Place the gel under UV light and take a photograph.

2. Validation and interpretation of results

a- Test validation
The test is considered to be **valid** if a single band, identical to band N° 5 of the Size Marker, is observed in the negative control lane (lane F on photo below). Each positive control should lead to the amplification of the corresponding group as described in the table page 10.



b- Interpretation of results
The sample is considered to be **negative** if there is a single band corresponding to band N° 5 of the Size Marker E1 (lane F on photo).
The sample is considered to be **positive** if one or more bands, corresponding to bands N° 1, 2, 3 or 4 of the Size Marker, are observed. Band N° 5 of the internal control may be co-amplified. For serotypes identification, see the table below.
A sample is considered to be **inhibited** if no band corresponding to the Size Marker is observed (lane G on photo). In this case, it is recommended to repeat the test, pure and ten-fold-diluted in sterile water.
Band N° 6 is specific of the Size Marker E1 (lane M on photo).
If signals appear that are different from those presented in the appended figure, we recommend that you contact your distributor.

Band n°	Presence of a group III APP	Presence of a group I APP	Presence of a group IV APP	Presence of a group II APP	Presence of the 4 groups I-II-III-IV APP	PCR negative result	Undetermined To be re-tested
6	-	-	-	-	-	-	-
5	+/-	+/-	+/-	+/-	+/-	+	-
4	+	-	-	-	+	-	-
3	-	+	-	-	+	-	-
2	-	-	+	-	+	-	-
1	-	-	-	+	+	-	-
Lane M	Lane A	Lane B	Lane C	Lane D	Lane E	Lane F	Lane G

PCR typing	Serotyping	Band	Lane
Group I	Serotypes 1-9-11-12*-14	3	B
Group II	Serotypes 5a-5b-10	1	D
Group III	Serotypes 2-4-7-8-12	4	A
Group IV**	Serotypes 3-4*-6-7-13-15	2	C

* only serotypes 4 and 12 reference strains are included in these groups

** Some *Actinobacillus lignieresii s*trains can also give false positive result.

VI. 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

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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

VII. 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
	For veterinary in vitro use only – For animal use only

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