ADIAVET™ CHLAM.SPP FAST TIME

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

Reference :
ADI611-100 (100 reactions)

VET

English Version
NE611-02
2021/09

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Revisions 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 change are not included in the revision historic

Release Date	Part Number	Change type	Change summary
2019/09	NE611-01	N/A	First publication
2021/09	NE611-02	Technical	Addition of fetal gastric fluid matrix

I. General informations

1. Purpose of the test

ADIAVET™ CHLAM.SPP FAST TIME kit allows to detect all bacteria of the genus Chlamydia using real-time Polymerase Chain Reaction (PCR) technology, from swab, tissue (bovine, ovine, goat, poultry, pigs and other animal species) as well as milk and fetal gastric fluid from ruminants.

2. Chlamydia

The family Chlamydiaceae contains a single genus Chlamydia *(C)* comprising 13 species : *C. abortus,* C*. avium, C. buteonis, C. caviae, C. felis, C. gallinacea, C. ibidis, C. muridarum, C. pecorum, C. pneumoniae, C. psittaci, C. suis* and *C. trachomatis* (Sachse and al., 2014 ; Laroucau and al., 2019).

These bacteria are obligate intracellular organisms and can induce depending of infected animal species, arthritis, conjunctivitis, pneumonia, abortions… They also could be the causative agent of zoonosis. *Chlamydia* have also been detected in horses, carnivorous, rabbits, mouse, guinea pigs and also in reptiles and fishes.

Chlamydia do not grow up out of eukaryotes cells, therefore, the isolation is not performed in routine.

The need of sensibility and specificity for *chlamydia* diagnosis conduct to the development of PCR tests.

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™ CHLAM.SPP FAST TIME kit enables the simultaneous detection of:

- *Chlamydia* (probe marked with FAM)
- an exogen control "EPC-Amp" added in the amplification reagent or "EPC-Ext" added during the extraction that allows validating extraction and amplification steps (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 and Macherey-Nagel). Other purification kits can be used if they have been validated by the user.

Analysis options accoriding to the specimen:

Specimen	Individual Analysis
Swab (placental, vaginal, cloacal, faeces, nasal…)	☑
Tissue (placental, fœtal tissues…)	☑
Milk	☑
Fetal gastric fluid	☑

II. Material and reagents

1. Reagents provided with the kit

REF ADI611-100

A5	Amplification Solution	2 x 500 µl tubes with green caps (Ready to use reagent)
CHLAM.SPP CTL+	Positif control of *Chlamydia*	1 tube with purple cap (deshydrated)
EPC-Amp	exogenous internal control of amplification	1 x 150 µl tube with white cape (Ready to use reagent)
EPC-Ext	exogenous internal control of extraction	2 x 300 µl tubes with yellow caps (Ready to use reagent)
NF-Water	Nuclease free water	1 x 1000 µl tube with white cap (Ready to use reagent)

Downloadable notice in 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 the differents controls provided with the kit

A. Use of « CHLAM.SPP CTL+ »

« CHLAM.SPP CTL+ » is a positive control of amplification.
Add **200 µl** of **NF-Water** in the tube **CHLAM.SPP CTL+** and vortex it at least 20 secondes until complete dissolution of the blue pellet.
Aliquot this solution by 6 or 12 µl and store it at <-15°C.
For each analysis, use **5 µl** of **CHLAM.SPP CTL+** in one of the wells.

B. Use of « EPC-Ext »

Aliquot and store this solution at <-15°C according to the size of extraction series. Do not defrost reagent more than tree times.
For each extraction, use **5 µl** d'**EPC-Ext** in each sample.

C. Use of « EPC-Amp »

Aliquot and store this solution at <-15°C according to the size of extraction series. Do not defrost reagent more than tree times.
If amplification of DNA with no **EPC-Ext**, we recommand to add 0.5 µl of **EPC-Amp** in each well.

4. Equipment required but not supplied in the kit

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
- Scalpel blades
- 96-100% ethanol solution
- Nuclease-free 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

- ADIAMAG (Bio-X Diagnostics, 200 tests: Ref. NADI003; 800 tests: Ref. NADI003-XL)

See the NEKF user manual available on 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 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 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.
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

See § IV for the extraction and purification of DNA.

4. Controls preparation

The use of controls allows to verify 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 exogenous internal control of amplification "EPC-Amp", added in A5 reagent, verifies the amplification steps of each sample.
- The exogenous internal control of extraction "EPC-Ext", added in all samples, verifies the extraction and amplification steps of each sample.
- The CHLAM.SPP CTL+ validates the amplification of the both targets.

Other controls must or could be added:

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

To verify the absence of cross-contamination, at least on negative control must be included per trial. The control is a negative sample, for example a buffer used for dilutions.

- **Positiv contrôle of extraction (recommended)**

A positiv control could be added in each trial. The control is a sample including *Chlamydia*. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of *Chlamydia*. 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 number 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**	**Milk, fetal gastric fluid**
Sample preparation	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.	Transfer **200 µl** in a microtube.
Lysis	Add **180 µl** of **ATL buffer**, **20 µl** of **proteinase K + 5 µl EPC-Ext**. 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 bing 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.*		
1er wash	Change the collection tube and add **500 µl** of **AW1 buffer** to the column. Centrifuge 1 minute at 10 000 g.		
2ème 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 with theNucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.

Particular case of placentas:
May contain a large number 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	Milk, fetal gastric fluid
Preparation of the sample	Mix the **swab** with **1 ml** of **1X PBS buffer.** Transfer **200 µl** in a microtube.	Put **20-30mg** of **tissue** in a microtube.	Transfer **200 µl** in a microtube.
Lysis	Add **180 µl** of **T1 buffer**, **25 µl** of **proteinase K+ 5 µl EPC-Ext**. 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 with DNA/RNA magnetics beads kit

See the NEKF user manual available on 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.
If EPC-Ext was added during extraction step:
Dispense **10 µl** of the A5 solution in each PCR tubes or PCR plate wells with a micropipette with a Nuclease-free tips.

If EPC-Ext was NOT added during extraction step:
Dispense (n+1) x **10 µl** of « A5 » reagent in microtube.
Add (n+1) x **0.5 µl** of **« EPC-Amp »**.
Dispense **10 µl** of the mix in each PCR tubes or PCR plate wells with a micropipette with a Nuclease-free tips.

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

d- For each sample, the extraction negative control (required) and the extraction positive control (recommended), add **5 µl** of purified extract to the **10 µl** of **A5** reagent.
For the CHLAM.SPP CTL+, add **5 µl** of the solution (§ II.3.) to the **10 µl** of **A5** reagent.
For the No Template Control (NTC), nothing is added to **A5** reagent.
Immediately replace the purified DNA 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 *Chlamydia* target is read in FAM. The internal control is read in VIC or HEX. The Quencher is no fluorescent. The solution contains a passive reference ROX for the ABI machines. The fluorescence is read during the elongation step at 60°C.

The following programs are defined for **QuantiStudio 5**, **ABI Prism** thermalcyclers (like 7500, StepOne…) from **Applied Biosystems** (check the "emulation 9600" option if available), for the **MX3005P** and **ARIAMX** of **Agilent,** the **LightCyclers** from **Roche Diagnostics** and for the **CFX96** of **BioRad**.

Standard Program		**Fast Program**	
2 min 50°C 10 min 95°C		2 min 95°C	
15 sec 95°C	45 cycles	5 sec 95°C	45 cycles
1 min 60°C		30 sec 60°C *	

*Note 32 secondes for the 7500 Thermofisher

Contact us if you wish to use other thermalcyclers.

VI. Interpretation of results

1. Definitions

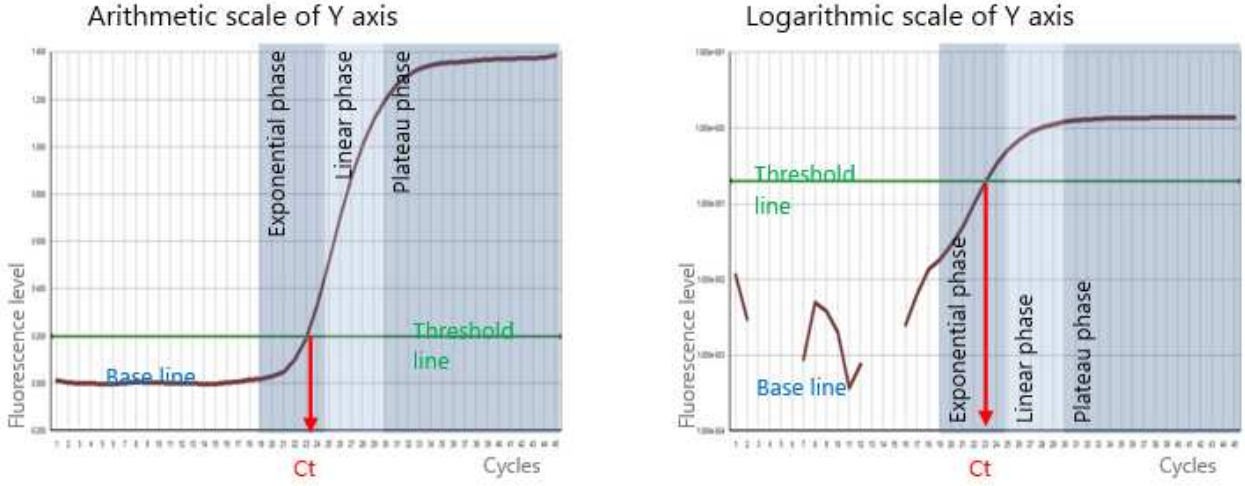
The **« base line »** corresponds to the background of fluorescence and qualifies the non characteristic part of the curve observed during the first cycles.

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



2. Reading 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.

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 (CHLAM SPP CTL+)	Extraction negative control	Extraction positive control*
FAM Amplification		no	yes	no	yes
HEX Amplification	If EPC-Ext used	no	no	yes	yes
	If EPC-Amp used	yes	yes	yes	yes
Validation of		Absence of contamination for amplification	Amplification of *Chlamydia* target	Absence of contamination for extraction	Extraction and amplification step

* Optional

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

B. Results interpretation

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

Example	A	B	C	D
FAM Amplification	No	Yes	Yes	No
HEX Amplification	Yes	Yes	No	No
Results	Negative	Positive	Positive	Undetermined

The sample is considered as **negative** if a characteristic amplification curve is observed in VIC/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 bec o-amplified (example C).

A total absence of characteristic amplification curve for a sample (exemple 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 NF-Water. Then, if the teste is still not valid, a new extraction is recommanded.

VII. References

AFNOR NF U47-600-2 guide – February 2015 - Requirements and recommendations for the implementation. the development and the validation of veterinary PCR for animal health analysis methods. – part 2; requirements and recommendations for the development and validation of PCR for animal health

K. Laroucau, F. Vorimore, R. Aaziz, L. Solmonson, R.C. Hsia, P.M. Bavoil, P. Fach, M. Hölzer, A. Wuenschmann, K. Sachse. Chlamydia buteonis, a new Chlamydia species isolated from a red-shouldered hawk. Syst. Appl. Microbiol. 2019

SACHSE K., LAROUCAU K., RIEGE K., WEHNER S., DILCHER M., CREASY HH., et al. Evidence for the existence of two new members of the family Chlamydiaceae and proposal of Chlamydia avium sp. nov. and Chlamydia gallinacea sp. nov. Syst. Appl. Microbiol.. 2014, 37, 79-88.

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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