ParoReal Kit *Neospora caninum*

Manual

For veterinary use only

REF **DVEP00511, DVEP00513** Σ **100**

REF **DVEP00551, DVEP00553** Σ **50**

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Explanation of symbols

LOT	Batch code		Use by
REF	Catalogue number		Manufactured by
Σ	Contains sufficient for <n> tests		Store at

1. Product description

ParoReal Kit *Neospora caninum* is a real-time PCR assay for detection of *Neospora caninum* DNA. This test was developed for the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific), LightCycler® 480 (Roche) and for Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments. This test allows the rapid and sensitive detection of DNA of *Neospora caninum* from samples purified from biopsies such as brain, heart, liver, placenta, and also body fluids (e.g. with the QIAamp DNA Mini Kit) or from feces (e.g. with the QIAamp DNA Stool Mini Kit).

ParoReal Kit *Neospora caninum* detects the mitochondrial NC5 gene (apparent in 2-3 copies/genome) of *Neospora caninum*. A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *Neospora caninum* specific DNA.
An internal positive control system (IPC) for detection in VIC/HEX channel, (554 nm, order no. DVEP00511 or DVEP00551) or Cy5 channel (667 nm; order no. DVEP00513 or DVEP00553) excludes false-negative interpretation of results due to inhibition of real-time PCR (see 8. Interpretation of PCR-data).
When using PCR-platforms not validated by ingenetix, an evaluation of the multiplex-PCR is recommended. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

BactoReal®, MycoReal, ParoReal and ViroReal® Kits are optimized to run under the same thermal cycling conditions. RNA and DNA material can be analysed in one run.

2. Pathogen information

Neospora caninum is a coccidian parasite and an important cause of spontaneous abortion in infected livestock. It has a heteroxenous life cycle, with the sexually reproductive stage occurring in the intestine of a definitive host (domestic dog, coyotes, gray wolves and dingos). Oocysts passed in the feces of the definitive host are ingested by intermediate hosts, such as cattle. Formation of cysts results in chronic infection of the intermediate host. Neosporosis is an infectious disease for many different canids and cattle. In canines it leads to neuromuscular degeneration. In dairy and beef cattle it is an important cause of reproductive problems and abortion. Once the cattle is infected, *N. caninum* is maintained as a life-long infection and is transmissed from cow to the fetus during pregnancy, resulting in abortion or weak or also normal calves. The occurrence of *N. caninum* infection in beef and dairy cattle has been reported worldwide.

3. Principle of real-time PCR

A specific DNA sequence of the pathogen genome is amplified and the generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows for a sequence-specific detection of PCR amplificates.

4. General Precautions

The user should always pay attention to the following:

- Always include a negative control per PCR-run (water instead of sample).
- Optional: for valid interpretation of results, a negative control should be included during DNA-extraction (for example extraction of water instead of sample material), in order to exclude false-positive results due to contamination with *Neospora caninum* DNA during extraction.
- Be careful when handling the positive control.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated workspace.
- Periodically decontaminate benches and devices.
- Use sterile pipette tips with filters.
- Thaw all components thoroughly at room temperature before starting an assay. When thawed, mix the components and centrifuge briefly.
- For MSDS, see www.ingenetix.com.

5. Contents of the Kit

5.1. ParoReal Kit *Neospora caninum* order no. DVEP00511 or DVEP00551

Labelling	Content	Amount		Storage
		DVEP00511	DVEP00551	
Neospora caninum Assay Mix (green cap)	Primer and probe (FAM) for detection of *Neospora caninum*	2 x 50 µl	1 x 50 µl	-20°C
CR-1 Assay Mix (yellow cap)	Primer, probe (VIC/HEX) and target for detection of IPC	2 x 50 µl	1 x 50 µl	-20°C
Neospora caninum Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl	1 x 25 µl	-20°C
DNA Reaction Mix (white cap)#	Reaction Mix	2 x 500 µl	1 x 500 µl	**-20°C until first use, then at +4°C**
Water (blue cap)	Water	1 x 1000 µl	1 x 1000 µl	-20°C to +4°C

#DNA Reaction Mix contains uracil-N glycosylase (UNG)

5.2. ParoReal Kit *Neospora caninum* order no. DVEP00513 or DVEP00553

Labelling	Content	Amount		Storage
		DVEP00513	DVEP00553	
Neospora caninum Assay Mix (green cap)	Primer and probe (FAM) for detection of *Neospora caninum*	2 x 50 µl	1 x 50 µl	-20°C
CR-3 Assay Mix (yellow cap)	Primer, probe (Cy5) and target for detection of IPC	2 x 50 µl	1 x 50 µl	-20°C
Neospora caninum Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl	1 x 25 µl	-20°C
DNA Reaction Mix (white cap)#	Reaction Mix	2 x 500 µl	1 x 500 µl	**-20°C until first use, then at +4°C**
Water (blue cap)	Water	1 x 1000 µl	1 x 1000 µl	-20°C to +4°C

#DNA Reaction Mix contains uracil-N glycosylase (UNG)

The components of ParoReal Kit *Neospora caninum* are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction
- PCR-grade water
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM and VIC/HEX or Cy5 channel
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material

7. Preparation of real-time PCR

Please make sure that at least one negative control (water, blue cap), as well as one positive control (red cap) and one extraction negative control (optional, recommended) are included per PCR run.
Ingenetix highly recommends performing PCR analyses in duplicates, which increases the probability of detection of the pathogen and facilitates interpretation of results.

7.1. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Water*	3.0 µl
	DNA Reaction Mix (2x)	10.0 µl
	Neospora caninum Assay Mix	1.0 µl
	CR Assay Mix	1.0 µl
	Total volume Master Mix	**15.0 µl**
Preparation of PCR assay	Master mix	15.0 µl
	Sample*	5.0 µl
	Total volume	**20.0 µl**

*1-8 µl of the sample can be used. When using a volume other than 5 µl, the volume of H_2O has to be changed accordingly.

Positive Control: As positive control use 1 µl of the *Neospora caninum* Positive Control + 4 µl H_2O.
Optional: a 1:10 dilution of the positive control can be used and defined as second standard value (approx. 1000 target copies/µl).

7.2. Programming of the temperature profile

Please find further information on programming the real-time PCR instrument in the respective operator's manual. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

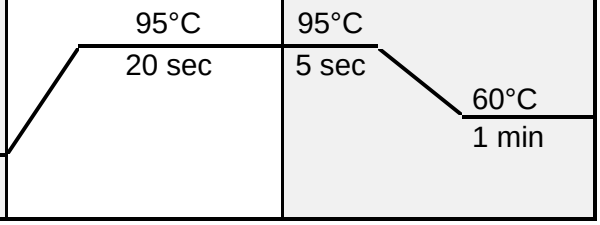
Select dyes: FAM-TAMRA for detection of *Neospora caninum*
Cy5-NONE (CR-3 Assay Mix) **or** VIC-TAMRA (CR-1 Assay Mix) for detection of IPC

Select reference dye (passive reference): ROX

Sample Volume: 20 µl

Temperature Profile:

Program 1	**Program 2**	**Program 3**
Cycles: 1 Analysis: None	Cycles: 1 Analysis: None	Cycles: 45 Analysis: Quantification Acquisition at 60°
50°C 2 min*	95°C 20 sec	95°C 5 sec 60°C 1 min



For ABI PRISM® 7500:
Ramp speed: Without "fast cycling" parameter

For LightCycler® 480 instrument:
Detection format: 2 Color Hydrolysis Probe (dyes see above)

***Note:** If viral RNA should be also detected in the same PCR run, program 1 has to be prolonged to 15 min at 50°C. This temperature profile can be used for all BactoReal®, MycoReal, ParoReal, and ViroReal® kits for the detection of DNA or RNA.

8. Interpretation of PCR-data

Examples for interpretation of positive reactions are shown in the amplification plots below.

For a valid interpretation, the following criteria must be fulfilled:

	Ct/Cp (FAM channel) *Neospora caninum* target	Ct/Cp IPC target	Interpretation
Negative control	Negative	36.0 ± 2	Valid
Positive control (undiluted, 1 µl/PCR)	28.0-31.0	36.0 ± 2	Valid
Extraction negative control (optional)	Negative	36.0 ± 2	Valid
Negative sample	Negative	36.0 ± 2	Valid
Positive sample	Positive	36.0 ± 2	Valid

For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with ParoReal Kit *Neospora caninum* please select fluorescence display options FAM channel for the *Neospora caninum* target and VIC/HEX channel (order no. DVEP00511, DVEP00551) or Cy5 channel (order no. DVEP00513, DVEP00553) for the internal positive control target (IPC). Samples with a positive Cp or Ct-value are considered positive. Please also check amplification-curves manually.

8.1. Signal in FAM channel:

→ DNA of *Neospora caninum* was amplified. The sample has to be interpreted as positive.
Neospora caninum DNA can lead to a reduced or absent fluorescence signal of the IPC.

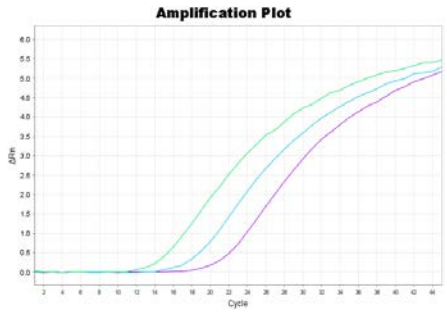
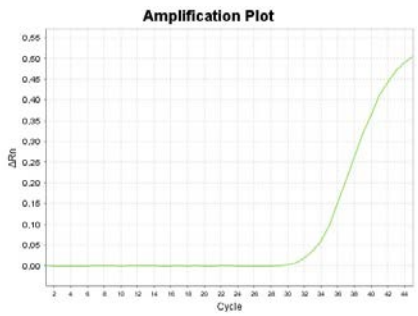
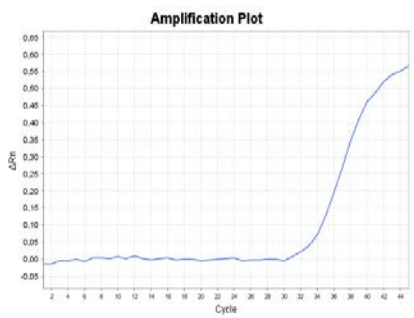
8.2. No signal in FAM channel but signal of the IPC:

→ No *Neospora caninum* DNA is detectable in the sample. The sample has to be interpreted as negative.
The positive signal of the internal positive control assay excludes a putative PCR inhibition.

8.3. No signals in FAM channel and no signal with the IPC:

→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

Amplification plots:

Detection of *Neospora caninum*	Detection of internal positive control **CR-3**	Detection of internal positive control **CR-1**
		
ABI Prism® 7500: FAM channel, 530 nm	**ABI Prism® 7500:** Cy5 channel, 667 nm	**ABI Prism® 7500:** VIC channel, 554 nm
1:10 serial dilution of *Neospora caninum* DNA	Internal positive control	Internal positive control

9. Troubleshooting

9.1. No *Neospora caninum* specific signal with positive control:

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 7. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 7. Preparation of real-time PCR) and repeat the PCR, if necessary.

9.2. No signal with IPC and no *Neospora caninum* specific signal with sample:

- The PCR reaction was inhibited. No interpretation can be made.
  → Make sure that you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during extractions can be retraced, it is recommended to repeat the PCR with lower amounts of DNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of H_2O).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.

9.3. *Neospora caninum* specific signal with negative control:

- A contamination occurred during preparation of the PCR.
  → Repeat the PCR with new reagents in replicates.
  → Strictly pipette the positive controls at last.
  → Make sure that work space and instruments are decontaminated at regular intervals.

9.4. *Neospora caninum* specific signal with negative control of extraction (optional):

- A contamination occurred during extraction.
  → Repeat the extraction and PCR using new reagents.
  → Make sure that work space and instruments are decontaminated at regular intervals.

10. Specifications

ParoReal Kit *Neospora caninum* was evaluated with the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific). For further validation data please contact ingenetix GmbH.

10.1. Analytical sensitivity

The analytical sensitivity is 5 target copies per PCR.

10.2. Analytical specificity

The specificity is ensured by the selection of highly specific primers and probes. The primers and probes were checked for possible homologies to currently published sequences by sequence comparison analyses. This also validated the detection of so far known *Neospora caninum* strains.