ADIAVET™ ANA PHA REAL TIME

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

References:
418028-50 (50 reactions)
418028 (100 reactions)

VET

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English version
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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 summaru
2014/09	NE113-01	N/A	First publication
2016/07	NE113-02	Administrative	Changing logos
2016/07	NE113-02	Administrative	Biosearch legal mention
2016/07	NE113-02	Administrative	Addition of table "Analysis options according to the specimen" §I.3.
2016/07	NE113-02	Administrative	Addition of 418028-50 reference (50 reactions)

I. General informations

1. Purpose of the test

ADIAVET™ ANA PHA REAL TIME kit is intended to detect *Anaplasma phagocytophilum* using real-time Polymerase Chain Reaction (PCR) technology from swab, tick and whole blood specimen of bovine, ovine, wild cervid and equine, as well as from tissue specimen of bovine, ovine and wild cervid.

2. *Anaplasma phagocytophilum*

Anaplasma phagocytophila is a new combination of biovars published on 15th November 2001 and corrected on 14th January 2002 in *phagocytophilum*. This taxon gathers *Ehrlichia phagocytophila* but also *Ehrlichia equi* and the agent of human granulocytic ehrlichiosis (HGE).
The *Anaplasma phagocytophilum* strains appear like small Gram negative bacteria, infecting mammalians myeloblastic cells, especially the neutrophil granulocytes, and to a lesser extent, eosinophil granulcytes.
Anaplasma phagocytophilum is a ticks and mammalians parasite, domestic and wild animals being the main germs reservoir. This bacteria is transmitted to healthy mammalians by ticks of *Ixodes* genus, especially *Ixodes ricinus* in Europe. By infected ticks, *Anaplasma phagocytophilum* is maily located in salivary glands.
The *Biovar Phagocytophilum* is the agent of a disease of the ruminants known to ovine races under the name of "tick-border fever" and, to the cattle, under the name of "pasture fever". The infection has been identified mainly in Europe (United Kingdom, Norway, Finland, Sweden, Ireland, Nederland, Austria, Germany, France, Spain, Switzerland) but also in India and in South Africa. The natural disease affects the wild ruminants (especially in cervids and bovine) and the domestic ruminants (especially in sheep and cattle). The incubation period is about 3 to 6 days to ovine races and 4 to 17 days to the cattle. Fever is the main symptom. The fever comes along with anorexia, a loss of weight and a fall of the milk production which can be rough and massive. Other clinical signs consist of locomotive confusions, respiratory deficiencies, abortions. The infected sheep remain carriers during a long period (until two years) while the carriage is shorter in the cattle.

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™ ANA PHA REAL TIME kit enables the simultaneous detection of:

- *Anaplasma phagocytophilum* (probe labelled in FAM),
- GAPDH, 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 (Qiagen and 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 (vaginal, placental…)	☑	☒
Tick	☑	Larvae: from 10 up to several hundreds Nymph: between 3 up to 40 Adult male ticks: up to 15 Non-engorged adult female ticks: up to 12 Engorged adult female ticks: up to 3 (following size)
Whole blood	☑	☒
Tissue (placenta, fœtal tissues…)	☑	☒

***** It depends on the epidemiological case and on the quality of the specimen.

II. Material & reagents

1. Reagents provided with the kit

Designation	Reagents	418028-50 (50R)	418028 (100R)
A5	Amplification solution	1 x 1000 µl green tube	2 x 1000 µl green tubes
ANA PHA CTL+	Positive control *Anaplasma phagocytophilum*	1 purple tube	1 purple tube
Exogeneous IPC	Exogeneous Internal Positive Controle	1 purple tube	1 purple tube

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 ANA PHA CTL+

ANA PHA CTL+ is a positive control of amplification.
Add **200 µl** of Nuclease-free water to the ANA PHA 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 ANA PHA CTL+ in a well.

4. Use of Exogeneous IPC

Exogenous IPC is a positive control of extraction used for analysis from tick.
Add **100 µl** of Nuclease-free water to the Exogenous IPC tube and vortex at least 20 seconds.
Aliquot this solution by 6 or 12 µl and store them to <-15°C.
For each tick, use 2 µl of Exogenous IPC in a sample.

5. 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
- Universal laboratory mixer mill
- 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
- Latex or nitrile powder-free gloves
- Stainless steel beads 3 mm
- Scalpel blades
- 96-100% ethanol solution
- Nuclease-free water
- Sterile distilled water
- PBS buffer

- 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)

or

- Automated DNA/RNA extraction kit using magnetic beads
See the NEKF user manual available of the web site mentioned on the certificate of analysis included in the used ADIAVET™ kit.

III. 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 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 demineralised and 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.
Samples of blood with anticoagulant reagent must not be frozen.
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. Samples preparation

Blood should be sampled on anti-coagulant, privilege EDTA (citrate and heparine may be inhibitor)

Ticks and larvae can be analysed by pool:
Larvae: from 10 to several hundred
Nymph: up to 40
Adult male ticks: up to 15
Non-engorged adult female ticks: up to 12
Engorged adult female ticks: up to 3 following size

Larvae, nymphs and male adults are put in a 1.5 ml-tube without dilacerations. On the other hand, for engorged females, only the head is used, dissection is achieved under a microbiological Safety Cabinet in a Petri dish with a scalpel.
Change blade and petri dish between each tick batch.

See § IV for the extraction and purification of DNA.

4. 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 kit.

- The internal endogenous control (GAPDH) naturally found in the samples verifies the extraction and amplification steps of each sample.
- The ANA PHA 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 *A. phagocytophilum*. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of *A. phagocytophilum*. 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.

IV. Extraction and Purification

1. Extraction using QIAamp® DNA Mini kit

All the centrifugations are performed at room temperature.

Particular case of placentas:
May contain a large amount of microorganisms, manipulate them with extreme precaution.
1st method
Cut the cotyledon with a scalpel, then rub inside with a swab.
Perform analysis according to swab protocol.
2nd method
Perform analysis according to tissue protocol.

<table>
<tr><th></th><th>Swab</th><th>Tissue</th><th>Blood</th><th>Larvae and tick</th></tr>
<tr><td>Preparation of the sample</td><td>Mix the swab with 1 ml of 1X PBS buffer.
Transfer 200 µl in a microtube.</td><td>Put 20-30 mg of tissue in a microtube.</td><td>Place 500 µl of bovine blood or 100 µl of equin blood in a microtube.
Add 1 ml of sterile distilled water.
Vortex, then incubate 10 minutes on melting ice (~0°C).
Centrifuge 5 minutes at 6 000 g
Discard the supernatant.
Add 1 ml of sterile distilled water.
Vortex, then centrifuge 5 minutes at 6 000 g
Discard the supernatant.</td><td rowspan="2">Place tick and larvae in microtube (see § III.3.).
Add 180 µl of ATL buffer, 20 µl of proteinase K and 2 µl « Exogeneous IPC ».
Grind*
Incube between 4 hours and 1 night at +55°C.</td></tr>
<tr><td rowspan="2">Lysis</td><td colspan="3">Add 180 µl of ATL buffer, 20 µl of proteinase K. Vortex.
Incubate 30 minutes at +70°C (or a night at +56°C).</td></tr>
<tr><td colspan="4">Add 200 µl of AL buffer. Vortex.
Incubate 10 minutes at +70°C.</td></tr>
<tr><td>Binding preparation</td><td colspan="4">Add 200 µl of ethanol 100%.
Homogenise the mixture by pipeting (~10 times) or by vortex (~15 secondes).</td></tr>
<tr><td>Transfer to columns and binding to the membrane</td><td colspan="4">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.</td></tr>
<tr><td>1st wash</td><td colspan="4">Change the collection tube and add 500 µl of AW1 buffer to the column.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>2nd wash</td><td colspan="4">Change the collection tube and add 500 µl of AW2 buffer to the column.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Column dry step</td><td colspan="4">Change the collection tube.
Centrifuge 3 minutes at 10 000 g.</td></tr>
<tr><td>Elution</td><td colspan="4">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.</td></tr>
<tr><td>Storage</td><td colspan="4">Close the tubes, identify and store at +2/8°C for 24 hours, then at <-15°C.</td></tr>
</table>

* For example, using a Mixer Mill: add 1 stailess steel beads (3 mm), grind 4 minutes at 30 Hz.

2. Extraction using NucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.

Particular case of placentas:
May contain a large amount of microorganisms, manipulate them with extreme precaution.
1st method
Cut the cotyledon with a scalpel, then rub inside with a swab.
Perform analysis according to swab protocol.
2nd method
Perform analysis according to tissue protocol.

	Swab	Tissue	Blood
Preparation of the sample	Mix the **swab** with **1 ml** of **1X PBS buffer.** Transfer **200 µl** in a microtube.	Put **20-30 mg** of **tissue** in a microtube.	Place **500 µl** of **bovine blood** or **100 µl** of **equin blood** in a microtube. Add **1 ml** of **sterile distilled water**. Vortex, then incubate 10 minutes on melting ice (~0°C). Centrifuge 5 minutes at 6 000 g Discard the supernatant. Add **1 ml** of **sterile distilled water**. Vortex, then centrifuge 5 minutes at 6 000 g. Discard 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 using DNA/RNA magnetic beads kit

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

V. 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 **20 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 20 µl of A5 solution.
For the CTL+, add **5 µl** of the solution obtained in § II-3 to the 20 µl of A5 solution.
For the No Template Control (NTC), nothing is added to the A5 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 *Anaplasma phagocytophilum* 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** 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** of **Stratagene**:
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.

VI. 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.

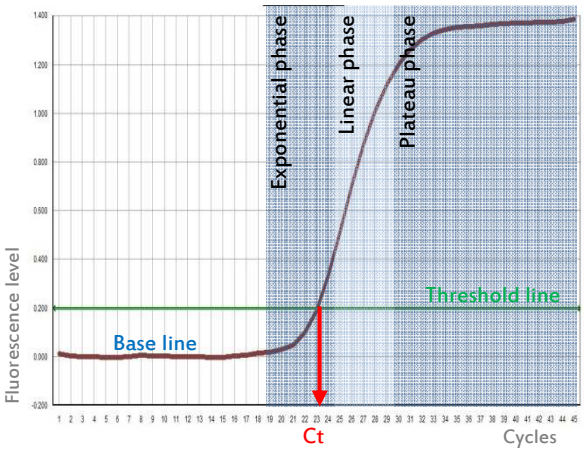
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

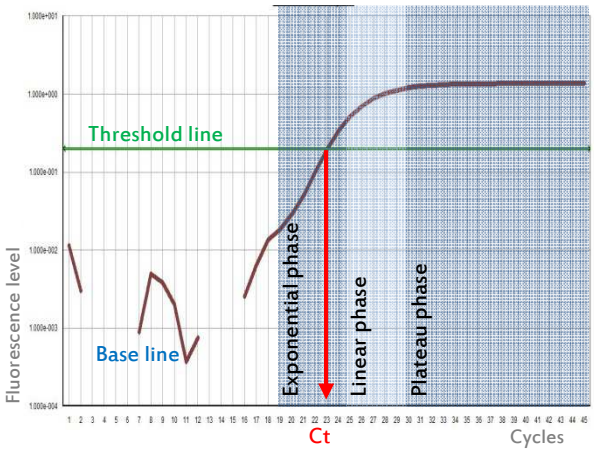
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present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

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
	Keep away from sunlight
	For veterinary in vitro use only – For animal use only

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