ADIAVET™ NEOSPORA REAL TIME

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

Reference:
418023 (100 réactions)

VET

NOTE
Dye compounds in this product are sold under license from Biosearch Technologies, Inc. and protected by U.S. and worldwide patents either issued or in application. The license covers Veterinary Molecular Diagnostics.

English version
NE243-04
2016/07

ADIAVET™ NEOSPORA REAL TIME

I. Revision history

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 history.

Release Date	Part Number	Change type	Change summary
2013/11	NE243-01	N/A	First publication
2014/09	NE243-02	Technical change	Addition of reference 418023 (100 reactions)
2014/12	NE243-03	Technical change	Removal of reference 416440 (50 reactions)
2016/07	NE243-04	Administrative	Changing logos
2016/07	NE243-04	Administrative	Biosearch legal mention
2016/07	NE243-04	Administrative	Addition of table "Analysis options according to the specimen" §I.3.

II. General information

1. Purpose of the test

ADIAVET™ NEOSPORA REAL TIME kit is intended to detect *Neospora caninum* using real-time Polymerase Chain Reaction (PCR) technology from swab, from tissue and brain specimens of bovine, ovine and equine.

2. Pathogen

Neospora caninum is an apicomplexan parasite first associated with bovine abortion in the USA in 1989 (Bjerkas *et al.*, 1984; Thilsted and Dubey, 1989). Since this observation *N. caninum* has been implicated as one major cause of infectious abortion of cattle worldwide.
Abortion due to neosporosis can occur at any stage of pregnancy but is most likely to occur between 5–7 months of gestation. Cows that aborted in a previous pregnancy due to neosporosis can abort again. They can also give birth to diseased calves, calves with a sub-clinical *N. caninum* infection, or uninfected calves.
Neospora caninum is a cyst-forming coccidian parasite. Two asexual stages of development occur in the intermediate host (cattle, horse), tachyzoite and bradyzoite and one sexual stage (oocyst) occurs in the definitive host (dog) (McAllister *et al.*, 1998).
Routine diagnosis of *N. caninum* infection is made by observation of clinical signs (abortion, peaking between the fifth and seventh month of gestation), by histology of fœtal tissue and by indirect fluorescent antibody (IFAT), and ELISA tests on maternal and foetal blood. But abortion is not always related to the presence of antibody.
In 1996, a shift of biologist of Institute of Parasitology of Berne conducted by the Professor Kaufmann identified a repetitive genomic fragment called Nc5, specific of *N. caninum* (Kaufmann *et al.*, 1996). Since, the sequence has been used as probe in molecular test as PCR test (Muller *et al.*, 1996; Yamage *et al.*, 1996).
The PCR test allows the specific and sensitive detection of the parasite from fœtal tissues.

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™ NEOSPORA REAL TIME kit enables the simultaneous detection of:

- *N. caninum* (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 recommands the test using 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 (placental, vaginal…)	☑
Tissue (placenta, fœtal tissues…)	☑
Brain	☑

III. Material and reagents

1. Reagents provided with the kit

Designation	Reagents	418023 (100R)
A5	Amplification solution	2 x 1000 µl green tubes
NEO CTL+	Positive control *Neospora caninum*	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 A5reagent is ready to use for PCR reaction.
Do not mix reagents of two different batches.

3. Use of NEO CTL+

Add **200 µl** of Nuclease-free water to the NEO CTL+ tube included in the kit. Homogenize tube contents using a mixer such as vortex, at least 20 seconds. Aliquot this solution by 6 or 12 µl and store them to <-15°C.
For each analysis, we recommend to use 5 µl of NEO CTL+ in a well.

4. 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
- Instrument for homogenous mixing of tubes
- 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
- Powder-free latex or nitrile gloves
- Scalpel blades
- 96-100% ethanol solution
- Sterile distilled water
- Sterile saline water (NaCl 8.5 g/l)
- PBS buffer
- MEM medium

- DNA extraction kit (individual 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 (magnetic beads)

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

IV. Recommendation 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 used 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 24h hours, then at <-15°C.

3. Samples preparation

See § IV for the extraction and purification of DNA.

4. Controls to include

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.
The use of the controls follows the recommendation of the the normative requirement and recommendation for the development and the validation of veterinary PCR (NF U47-600).

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 allows verifying the extraction and amplification steps of each sample.
- The NEO CTL+ allows validating the amplification of the target.

Other controls must or could be added:

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

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 *N. caninum*. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of *N caninum*. 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.

	Brain	Placenta swab	Placenta
Preparation of the sample	Put **one volume of brain** and **one volume of MEM or sterile saline water.** Homogenise.[1] Transfer 200 µl supernatant in microtube	Homogenise swab in microtube contain **1 ml of 1X PBS buffer.**	Place **20 mg of placenta** in microtube
Lysis	Add **180 µl** of **ATL buffer**, **20 µl** of **proteinase K**. Homogenize. Incubate **30 minutes** at **+70°C** (or a night at +56°C).		
	Add **200 µl** of **AL buffer**. Homogenize. Incubate **10 minutes** at **+70°C**.		
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenise the mixture by pipeting (~10 times) or by using a mixer such as vortex (~15 seconds).		
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 24h hours, then at <-15°C.		

[1] When more than 10 g of brain sample is analysed, it is possible to put the sample in a bag for blender (Mix 2 or Blender with paddles).

2. Using NucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.

	Brain	Placenta swab	Placenta
Preparation of the sample	Put **one volume of brain** and **one volume of MEM or sterile saline water**. Homogenise.[1] Transfer 200 µl supernatant in microtube	Homogenise swab in microtube contain **1 ml of 1X PBS buffer.**	Place **20 mg of placenta** in microtube
Lysis	Add **180 µl** of **T1 buffer**, **25 µl** of **proteinase K**. Homogenize. Incubate **30 minutes** at **+70°C** (or a night at +56°C).		
	Add **200 µl** of **B3 buffer**. Homogenize. Incubate **10 minutes** at **+70°C**.		
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenise the mixture by pipeting (~10 times) or by using a mixer such as vortex (~15 seconds).		
Transfer to columns and binding to the membrane	Identify columns, apply the 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 24h hours, then at <-15°C.		

[1] When more than 10 g of brain sample is analysed, it is possible to put the sample in a bag for blender (Mix 2 or Blender with paddles).

3. 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.

VI. Amplification

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

b - Defrost the A5 solution at room temperature. Homogenize. 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 PCR reagent 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 real-time PCR amplification.

The *Neospora caninum* 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.

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.

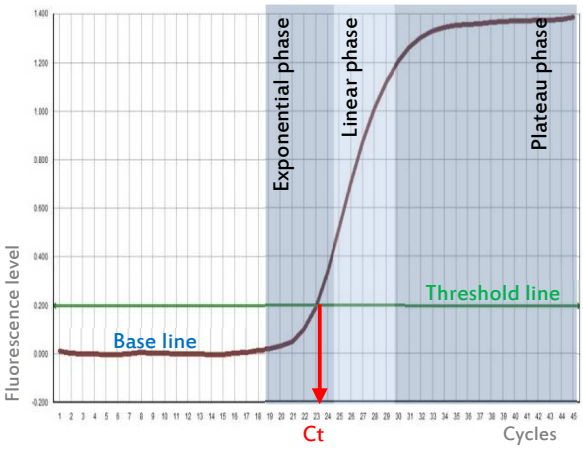
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

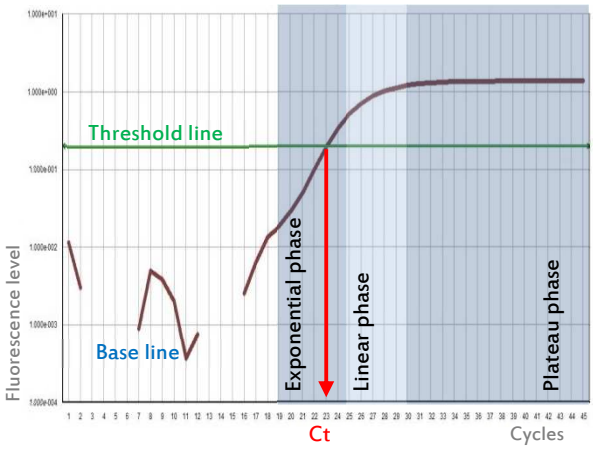
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present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

A. Validation of the run

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

Controls	Reagent control (NTC)	NEO 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 NEO CTL+ are indicated in the certificate of analysis of the kit.

B. Result interpretation

DNA extraction and amplification for each sample are considered to be **valid** if at least a characteristic amplification curve is observed for *Neospora caninum* (FAM) or for the internal control (VIC or 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 (B example). Internal control can be co-amplified (C example).

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

Bjerkas I, Mohn SF and Presthus J. Unidentified cyst-forming sporozoon causing encephalomyelites and myositis in dog. Zparasitenkd. 1984. 70(2): 271-274

Dubey J. P and Lindsay D.S. A review of *Neospora caninum* and neosporosis. Vet Parasitol. 1996, 67: 1-59.

Kaufmann H., Yamage M., Roditi I *et al*. Discrimination of *Neospora caninum* from *Toxoplasma gondii* and other apicomplexan parasites by hybridation and PCR. Mol. Cell. Probes. 1996, 10: 289-297.

McAllister M.M, Dubey J.P, Lindsay D.S, Holley W.R, Wills R.A and A.M McGuire. Dogs are definitive host of *Neospora caninum*. Int J. Parasitol. 1998, 28: 1473-1478.

Müller N., Zimmermann V., Hentrich B., Gottstein B. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* Infection by PCR and DNA Hybridization Immunoassay. J. Clin. Microbiol. 1996, 34: 2850-2852.

Thilsted J.P and Dubey J.P. Neosporosis-likeabortions in a herd of dairy cattle. J. Vet. Diagn. Invest. 1989.1(3): 205-209.

Yamage M., Fletchner O., Gottstein B. *Neospora caninum*: Specific oligonucleotides primers for the detection of brain "cyst" DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). J. Parasitol., 1996, 82: 272-279.

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 – 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