# MycoReal Kit
# *Ascosphaera apis*

## Manual

**For use with the**

- **Applied Biosystems® 7500 (Fast)**
- **Mx3005P®**
- **LightCycler® 480**


**For veterinary use only**

**REF DVEF00211, DVEF00213** Σ **100**

**REF DVEF00251, DVEF00253** Σ **50**

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## 1. Product description

MycoReal Kit *Ascosphaera apis* is a real-time PCR kit for detection of *Ascosphaera apis* DNA. This test was developed for the Applied Biosystems® 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 *Ascosphaera apis* from samples purified from diseased larvae and/or honey samples (spores) (e.g. with the QIAGEN DNeasy Plant Mini Kit). Cell/spore suspensions and suspensions containing only spores have to be differentiated. Suspensions containing only spores require a more complex DNA extraction step [1].

MycoReal Kit *Ascosphaera apis* detects the ITS1 region of *Ascosphaera apis.* A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *Ascosphaera apis* specific DNA.
An internal positive control system (IPC) for detection in VIC/HEX channel, (554 nm, order no. DVEF00211 or DVEF00251) or Cy5 channel (667 nm; order no. DVEF00213 or DVEF00253) 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.

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

## 2. Pathogen information

*Ascosphaera apis* is a fungus belonging to the family *Ascophaeraceae* and affecting honeybee brood. This fungus causes the larval disease chalkbrood of honeybees (*Apis mellifera*). Larvae infected by ingesting fungal spores that germinate within the digestive tract and compete with the larvae for food leads to starvation. The fungus consumes the body of the larvae, resulting in a white and “chalky” appearance, hence the designation chalkbrood, and is most visible during wet springs with cooler outside temperatures. The chalky mummies are highly infectious and spores of this fungus often re-infect colonies. *Ascosphaera apis* causes large economic losses, particularly in combination with other pathogens [2].

**References:**
[1] Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. World Organization of Animal Health. http://www.oie.int/en/international-standard-setting/terrestrial-manual/
[2] E. Garrido-Baitón et al. (2013). The prevalence of the honeybee brood pathogens *Ascosphaera apis*, *Paenibacillus larvae* and *Melissococcus plutonius* in Spanish apiaries determined with a new multiplex PCR assay. Microbial Biotechnology, 6, 731-739.

## 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 *A. apis* 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. MycoReal Kit *Ascosphaera apis* order no. DVEF00211 or DVEF00251

| Labelling | Content | Amount | | Storage |
|---|---|---|---|---|
| | | DVEF00211 | DVEV00251 | |
| *Ascosphaera apis* Assay Mix (green cap) | Primer and probe (FAM) for detection of *A. apis* | 2 x 50 µl | 1 x 50 µl | -15°C to -25°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 | -15°C to -25°C |
| *Ascosphaera apis* Positive Control (red cap) | Control-DNA (approx. 10,000 target copies/µl) | 1 x 25 µl | 1 x 25 µl | -15°C to -25°C |
| DNA Reaction Mix (white cap) # | Reaction Mix | 2 x 500 µl | 1 x 500 µl | **-15°C to -25°C until first use, then at +4°C** |
| Water (blue cap) | Water | 1 x 1000 µl | 1 x 1000 µl | -25°C to +4°C |

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

### 5.2. MycoReal Kit *Ascosphaera apis* order no. DVEF00213 or DVEF00253

| Labelling | Content | Amount | | Storage |
|---|---|---|---|---|
| | | DVEF00213 | DVEV00253 | |
| *Ascosphaera apis* Assay Mix (green cap) | Primer and probe (FAM) for detection of *A. apis* | 2 x 50 µl | 1 x 50 µl | -15°C to -25°C |
| CR-3 Assay Mix (yellow cap) | Primer, probe (Cy5) and target for detection of IPC | 2 x 50 µl | 1 x 50 µl | -15°C to -25°C |
| *Ascosphaera apis* Positive Control (red cap) | Control-DNA (approx. 10,000 target copies/µl) | 1 x 25 µl | 1 x 25 µl | -15°C to -25°C |
| DNA Reaction Mix (white cap) # | Reaction Mix | 2 x 500 µl | 1 x 500 µl | **-15°C to -25°C until first use, then at +4°C** |
| Water (blue cap) | Water | 1 x 1000 µl | 1 x 1000 µl | -25°C to +4°C |

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

The components of MycoReal Kit *Ascosphaera apis* 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
- Nuclease-free 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 |
| | *Ascosphaera apis* 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 *Ascosphaera apis* 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 *A. apis*
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<br>Analysis: None | Cycles: 1<br>Analysis: None | Cycles: 45<br>Analysis: Quantification<br>Acquisition at 60° |
| 50°C<br>2 min* | 95°C<br>20 sec | 95°C<br>5 sec<br>60°C<br>1 min |

<u>For Applied Biosystems® 7500:</u>
Ramp speed: Without "fast cycling" parameter

<u>For LightCycler® 480 instrument:</u>
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

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

| | Ct/Cp (FAM channel) *A. apis* 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 | Positive/Negative* | Valid |

**For analysis of PCR data please proceed as follows:**
For analysis of PCR results gained with MycoReal Kit *Ascosphaera apis* please select fluorescence display options FAM channel for the *A. apis* target and VIC/HEX channel (order no. DVEF00211, DVEF00251) or Cy5 channel (order no. DVEF00213, DVEF00253) 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 *A. apis* was amplified. The sample has to be interpreted as positive.
*) *Ascosphaera apis* DNA can lead to a reduced or absent fluorescence signal of the IPC.

### 8.2. No signal in FAM channel but signal of IPC
→ No *A. apis* DNA is detectable in the sample. The sample has to be interpreted as negative.
The positive signal of the IPC assay excludes a putative PCR inhibition.

### 8.3. No signals in FAM channel and no signal with IPC
→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

## 9. Troubleshooting

### 9.1. No *A. apis* 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 *A. apis* 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. *A. apis* 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 workspace and instruments are decontaminated at regular intervals.

### 9.4. *A. apis* specific signal with negative control of extraction (optional)
- A contamination occurred during extraction.

→ Repeat the extraction and PCR using new reagents.
→ Make sure that workspace and instruments are decontaminated at regular intervals.

## 10. Specifications and performance evaluation

MycoReal Kit *Ascosphaera apis* was evaluated with the Applied Biosystems® 7500 (Fast) instrument (Thermo Fisher Scientific). For further validation data please contact ingenetix GmbH.

### 10.1. Analytical sensitivity and linearity

MycoReal Kit *Ascosphaera apis* was tested with a 10-fold dilution series of a plasmid containing a fragment of *Ascosphaera apis* DNA. Analytical sensitivity is 10 target copies/PCR reaction.
The assay shows **linearity** over the range of 10 to 10,000,000 target copies/reaction with a slope of -3.7 and a $R^2$ of > 0.99 as shown in Figure 1.

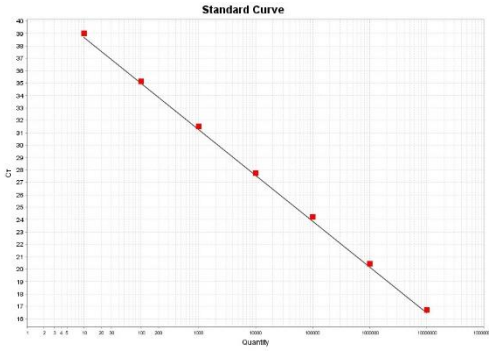



**Figure 1** Ten-fold dilution series of an *A. apis* DNA standard plotted against Ct

### 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 *Ascosphaera apis* strains.

## 10.3. Kit performance

Performance of MycoReal Kit *Ascosphaera apis* with an Applied Biosystems® 7500 Fast Real-time PCR System (Thermo Fisher Scientific) is shown in Figure 2.

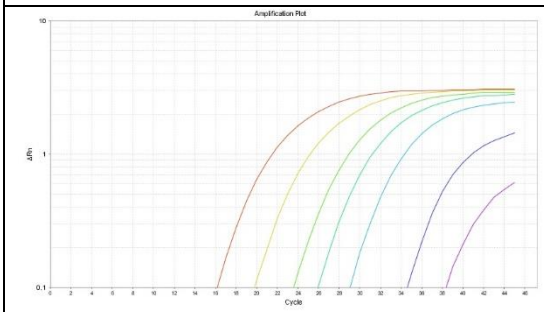
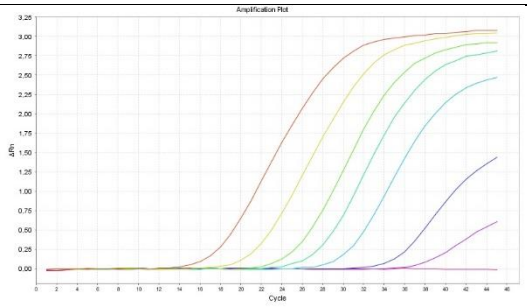
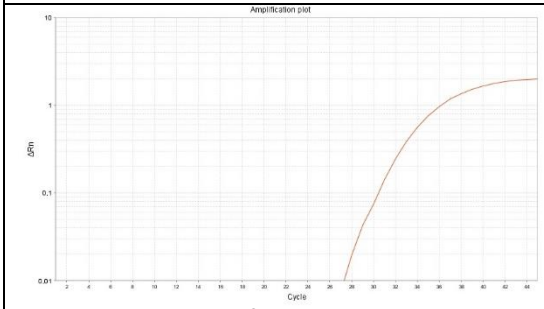
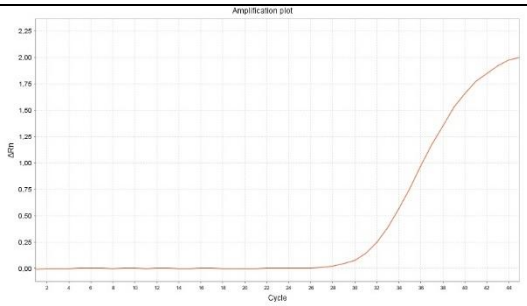
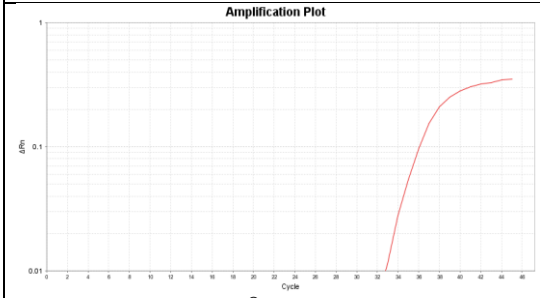
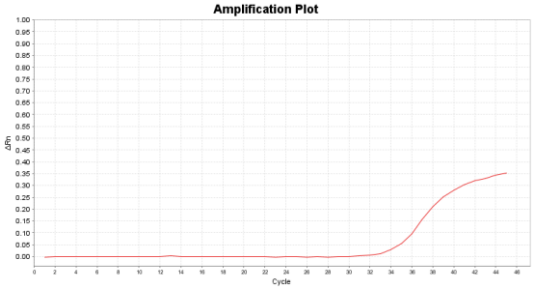
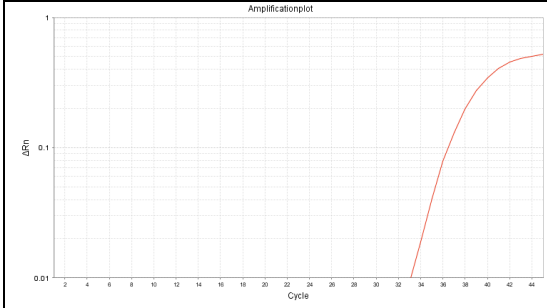
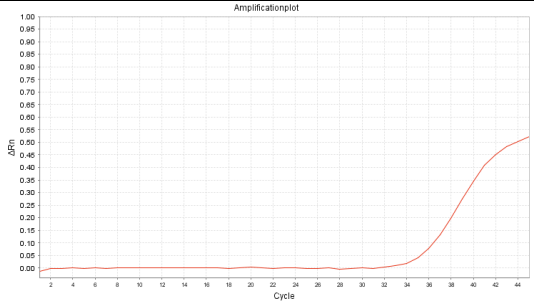
| Logarithmic view | Linear view |
|---|---|
|  |  |
| **Applied Biosystems® 7500:** FAM channel, 530 nm<br>1:10 serial dilution of a *Ascosphaera apis* DNA target | |
|  |  |
| **Applied Biosystems® 7500:** FAM channel, 530 nm<br>*Ascosphaera apis* positive control | |
|  |  |
| **Applied Biosystems® 7500:** Cy5 channel, 667 nm<br>Internal DNA positive control (ControlReal 3 or Internal Positive Control Assay 3) | |
|  |  |
| **Applied Biosystems® 7500:** VIC/HEX channel, 554 nm<br>Internal DNA positive control (ControlReal 1 or Internal Positive Control Assay 1) | |

**Figure 2** Performance of MycoReal Kit *Ascosphaera apis*

## 11. Annex – symbols

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