MycoReal Kit
Nosema apis & ceranae

Manual

For use with the

- **ABI PRISM® 7500 (Fast)**
- **Mx3005P®**
- **LightCycler® 480**

For veterinary use only

REF	**DVEF00113**	Σ	**100**
REF	**DVEF00153**	Σ	**50**

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1. Product description

MycoReal Kit *Nosema apis & ceranae* is a real-time PCR assay for detection and differentiation of DNA of *Nosema apis* and *Nosema ceranae*. This test was developed and validated for the ABI PRISM® 7500 (Fast) instrument (Life Technologies), LightCycler® 480 (Roche) and Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments. The test facilitates the rapid and sensitive detection of DNA of *Nosema apis* and *Nosema ceranae* from samples purified from bees (e.g. with the QIAamp DNA Mini Kit extraction methods).

MycoReal Kit *Nosema apis & ceranae* is based on the detection and differentiation of the 18S rRNA gene of *Nosema apis* (detection in VIC/HEX channel) as well as of the 18S rRNA gene of *Nosema ceranae* (detection in FAM and VIC/HEX channel) using multiplex real-time PCR. Thus, samples showing a positive signal in FAM as well as in VIC/HEX channel are positive for *N. ceranae.* Samples showing a positive signal in VIC/HEX channel are positive for *N. apis.*
An internal positive control system for detection in Cy5 channel (667 nm) 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

Nosema apis and *Nosema ceranae* are microsporidian parasites recently reclassified as a fungus. Both species cause nosema disease in honey bees (*Apis mellifera*). The dormant stages are long-lived spores. The most notable symptom is dysentery. *Nosema ceranae* can be detected in all four seasons, while *Nosema apis* occurs mostly in the milder seasons of autumn and spring. There is a higher mortality of bees when they are infected by *Nosema ceranae* than when infected with *Nosema apis*. Double infection is also possible.

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 *Nosema* 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

Labelling	Content	Amount		Storage
		DVEF00113	DVEF00153	
Nosema apis Assay Mix (purple cap)	Primer and probe (VIC/HEX) for detection of *N. apis*	2 x 50 µl	1 x 50 µl	-20°C
Nosema ceranae Assay Mix (green cap)	Primer and probe (FAM) for detection of *N. ceranae*	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
Nosema apis Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl	1 x 25 µl	-20°C
Nosema ceranae 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 MycoReal Kit *Nosema apis & ceranae* 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, VIC/HEX and 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*	2.0 µl
	DNA Reaction Mix (2x)	10.0 µl
	Nosema apis Assay Mix	1.0 µl
	Nosema ceranae Assay Mix	1.0 µl
	CR-3 Assay Mix	1.0 µl
	Total volume Master Mix	**15.0 µl**
Preparation of PCR	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 an amount other than 5 µl of the sample, the amount of H_2O has to be changed accordingly.

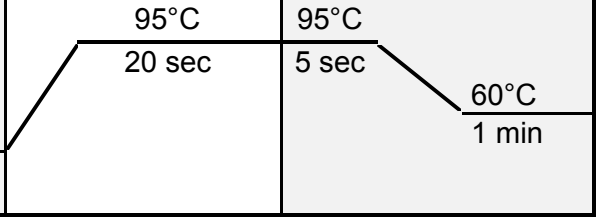
Positive Control: As positive control use 1 µl of the *Nosema apis* Positive Control and 1 µl of the *Nosema ceranae* Positive Control + 3 µl H_2O.
Optional: a 1:10 dilution of the positive control can be used and defined as second standard value (approx. 1000 target copies/µl).

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 *Nosema ceranae*
VIC/HEX-TAMRA for detection of *Nosema apis*
Cy5-NONE for detection of internal positive control
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: 3 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	Ct/Cp VIC/HEX channel	Ct/Cp IPC target	Interpretation
Negative control	Negative	Negative	36.0 ± 2	Valid
Positive control *N. ceranae*	26.0-28.0	27.0-29.0 (low fluorescence)	36.0 ± 2	Valid
Positive control *N. apis*	Negative	26.0-28.0	36.0 ± 2	Valid
Extraction negative control (optional)	Negative	Negative	36.0 ± 2	Valid
Negative sample	Negative	Negative	36.0 ± 2	Valid
Positive sample *N. ceranae*	Positive	Positive (low fluorescence)	Pos./Neg.	Valid
Positive sample *N. apis*	Negative	Positive	Pos./Neg.	Valid
Positive sample *N. apis* + *N. ceranae*	Positive	Positive	Pos./Neg.	Valid

For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with MycoReal Kit *Nosema apis & ceranae* please select fluorescence display options FAM, VIC/HEX channels for the *Nosema* targets and Cy5 channel for the internal positive control target. Samples with a positive Cp or Ct-value are considered positive. Please also check the presence of amplification-curves manually.

Once the analysis is completed, the following results are possible:

1. Signal in FAM channel and signal in VIC/HEX channel; the signal in VIC/HEX channel has low fluorescence comparable to fluorescence of CR3 assay:
→ DNA of *Nosema ceranae* was amplified. The sample has to be interpreted as positive.
Nosema DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

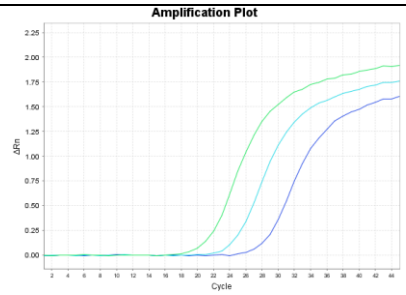
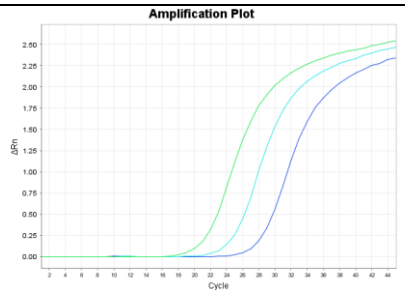
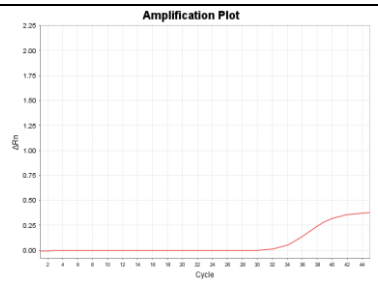
2. Signal in FAM channel and in VIC/HEX channel; the signal in VIC/HEX channel has high fluorescence
→ DNA of *Nosema ceranae* + *Nosema apis* was amplified. The sample has to be interpreted as positive.
Nosema DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

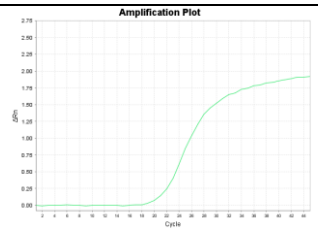
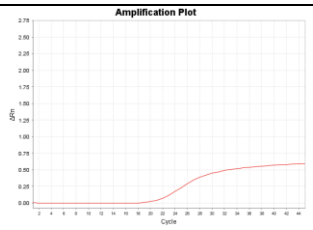
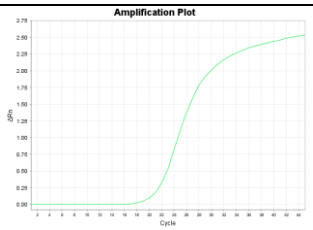
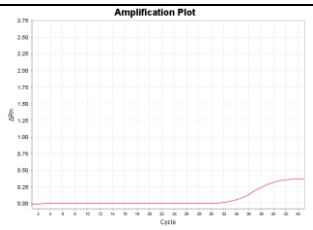
3. Signal in VIC/HEX channel, no signal in FAM channel:
→ DNA of *Nosema apis* was amplified. No DNA of *Nosema ceranae* is detectable in the sample. The sample has to be interpreted as positive.
Nosema DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

4. No signal in FAM or VIC/HEX channel:
→ No DNA of *Nosema apis* or *Nosema ceranae* is detectable in the sample. The sample has to be interpreted as negative.
An inhibition of PCR cannot be excluded.

4a. No signal in FAM or VIC/HEX channel but signal of the internal positive control:
→ No DNA of *Nosema apis* or *Nosema ceranae* 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.

4b. No signals in FAM or VIC/HEX channel and no signal with internal positive control:
→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

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

Detection of ***Nosema ceranae***		Detection of ***Nosema apis***	Detection of internal positive control **CR-3**
			
ABI Prism® 7500: FAM channel, 530 nm *N. ceranae* DNA, approx. 60,000 copies	**ABI Prism® 7500:** VIC channel, 554 nm *N. ceranae* DNA, approx. 60,000 copies	**ABI Prism® 7500:** VIC channel, 554 nm *N. apis* DNA, approx. 60,000 copies	**ABI Prism® 7500:** Cy5 channel, 667 nm Internal positive control

9. Troubleshooting

1. No *Nosema* specific signal with positive controls:

- 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.

2. No signal with the internal positive control and no *Nosema* specific signal with the 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 amount of H_2O).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.

3. *Nosema* specific signal with the 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.

4. *Nosema* specific signal with the negative control of DNA-extraction:

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

10. Specifications

MycoReal Kit *Nosema apis & ceranae* was evaluated with the ABI PRISM® 7500 (Fast) instrument (Life Technologies), with the LightCycler® 480 (Roche) and the Mx3005P® (Agilent). For further validation data please contact ingenetix.

10.1. Analytical sensitivity

The analytical sensitivity is approx. 3 target copies/PCR reaction.

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 *Nosema apis* and *Nosema ceranae* strains.

11. Annex – symbols

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