ADIAVET™ CHLAMCOX REAL TIME

TEST FOR THE DETECTION OF *CHLAMYDOPHILA ABORTUS* AND *COXIELLA BURNETII* BY REAL-TIME ENZYMATIC GENE AMPLIFICATION (PCR TEST)

Reference:
418025 (100 reactions)

VET

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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	NE313-01	N/A	First publication
2016/07	NE313-02	Administrative	Changing logos
2016/07	NE313-02	Administrative	Biosearch legal mention
2016/07	NE313-02	Administrative	Addition of table "Analysis options according to the specimen" §I.3.
2016/07	NE313-02	Correction	Modification of NucleoSpin Tissue §IV.2

I. General informations

1. Purpose of the test

ADIAVET™ CHLAMCOX REAL TIME kit is intended to detect *Chlamydophila abortus* and to detect and/or to quantify *Coxiella burnetii*, using real-time Polymerase Chain Reaction (PCR) technology, from swab, tissue and milk specimens of bovine, ovine and caprine.

2. *Chlamydophila abortus* and *Coxiella burnetii*

Chlamydophila abortus is an intracellular bacterium whose main target organ is the ruminant placenta (bovine, caprine and ovine). This bacterium is the agent of abortion (enzootic abortive small ruminant) and neonatal death. It also represents an emerging zoonotic risk for pregnant women. The disease can also affect, less frequently, horses, carnivores, rabbits, swine, mousses and guinea pig. Clinical signs induced by *C. abortus* infection don't allow a clear diagnostic.
Detection of *C. abortus* by cell culture isolation is still regarded as the definitive test; however it is time consuming and requires cell culture facilities. The most often achieved diagnosis is a bacterioscopic examination of placental stamp.
The lack of sensitivity and specificity of this method has lead to the development of PCR tests.

Coxiella burnetii is a strictly intracellular Gram negative bacterium, agent of the Q fever (Burnet and Freeman, 1937; Cox, 1938). The disease is present worldwide. *C. burnetii* is able to infect a large range of hosts including humans. Q fever is a **zoonose** (pathology which can be transmitted to human) mainly transmitted by ovine, bovine and caprine. Q fever usually results from inhalation of contaminated aerosols originating mostly from dropping and body fluids of infected animals.
C. burnetii is present in reproductive apparatus, placenta and fluids produced during parturition or abortion. The bacterium is excreted in milk and faeces of infected animals presenting no clinical signs. Most infections are asymptomatic or subclinical by Human, but can be manifested as a flu-like disease, or as hepatitis. A neurological involvement is also possible. Pericarditis and myocarditis are rare. *C. burnetii* can induce abortions, causing economic losses in cattle. Prevention mostly consists in precautions with pregnant females and with fluids from parturition.
Detection of *C. burnetii* by culture is long and difficult. Detection of antibodies doesn't give information on sanitary status of the animal.
The real-time PCR ADIAVET™ COX REALTIME allows the identification of the bacteria *C. burnetii*. It is applicable to a large number of samples and various biological matrixes.

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™ CHLAMCOX REAL TIME kit enables the simultaneous detection of:

- *Chlamydophila abortus* (probe labelled in FAM),
- *Coxiella burnetii* (probe labelled in VIC),
- GAPDH, an internal control of extraction and amplification steps specific from an endogenous DNA (probe labelled in Cy5).

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
Swab (vaginal, cervical, placental…)	☑
Tissue (placental, foetal tissues…)	☑
Milk	☑

II. Material & reagents

1. Reagents provided with the kit

Designation	Reagents	418025 (100R)
A5	Amplification solution	2 x 1000 µl green tubes
CHLAM.A CTL+	Positive control *Chlamydophila abortus*	1 purple tube
COX CTL+	Positive control *Coxiella burnetii*	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 CHLAM.A CTL+

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

4. Use of COX CTL+

COX CTL+ is a positive control of amplification.
Carefully use this CTL+. It contains a large amount of DNA that could be a source of contamination.
The tube contains a titrated solution of *C. burnetii* DNA as EG/ml or as *C. burnetii*/ml.

Add **200 µl** of Nuclease-free water to the COX CTL+ tube and vortex at least 20 seconds.
Aliquot this solution by 6 or 12 µl and store them to <-15°C.

Realise, just before using, a standard range with 10-fold serial dilutions of "COX CTL+" with Nuclease-free water.

A standard range	***DNA Concentration (C. burnetii/ml)***
pure	$4x10^6$
1/10	$4x10^5$
1/100	$4x10^4$
1/1000	$4x10^3$
1/10000	$4x10^2$

For a **qualitative** PCR analysis, it is recommended to use **5 µl** of the range 1/1000.

For a **quantitative** PCR analysis**, 5 µl** of each range will be used per analysis.

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
- 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

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 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 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 CTL+ validate 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 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 *Chlamydophila abortus* and/or *Coxiella burnetii*. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of *Chlamydophila abortus* and/or *Coxiella burnetii*. 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.

	Swab	**Tissue**	**Milk**
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 **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.

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**	**Milk**
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**. 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 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. 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 or § II.4. 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 *Chlamydophila abortus* target is read in FAM. The *Coxiella burnetii* target is read in VIC or HEX. The Internal Control is read in Cy5. 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.

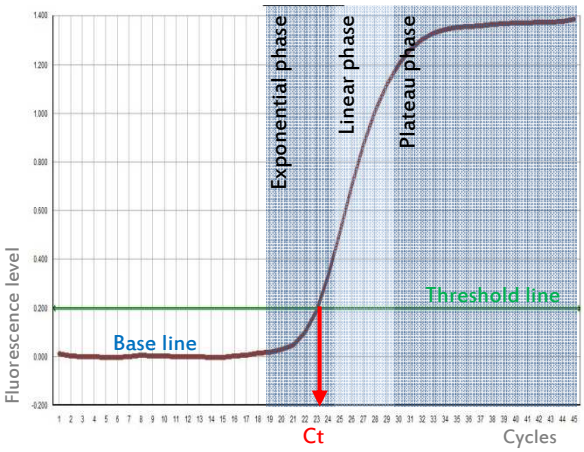
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

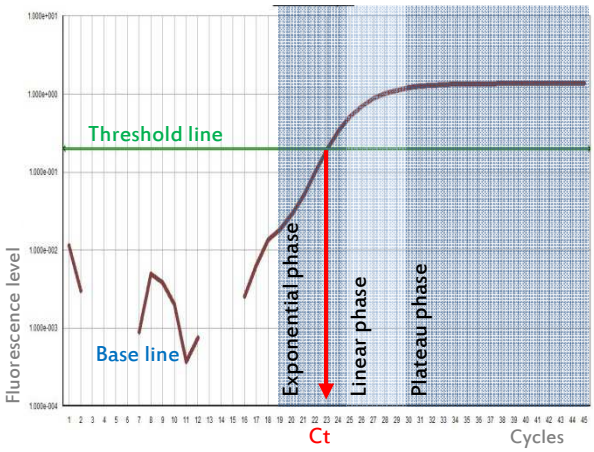
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present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

Example of characteristic amplification curve

Arithmetic scale of Y axis



Logarithmic scale of Y axis



2. Reading and validation of qualitative 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 and Cy5 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 (CHLAM.A CTL+)	Amplification positive control (COX CTL+)	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	no	yes
VIC/HEX amplification	no	no	yes	no	yes
Cy5 amplification	yes	yes/no	yes/no	yes/no	yes/no
Validation of	Absence of contamination for amplification	Amplification of the target *C. abortus*	Amplification of the target *C. burnetii*	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM, Cy5 and VIC/HEX dyes) of the positive controls (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 *C. abortus* (FAM), for *C. burnetii* (VIC/HEX) or for the internal control (Cy5).

Example	A	B	C	D	E
FAM amplification	No	Yes	No	Yes	No
Cy5 amplification	No	No	Yes	Yes	No
VIC/HEX amplification	Yes	Yes/No	Yes/No	Yes/No	No
Results	Negative	Positive for *C. abortus*	Positive for *C. burnetii*	Positive for *C. abortus*and *C. burnetii*	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 (examples B and D) and/or in Cy5 (examples C and D). Internal control can be co-amplified.

A total absence of characteristic amplification curve for a sample (example E) 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.

3. Validation and interpretation of quantitative results for *C. burnetii*

Display the VIC/HEX curves from the plate and set the threshold value as indicated above. Proceed in the same mean for the Cy5 curves.

A. Validation of the standard curve

Controls	DNA Concentration (*C. burnetii*/ml)	VIC/HEX amplification	Cy5 amplification
COX CTL+ pure	$4x10^6$	Yes	No
COX CTL+ 1/10	$4x10^5$	Yes	No
COX CTL+ 1/100	$4x10^4$	Yes	No
COX CTL+ 1/1000	$4x10^3$	Yes	No
COX CTL+ 1/10000 (LQ_{PCR})	$4x10^2$	Yes	No
No Template Control (NTC)	0	No	No
Extraction negative control	0	No	No
Extraction positive control *	1 to 100 X LD_{METHOD}	Yes	Yes/No

* Optional

The indicative Ct values (Cy5 and VIC/HEX dyes) of the positive control (CTL+) are indicated in the certificate of analysis of the kit.

For the quantitative interpretation, check the PCR efficiency. The values of the obtained Ct should increase in proportion with the DNA dilution (about 3.33 Ct for 1/10 dilution). In general the software supplied with the thermalcyclers establishs a calibration line (cycles number = f (log concentration)), calculate the standard curve ($y = ax + b$) and PCR efficiency ($Eff\% = \left(10^{\left(\frac{-1}{a}\right)} - 1\right) \times 100$

Before the results analysis, the standard curve should be validate:

- the 5 points of the standard range should be amplified; however, one point of the range may be remove if this point is not one the two extremes
- $R^2>0.9$
- 85%<Efficacity<115%
- homogenous distribution of the points

Interpretation of the results

The interpretation of the result of a sample is represented in the below table:

	VIC/HEX amplification			
	No signal	***Signal <$LQ_{METHODE}$***	***Signal between LQ_{METHOD} and LQmax***	***Signal >LQmax***
Interpretation of the résultat	*Negative* DNA no detected	*Positive:* DNA detected in a quantity less than the LQmethod	*Positive :* DNA quantifiable	*Positive:* DNA detected in a quantity greater than the LQmax

The sample is considered as **positive** for *C. burnetii* if a characteristic amplification curve is observed in VIC/HEX. The internal control can be co-amplified in Cy5.

The sample is considered to be **negative** if a characteristic amplification curve is observed in Cy5 without any amplification curve in VIC/HEX.

A total absence of characteristic amplification curve for a sample shows a deficient PCR (inhibitors in the sample, program error). In this case, we recommend repeating the test with DNA pure and diluted tenfold in Nuclease-free water. Then, if the test is still not valid, a new extraction is recommended.

The direct quantification of positive samples is possible with the aid of the standard range. The equation of the PCR standard range allows determining concentration in *C. burnetii* :

$$x = 10^{\left(\frac{y-b}{a}\right)} \times F$$

With: x: Concentration of *C. burnetii*
y: Ct value FAM of positif sample
b: Intercept
a: Slope
F: multiplying coefficient

The multiplying coefficient is determined according to the type of matrix and extraction method :

Matrix	Multiplying coefficient *(F)* according to extraction method		Units
	QIAamp® DNA Mini Kit or NucleoSpin® Tissue	RNA/DNA magnetic beads	
Vaginal swab, placenta swab, milk, fœtal liquide	1	0,6	*C. burnetii* / ml
Tissue	10	3	*C. burnetii* / g

VII. Literature references

Burnet, F. M., and M. Freeman (1937). Experimental studies on the virus of Q fever. Med. J. Aust. 2:299-302

Cox, H. R. (1938). A filter-passing infectious agent isolated from ticks. III. Description of organism and cultivation experiments. Public Health Rep. 53:2270-2276

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

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