MycoReal® Kit *Aspergillus*

Kit version 1.1

Instructions for Use

CE

IVD **For *in-vitro* diagnostic use**

REF **DHUF00253**

Σ **50 reactions**

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Explanation of symbols

LOT	Batch code		Use by date
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Temperature limit (Store at)
CE	This product fulfils the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices	IVD	In vitro diagnostic medical device
	Consult instructions for use	UDI	Unique device identifier
	Keep away from sunlight	QTY	Contents

1 Intended purpose

MycoReal® Kit *Aspergillus* is a non-automated IVD real-time PCR test for the qualitative detection of DNA (ITS2-region) of *Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus, Aspergillus niger* and *Aspergillus nidulans*. Furthermore, it detects some other closely related *Aspergillus/Emericella* species belonging to *Aspergillus* section *Fumigati, Flavi, Terrei, Nigri, Nidulantes, Usti* and *Versicolori*.

Proper specimens are DNA extracts isolated from human samples of EDTA-blood, bronchoalveolar lavage (BAL), aspirate, cerebrospinal fluid (CSF), tissue and paraffin-embedded tissue.

This test is suitable for patients with suspected infection with *Aspergillus* and is intended as an aid in the diagnosis of infection with this pathogen in combination with patient history and additional clinical information.

The test is intended for professional use and is limited to qualified personnel instructed in the procedures of real-time PCR and *in vitro* diagnostic procedures.

2 Product description

MycoReal® Kit *Aspergillus* is a real-time PCR test and detects the internal transcribed spacer 2 (ITS2-region, multicopy gene) of *Aspergillus.* Due to the use of specific probes (FAM and VIC labelled) the test is highly specific for *Aspergillus.*

This test detects clinically relevant *Aspergillus* species including *A. fumigatus, A. flavus, A. terreus, A. niger* and *A. nidulans*. The analysis is performed with 2 different assay mixes in two parallel PCR reactions (see Table 1) and thus allows the differentiation of individual species, enabling targeted antifungal therapy.
This test detects also other *Aspergillus* and *Emericella* species belonging to *Aspergillus* section *Fumigati, Flavi, Terrei, Nigri, Nidulantes, Usti* and *Versicolori*. It does not cross react with other fungi (see 13.4. Analytical Specificity).

A probe-specific amplification-curve in the FAM or VIC channel indicates amplification of *Aspergillus* specific DNA (Table 1). The Internal Positive Control (IPC) is detected in the fluorescence channel Cy5 and serves as a control for DNA extraction and possible real-time PCR inhibition. The target for the IPC (artificial target DNA) is added during sample extraction.

Table 1 Detection channels

	Target 1 FAM	**Target 2 VIC**	**Target 3 CY5**
Aspergillus 1 Assay Mix	*A. flavus*	*A. fumigatus*	IPC
Aspergillus 2 Assay Mix	*A. niger* and *A. nidulans*	*A. terreus*	IPC

This test has been validated with the ABI® 7500 Fast instrument (Thermo Fisher Scientific, fast cycle parameters are not supported) and tested with a cobas z 480 Analyzer (Roche).
The test is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM, VIC and Cy5 channel (e.g., QuantStudio™ 5, QuantStudio™ 7 real-time PCR system (Thermo Fisher Scientific), qTOWER^{3}G (Analytik Jena), Mic instrument (bio molecular systems), cobas z 480 Analyzer (Roche)).
When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR shall be done. Keep in mind that some PCR-platforms first must be calibrated with the corresponding dye before performing multiplex-PCR.

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

3 Pathogen information

Aspergillus is a fungal genus consisting of several hundred species. *Aspergillus* species are ubiquitously found and some species can cause opportunistic infections such as allergic bronchopulmonary aspergillosis, pulmonary aspergilloma and invasive aspergillosis. Invasive aspergillosis is increasingly recognized as a primary cause of morbidity and mortality especially in immunocompromised patients. *Aspergillus fumigatus* is the predominant disease-causing species. However, other species than *A. fumigatus*, in particular *A. terreus* and *A. flavus* but also *A. niger, A. nidulans* and *A. ustus* have gained greater clinical significance.
Not only the detection, but also the identification of the fungal species is important. The species might provide information on a potential contamination, colonisation or infection and moreover antifungal resistance.

4 Principle of real-time PCR

The test is based on multiplex real-time PCR by 5´-nuclease-assay technology. Specific DNA sequences are amplified and the generated PCR-products are detected by oligonucleotide-probes labelled with fluorescent dyes. This allows a sequence-specific detection of PCR amplificates.
During PCR, primers are extended by *Taq* polymerase and probes hybridized to the target are cleaved by the 5'-exonuclease activity of *Taq* polymerase. According to the accumulation of PCR product, the fluorescence of the probe increases with each PCR cycle. The change in fluorescence of the different dyes is recorded cycle by cycle in the real-time PCR instrument in the closed reaction tube at different fluorescence wavelengths.
The Cq value (Cq = Quantification cycle, Ct = Cycle threshold, Cp = Crossing point) describes the cycle at which the fluorescence first rises significantly above the background fluorescence.

5 Kit contents, stability, and storage

Table 2

Labelling	**Content**	**Amount**	**Storage**
Aspergillus 1 + IPC3 Assay Mix (green cap)	Primer and probe for detection of - *A. flavus* (FAM) - *A. fumigatus* (VIC) - IPC (Cy5)	1 x 50 µl	-25 to -15 °C
Aspergillus 2 + IPC3 Assay Mix (green cap)	Primer und probe for detection of - *A. nidulans* and *A. niger* (FAM) - *A. terreus* (VIC) - IPC (Cy5)	1 x 50 µl	-25 to -15 °C
IPC-Target (DNA) (orange cap)	Target for DNA IPC (internal DNA positive control system)	1 x 200 µl	-25 to -15 °C
Aspergillus Positive Control (red cap)	DNA positive control for *A. fumigatus* + *A. flavus* + *A. nidulans* + *A. terreus* (à approx. 1000 target copies/µl)	1 x 300 µl	-25 to -15 °C
DNA Reaction Mix (white cap)	PCR reaction mix for DNA amplification	2 x 500 µl	**-25 to -15 °C after first opening 2 to 8 °C**
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	-25 to -15 °C

DNA Reaction Mix
The Master Mix provided with the kit has been designed for reliable, high-sensitivity real-time PCR. The Master Mix contains a highly purified Taq Polymerase for rapid hot-start PCR, dNTPs with dUTP and Uracil-N glycosylase (UNG) to eliminate amplicon carryover, ROX™ dye (passive reference) and buffer components – additives optimized to handle RT-PCR inhibitors.

Delivery and Storage
Shipment is with dry ice or with coolpacks. When stored properly, the kit components are stable until the specified expiration date. This also applies after opening. Store kit protected from light.

Quality Control Release Testing
In accordance with the ISO 13485 certified Quality Management System of ingenetix, each lot is tested against predetermined specifications to ensure consistent product quality. Quality control is performed with a plasmid containing parts of the pathogen DNA.

6 Additionally required materials and devices

- Reagents and devices for DNA-extraction which are appropriate for the listed sample material (see 9. Preparation of the samples)
- Nuclease-free water
- Powder-free disposable gloves
- Pipettes (adjustable)
- Filter pipette tips
- Vortex-Mixer
- Benchtop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument detecting and differentiating fluorescence in FAM, VIC, Cy5 channel (see 2. Product description)
- Appropriate 96 well optical reaction plates or reaction tubes with corresponding (optical) closing material
- Optional: Laminar flow box
- Optional: PCR workstation

7 Precautions and safety information

7.1 General information

- For *in vitro* diagnostic use. The use of this kit is limited to qualified personnel instructed in real-time PCR and *in vitro* diagnostic procedures.
- Transportation of clinical specimens must comply with local regulations for the transport of biological materials.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective powder-free disposable gloves when handling kit reagents and specimens.
- Discard specimens, reagents, and waste according to your local safety regulations.
- Improper collection, transport or storage of specimens may hinder the ability of the assay to detect the target sequences.
- Quality of DNA has a profound impact on the test performance. Ensure that the used DNA extraction system is compatible with real-time PCR technology.
- The real-time PCR instrument should be calibrated, serviced and cleaned regularly.
- Protect kit components from light.
- Avoid mixing reagents of different kits and lots and check expiry date of the kit.

7.2 Specific information

A workflow must be followed to avoid false positive results due to detection of contaminating DNA.

<u>Recommended measures to avoid DNA contamination:</u>

- Use separated workspaces for specimen preparation, set-up of real-time PCR and amplification. Supplies and equipment must be dedicated to each of these separate workspaces to ensure workflow in the laboratory from pre- to post-PCR.
- Laboratory benches and devices must be cleaned regularly.
- Sample preparation should be performed in a laminar flow box. Clean laminar flow box regularly in all areas.
- If possible, leave consumables and pipettes in the laminar flow sterile bench and PCR workstation.
- Preparation of real-time PCR should be done in a PCR workstation.
- The use of sterile aerosol-resistant pipette tips is required.
- Use only DNA-free consumables.
- Wear lab coat.

- Work only with powder-free disposable gloves, do not touch the outer surface of the palm and fingers of the gloves when putting them on. Change gloves frequently. To avoid skin contact, wear gloves over the sleeves of the lab coat. Use disposable sleeve protectors if possible.
- Do not touch the rim or threads of open vials.
- Caution is advised when handling specimens and positive control to avoid cross-contamination.
- Store positive and potentially positive material separately from all other reagents.
- For a valid interpretation of results, a negative control shall be included during DNA-extraction (e.g., extraction of water instead of sample material), in to exclude false-positive results due to contamination with DNA of pathogen during extraction.
- Optional: include a negative control of PCR per PCR-run (nuclease-free water instead of sample, NTC).

8 Limitations

- Reliable results with this test are only achieved by appropriate specimen collection, transport, and storage, as well as an appropriate DNA extraction procedure.
- The extraction system must ensure the extraction of fungal cells and shall be free of fungal DNA.
- This kit is only suitable for specimens taken from normally sterile/semi-sterile sites.
- This kit has been used to validate the detection of fungal DNA from EDTA-blood, bronchoalveolar lavage (BAL), aspirate, cerebrospinal fluid (CSF), tissue and paraffin-embedded tissue. Test performance with other specimen types has not yet been assessed.
- A negative test result does not exclude the possibility of an *Aspergillus* infection, because test results may be affected by improper specimen collection, technical error, specimen mix-up or pathogen quantities below the assay sensitivity. PCR inhibitors may lead to invalid results.
- Sequence variabilities in the target-region of some subtypes (strains) may lead to false-negative or less sensitive results.
- Results should be interpreted in context of other clinical and laboratory findings.

9 Preparation of samples

MycoReal® Kit *Aspergillus* is suitable for analysis of DNA extracted from human EDTA-blood, BAL, aspirate, CSF, tissue, and paraffin-embedded tissue.

Sample preparation should be performed with the recommended measures to avoid DNA contamination (see precautions and safety information). Always include a DNA extraction negative control (e.g., extraction of water).

Purified DNA should be stored at -25 to -15 °C.

9.1 Sample collection and storage

Aspirates and CSF can be stored in microcentrifuge tubes. It is recommended to process samples immediately after collection. Store samples at 2-8 °C for no longer than 48 hours or freeze at -20/-80 °C.

Blood has to be stored in EDTA blood collection tubes. It is recommended to process samples immediately after collection. Store samples at 2-8 °C for no longer than 48 hours or freeze at -20/-80 °C.

Tissue can be stored in microcentrifuge tubes containing sterile physiological saline (approx. 100 µl) at 2-8 °C for up to 48 h. If it is not guaranteed that the sample will be processed within 48 h, the sample shall be immediately frozen dry without additives at -20/-80 °C.

9.2 Recommended extraction methods

Ensure that the extraction system used is not contaminated with DNA of pathogens detected with MycoReal® Kit *Aspergillus.* Extract the sample using a DNA extraction system compatible with real-time PCR technology and appropriate for the sample material. The extraction system must ensure the extraction of fungal cells. The sensitivity of extraction of fungal DNA can be significantly increased by mechanical lysis with beads or with 3 to 5 “freeze/boil cycles” using liquid nitrogen or -80 °C and a heating block during extraction.
When testing paraffin-embedded tissue: mind to use only tissue-containing paraffin sections.

For manual extraction recommended
- Modified protocol of the High Pure PCR Template Preparation Kit, Roche Diagnostics. After incubation with proteinase K: Freeze reaction tube in liquid nitrogen or at -80 °C (liquid nitrogen is more effective), then put for 1 min at 95 -100 °C. Repeat these steps three times.

For automated extraction recommended
- innuPREP AniPath DNA RNA – KFFLX Kit (Analytik Jena) with the KingFisher FLEX instrument (Thermo Fisher Scientific)

9.2.1 Extraction of EDTA blood

The extracted DNA analyzed per module should be equivalent to 50 µl of blood at least (e.g., 500 µl of blood eluted in 100 µl). Extraction of 1 ml EDTA blood in combination with mechanical lysis by beads is recommended.

For automated extraction of EDTA blood recommended
- Mechanical lysis of 1.3 ml EDTA blood with beads (e.g., MP Biomedicals™ Lysing Matrix E, 2 ml, mpbio) on the Magnalyser or another beadbeater (70 sec, 7000 rpm).
- Subsequently, automated extraction of supernatant (approx. 800 µl) with the innuPREP AniPath DNA RNA KFFLX Kit (Analytik Jena) with a modified protocol for 800 µl sample volume using the KingFisher FLEX extraction device (Thermo Fisher Scientific).

When using extraction methods not recommended by ingenetix, an evaluation of the extraction method must be performed.

9.3 Quality control for DNA extraction and PCR inhibition with IPC

The DNA IPC system (internal DNA positive control) is used as a control for DNA extraction, identifies possible PCR inhibition and confirms the integrity of kit reagents.
For this, an artificial target DNA (IPC-Target (DNA), approx. $6x10^5$ copies/µl) is added during extraction.

Note: The Cq values of the IPC depend on the extraction method and on the type of sample material. Negative samples should show Cq values of the IPC between 27-30. The extraction method used must be validated accordingly with sample material. Use the IPC-Target (DNA) freshly diluted (1:10 with nuclease-free water) in the extraction, if Cq values < 27 are determined during validation with samples.

9.3.1 Application of IPC as control of extraction and real-time PCR

The IPC-Target (DNA) must be added during extraction.

→ Per sample spike 1 µl IPC-Target (DNA) (orange cap) directly into the appropriate volume of lysis buffer (or spike it to the sample after the lysis buffer has been added to the sample), then continue the extraction procedure.

Caution: The IPC-Target (DNA) shall not be added to sample material in the absence of lysis buffer, as degradation may occur. It must be added to the lysis buffer.

9.3.2 Application of IPC as quality control for PCR reaction

If the IPC-Target (DNA) has not been added during extraction, it can be added at a later stage to the PCR master mix.

→ Freshly dilute the IPC-Target (DNA) 1:100 with nuclease-free water and add 1 µl of the dilution/PCR reaction (approx. 6000 target copies).

Caution: The IPC-Target (DNA) shall not be added to the master mix undiluted.

10 Preparation of real-time PCR

- Include one positive control (red cap), one extraction negative control and optionally one PCR negative control (NTC, e.g., nuclease-free water) per PCR run.
- It is generally recommended to analyse samples in duplicates, which increases the probability of pathogen detection and facilitates interpretation of results.
- Thaw DNA samples on ice.
- Thaw kit components completely at room temperature. When thawed, mix components carefully, centrifuge briefly with low speed.
- Mix the DNA Reaction Mix gently to ensure homogeneity of solution.
- **Positive Control**
  → Use 9 µl of Positive Control (red cap). Always, pipette positive control at last

  Aspergillus Positive Control is a pool of four DNA target sequences for *A. fumigatus* + *A. flavus* + *A. nidulans* + *A. terreus*.

10.1 Pipetting scheme

Samples as well as the positive control and the extraction negative control must be analyzed with Aspergillus 1 + IPC3 Assay Mix and Aspergillus 2 + IPC3 Assay Mix, respectively. For blood and CSF samples it is recommended to use 9 µl DNA sample.

Aspergillus 1 + IPC3 Assay Mix:

		Per sample
Preparation of Master Mix 1 (mix well)	DNA Reaction Mix	10,0 µl
	Aspergillus 1 + IPC3 Assay Mix	1,0 µl
	Total volume of Master Mix 1	**11,0 µl**
Preparation of PCR	Master Mix	11,0 µl
	DNA-sample*	9,0 µl
	Total volume	**20,0 µl**

Aspergillus 2 + IPC3 Assay Mix:

		Per sample
Preparation of Master Mix 2 (mix well)	DNA Reaction Mix	10,0 µl
	Aspergillus 2 + IPC3 Assay Mix	1,0 µl
	Total volume of Master Mix 2	**11,0 µl**
Preparation of PCR	Master Mix	11,0 µl
	DNA-sample*	9,0 µl
	Total volume	**20,0 µl**

*1-9 µl of the sample can be used. For ≠ 9 µl sample, the volume must be adjusted with nuclease-free water.

→ **If IPC-Target (DNA) was not added during extraction:** Freshly dilute the IPC-Target (DNA) (orange cap) 1:100 with nuclease-free water and add 1 µl per sample directly to the master mix. In this case, the IPC is used for quality control of the PCR reaction. Only 8 µl of DNA sample can be analyzed.
Caution: The use of more than 1 µl diluted IPC-Target (DNA) per reaction might cause inhibition of the real-time PCR reaction.

- Prepare the Master Mix according to the number of samples, calculating an additional volume of approx. 10% for pipetting loss.
- Pipette 11 µl of the prepared Master Mix per sample into the well of the optical reaction plate.
- Then add 9 µl of the extracted sample or controls. Pipette the positive control at last.
- Seal the plate with a suitable optical sealing material.
- Vortex the sealed plate for 1-2 seconds and briefly centrifuge the plate.

10.2 Programming of temperature profile

Please find further information on programming of the real-time PCR instrument in the respective operator's manual. Keep in mind that some PCR-platforms must be calibrated with the corresponding dye before performing a multiplex-PCR.

Sample Volume: 20 µl
Ramp speed: "Standard" without "fast cycling" parameter for ABI® 7500 Fast Instrument, QuantStudio™ (Thermo Fisher Scientific)

Program 1 Cycles: 1 Analysis: None	**Program 2** Cycles: 1 Analysis: None	**Program 3** Cycles: 45 Analysis: Quantification Acquisition at 60 °C
50 °C 2 min	95 °C 2 min	95 °C 5 sec 60 °C 30 sec

Ad program 1: If viral RNA should be also detected in the same PCR run, program 1 must be prolonged to 15 min at 50 °C. This temperature profile can be used for all ingenetix ViroReal®, BactoReal®, MycoReal®, PanReal and ParoReal® kits for the detection of DNA or RNA.
Ad program 2: the previous temperature profile with 20 sec in program 2 can still be used.
Ad program 3: 1 minute possible in combination with other ingenetix kits.

Select detection channels

	Target 1 FAM-NONE	**Target 2 VIC-NONE**	**Target 3 Cy5-NONE**
Assay Mix 1	*Aspergillus flavus*	*Aspergillus fumigatus*	IPC
Assay Mix 2	*A. niger* and *A. nidulans*	*A. terreus*	IPC

For ABI® 7500 Instrument, QuantStudio™ 5/6/7 (Thermo Fisher Scientific), Mx3005P® (Agilent)
Passive reference dye: ROX

For cobas z 480 Analyzer / LightCycler® 480 II (Roche)
Detection format: 3 Color Hydrolysis Probe
FAM: Excitation at 465 nm, Emission at 510 nm
VIC: Excitation at 540 / 533 nm, Emission at 580 nm
Cy5: Excitation at 610 / 618 nm, Emission at 670 / 660 nm
Passive reference dye: no ROX needed

The color compensation for FAM and VIC must be selected from the Roche database after the analysis of Cy5.

11 Interpretation of data

For the analysis of the PCR results, select the fluorescence display options FAM and VIC channel for the pathogen target and Cy5 channel for the IPC.
Please note that some PCR platforms require Color Compensation when using a multiplex PCR.

IMPORTANT: Samples should be checked in both logarithmic (Roche instrument: Abs Quant/Fit Points) and linear view and compared to the negative controls. In addition to the Cq values, also check the amplification curves and adjust the threshold (noise band) if necessary.
After you have saved the new settings, export the data. For the cobas z 480 Analyzer, export tables per dye.

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid. If results of controls are not valid, no interpretation of results with clinical samples is possible.

Negative samples:
Samples without amplification curves (no Cq values, undetermined) are regarded as negative. No DNA was detected in these samples because there is no infection with the corresponding pathogen or the pathogen DNA concentration is below the detection limit of the test.

Positive samples:
Since *Aspergillus* spp. are environmental organisms, contamination with DNA thereof is possible during sampling, extraction, and PCR. Cq values of the samples in the FAM and VIC channel must be interpreted in comparison to Cq values of the negative control of DNA extraction also. Defined cut-off Cq values apply to the sample materials listed below. Samples with Cq values above the cut-off must be noted in the report as possibly being contaminated.

Cut-off Cq-values:

- Normally sterile samples such as blood, CSF, punctates, aspirates, tissue: Cut-off = Cq ≥ 45.0
- Semisterile samples such as BAL, punch biopsies: Cut-off = Cq ≥ 38.0
- Paraffin embedded tissue: Cut-off = Cq ≥ 34.0

Table 3 shows the criteria for valid positive and negative controls. Table 4, Table 5 show interpretation of data with clinical samples.

11.1 Controls

Table 3 Criteria for valid controls, IPC-Target (DNA) was added during extraction. Applies to the analysis with Aspergillus 1 + IPC3 Assay Mix and Aspergillus 2 + IPC3 Assay Mix

	Cq FAM channel Aspergillus	Cq VIC channel Aspergillus	Cq Cy5 channel IPC[1]	Interpretation	Action
Positive control	< 30	< 30	Negative	Valid	-
Positive control	Negative	Negative	Negative	Invalid	See 12.1
Positive control	< 30	Negative	Negative	Invalid	See 12.1
Positive control	Negative	< 30	Negative	Invalid	See 12.1
Extraction negative control	Negative	Negative	27-30[2]	Valid	-
Extraction negative control	Negative	Negative	Negative	Invalid	See 12.1
Extraction negative control	Positive	Positive	27-30[2]	Invalid	See 12.3
Extraction negative control	Positive	Negative	27-30[2]	Invalid	See 12.3
Extraction negative control	Negative	Positive	27-30[2]	Invalid	See 12.3
NTC PCR (optional)	Negative	Negative	Negative	Valid	-
NTC PCR (optional)	Positive	Positive	Negative	Invalid	See 12.2
NTC PCR (optional)	Negative	Positive	Negative	Invalid	See 12.2
NTC PCR (optional)	Positive	Negative	Negative	Invalid	See 12.2

[1] If the IPC-Target (DNA) was added directly to the master mix, all samples in the Cy5 channel must be positive.
[2] Cq values of the IPC are dependent on the extraction method and should show Cq values of 27-30 (see 9. preparation of samples).
[3] Negative control of the extraction

.

11.2 Clinical samples

Table 4 Interpretation of data with clinical samples of **Aspergillus 1 + IPC3 Assay Mix**

	Cq FAM channel *Aspergillus*	Cq VIC channel *Aspergillus*	Cq Cy5 channel IPC	Interpretation	Action
Clinical sample	Negative	Negative	27-30[1]	Negative	-
Clinical sample	Positive	Positive	Positive/Negative[2]	Positive for *A. flavus* (+*Aspergillus* section *Flavi)* and *A. fumigatus*	-
Clinical sample	Negative	Positive	Positive/Negative[2]	Positive for *A. fumigatus*	-
Clinical sample	Positive	Negative	Positive/Negative[2]	Positive for *A. flavus* (+*Aspergillus* section *Flavi)*	-
Clinical sample	Negative	Negative	Negative	Invalid	See 12.5

[1] A positive signal of the IPC excludes potential PCR inhibition. However, IPC Cq-values should show comparable results among samples. A shift of Cq- values can indicate a partial inhibition of PCR. Cq values of the IPC are dependent on the extraction method and should show Cq values of 27-30 (see 9. preparation of samples).
[2] High pathogen loads in the sample can lead to a reduced or absent fluorescence signal of the IPC.

For interpretation, see also Table 7.

Table 5 Interpretation of data with clinical samples of **Aspergillus 2 + IPC3 Assay Mix**

	Cq FAM channel *Aspergillus*	Cq VIC channel *Aspergillus*	Cq Cy5 channel DNA IPC Target	Interpretation	Action
Clinical sample	Negative	Negative	27-30[1]	Negative	-
Clinical sample	Positive	Positive	Positive/Negative[2]	Positive for *A.niger/A. nidulans* (+*Aspergillus section Nidulantes, Usti, Versicolores)* and *A. terreus/citrinoterreus/ floccosus/hortai*	-
Clinical sample	Negative	Positive	Positive/Negative[2]	Positive for *A. terreus/citrinoterreus/ floccosus/hortai*	-
Clinical sample	Positive	Negative	Positive/Negative[2]	Positive for *A.niger/A. nidulans* (+*Aspergillus section Nidulantes, Usti, Versicolores)*	-
Clinical sample	Negative	Negative	Negative	Invalid	See 12.5

[1] A positive signal of the IPC excludes potential PCR inhibition. However, IPC Cq-values should show comparable results among samples. A shift of Cq- values can indicate a partial inhibition of PCR. Cq values of the IPC are dependent on the extraction method and should show Cq values of 27-30 (see 9. preparation of samples).
[2] High pathogen loads in the sample can lead to a reduced or absent fluorescence signal of the IPC.

For interpretation, see also Table 7.

In case of invalid data, analysis must be repeated with the remaining or newly extracted DNA sample (see 12. Troubleshooting).

12 Troubleshooting

12.1 No pathogen specific signal with positive control and with IPC

- Incorrect programming of the temperature profile or incorrect setting of detection channels on the real-time PCR instrument.
  → Compare temperature profile and setting of detection channels with details specified in the protocol
- Incorrect configuration of PCR reaction.
  → Check your pipetting steps with the pipetting scheme and repeat PCR, if necessary.
  → The DNA may have been degraded.
- The IPC-Target (DNA) was added undiluted directly to the master mix and not freshly diluted 1:100. The PCR reaction is therefore inhibited.
  → Freshly dilute IPC-Target (DNA) 1:100 and repeat PCR.
- No Positive Control was added.
  → Repeat PCR in case all clinical samples are negative.
- For control of real-time PCR only: 1 µl of freshly 1:100 diluted IPC-Target (DNA) must be added to the master mix. If the addition of IPC-Target has been forgotten:
  → Freshly dilute IPC-Target (DNA) and repeat PCR.
- For control of DNA extraction and PCR inhibition, the undiluted IPC-Target (DNA) must be added during extraction to the lysis buffer. If the addition of IPC-Target has been forgotten:
  → Repeat DNA extraction.

12.2 Pathogen signal with negative control

- If there are Cq values in the negative control below the cut-off specified in the Instructions for Use, a contamination occurred during the preparation of the PCR.
  → Repeat PCR with new reagents in replicates.
  → Strictly pipette positive control at last.
  → Make sure that workspace and instruments are cleaned at regular intervals.

12.3 Pathogen signal with extraction negative control

- If there are Cq values in the negative control of extraction below the cut-off specified in the Instructions for Use, a contamination occurred during the preparation of the PCR or the DNA extraction.
  → Repeat the PCR with unused reagents in replicates.
  → If repeatedly positive: Repeat DNA extraction and PCR using new reagents.
  → Strictly pipette positive control at last.
  → Make sure that workspace and instruments are cleaned at regular intervals and that workstations are separated from samples with high pathogen concentration.

12.4 IPC specific signal with PCR negative control and positive control

- The IPC-Target (DNA) has been added to lysis buffer during extraction, but there is IPC specific signal with PCR-NTC and positive control: Contamination with the IPC-Target (DNA).
  → Make sure that workspace and instruments are cleaned at regular intervals.

12.5 No signal with IPC and no pathogen specific signal with sample

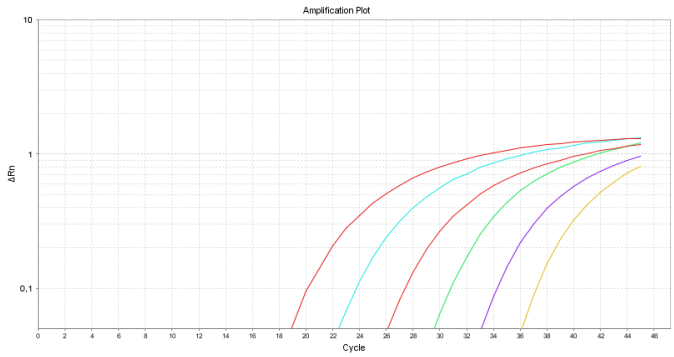
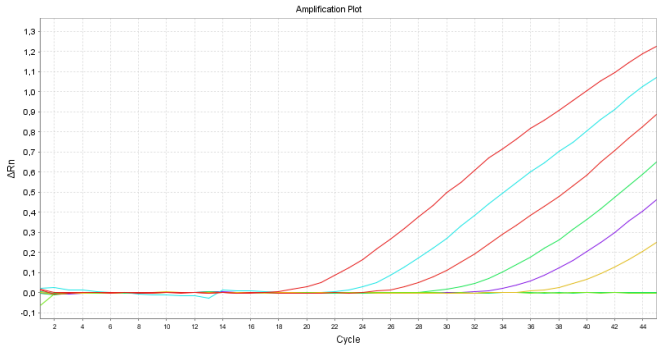
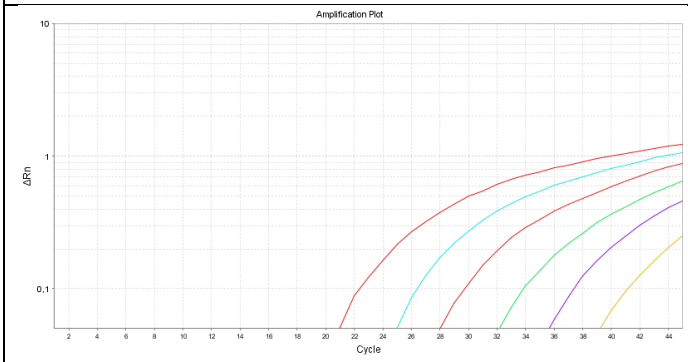
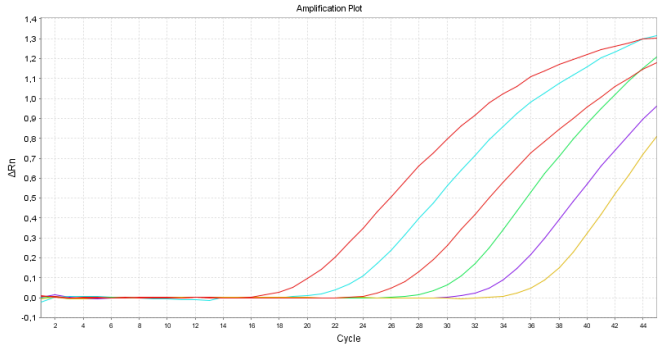
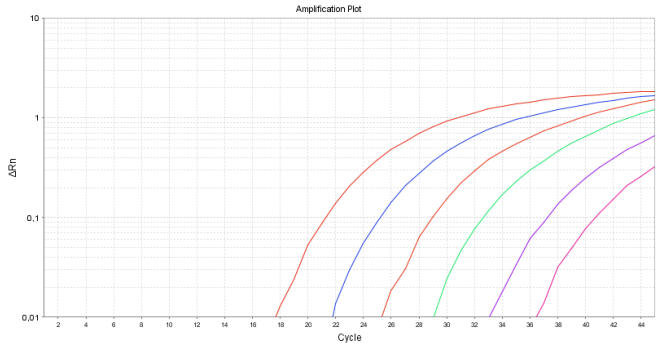
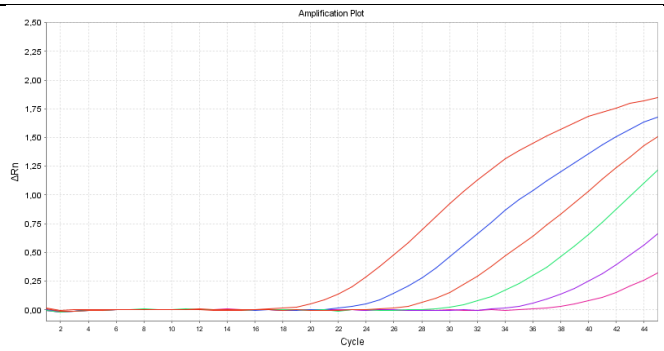
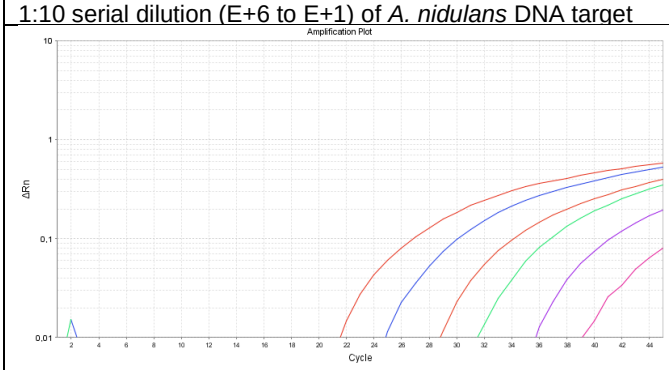
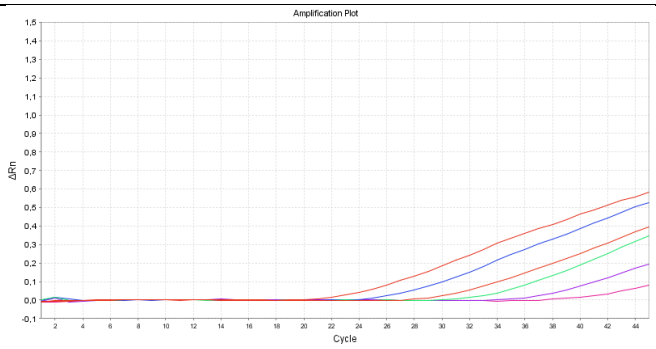
- Incorrect assignment of detection channels in sample.
  → Please verify the correct assignment of detection channels.
- The DNA might be degraded.
- If the IPC-Target (DNA) was added during extraction:
  - Inhibition of PCR may have occurred.
  - DNA extraction was unsuccessful.
  - The IPC-Target (DNA) was not added to the lysis buffer of the sample.
  - The extracted sample was not added to the PCR-reaction.

  → No statement is possible. Verify you use a recommended method for DNA isolation and re-examine the single steps of the DNA extraction.
  → If no operating mistakes during DNA 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 nuclease-free water).

13 Specifications and performance evaluation

13.1 Test performance on different real-time PCR instruments

Performance of MycoReal® Kit *Aspergillus* with an ABI® 7500 instrument (Thermo Fisher Scientific) is shown in Figure 1.

Logarithmic view	Linear view
	
ABI® 7500: FAM channel, Assay Mix 1 1:10 serial dilution (E+6 to E+1) of *A. flavus* DNA target	
	
ABI® 7500: VIC channel, Assay Mix 1 1:10 serial dilution (E+6 to E+1) of *A. fumigatus* DNA target	
	
ABI® 7500: FAM channel, Assay Mix 2 1:10 serial dilution (E+6 to E+1) of *A. nidulans* DNA target	
	
ABI® 7500: VIC channel, Assay Mix 2 1:10 serial dilution (E+6 to E+1) of *A. terreus* DNA target	

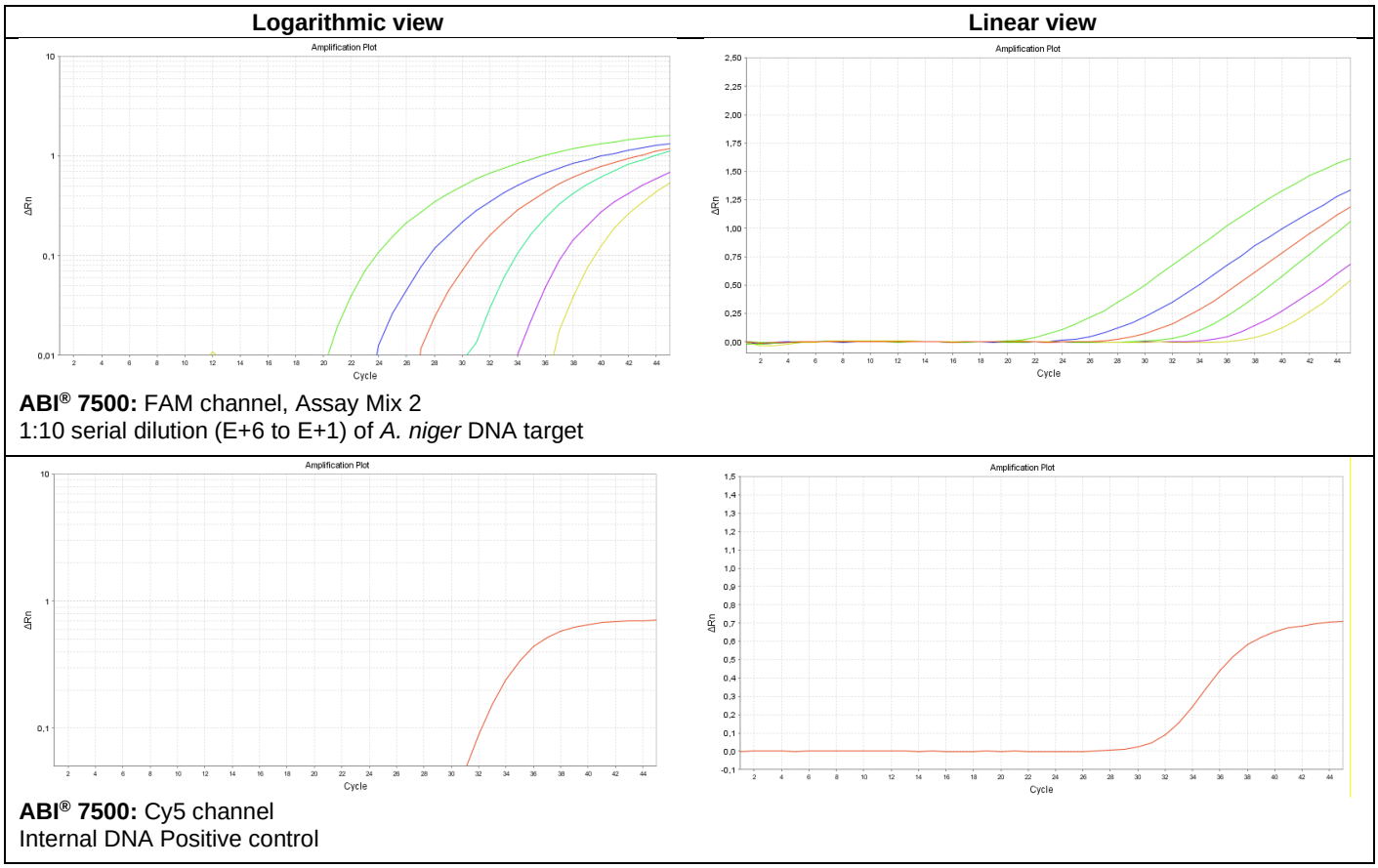


Figure 1 Performance of MycoReal® Kit *Aspergillus*

This kit was validated with the ABI® 7500 instrument (Thermo Fisher Scientific, fast cycle parameters are not supported) and with cobas z 480 Analyzer (Roche).

13.2 Limit of detection, LoD95%

Method: The detection limit (LoD95%: number of copies positively detected in 95% of cases) was determined with nine replicates at four different concentrations around the detection limit (1, 5, 10, 20 copies) with plasmid DNA containing a fragment of the ITS 2 region of *A. fumigatus, A. flavus, A. terreus, A. niger* and *A. nidulans.* LoD95% was then calculated with a non-linear (logistic) curve fit using Graph Pad Prism Software. The fungal ITS2 region is a multiocopy gene.

Results: seeTable 6. LoD95% ranges from 2 to 11 copies, depending on the species.
Note: Existing primer or probe region sequence variabilities may lead to false-negative or less sensitive results for some *Aspergillus* strains.

13.3 Linearity and dynamic range

Method: The linearity was determined with 10-fold dilution series (100 - 1,000,000 target copies/reaction) of the plasmids. The number of determinations (n) per dilution is nine.

Results are shown in Table 6. The R^2 for all species was 0.99 and the slope was between -3.3 and -3.8, indicating good test performance. The test shows linearity over the range of 100 to 1,000,000 target copies/reaction.

Table 6 Summary of LOD95% and linearity

	LoD95%	Linearity – gradient	Linearity – correlation coefficient R^2
Aspergillus fumigatus	6	-3.5 ± 0.02	0.998
Aspergillus flavus	7	-3.56 ± 0.04	0.991
Aspergillus terreus	11	-3.34 ± 0.05	0.9889
Aspergillus niger	2	-3.34 ± 0.03	0.995
Aspergillus nidulans	7	-3.84 ± 0.04	0.995

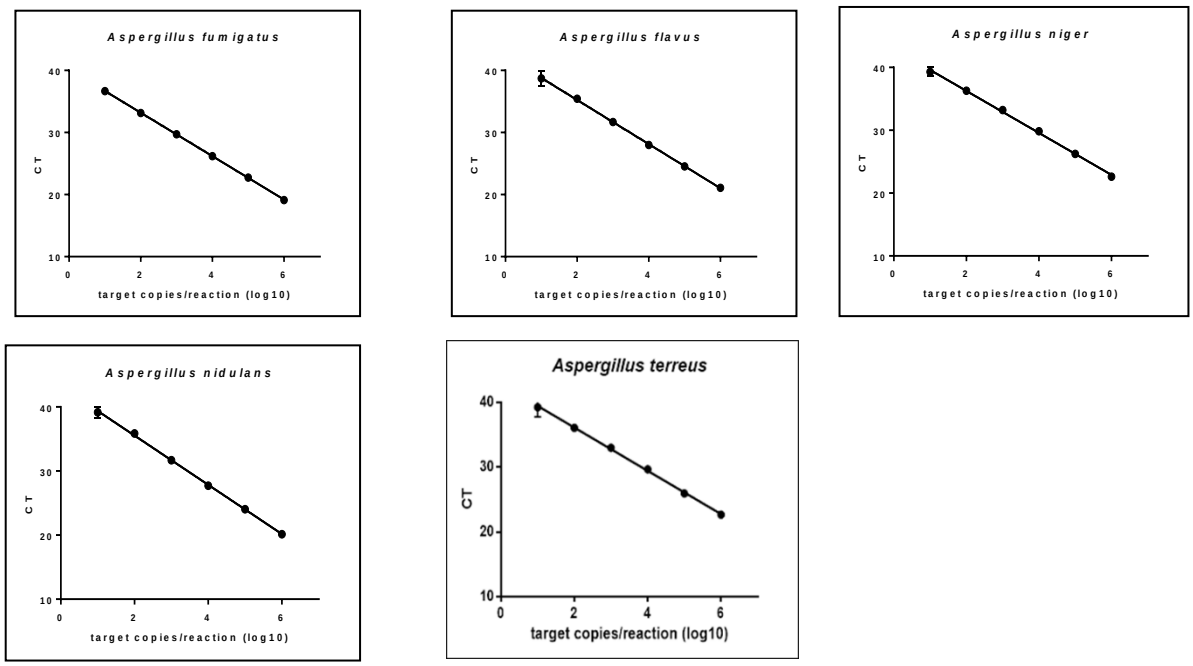


Figure 2 Ten-fold dilution series of DNA standards plotted against Cq

13.4 Analytical specificity

Method: The ITS region is a multi-copy gene and due to its high species specificity, is suited for the differentiation of *Aspergillus* groups. To investigate potential cross-reactivity, primers and probes were analyzed for potential homologies to currently published sequences. Specificity of primers and probes were validated *in silico* by carrying out the basic local alignment search tool (BLAST) against the NCBI database. This database analysis (BLAST analysis) validated the specific detection of *Aspergillus*. Furthermore, specificity was tested on several fungal strains.

Result: The test detects *A. fumigatus, A. flavus, A. terreus, A. niger, A. nidulans* and other phylogenetically closely related *Aspergillus* and *Emericella* species belonging to *Aspergillus* section *Fumigati, Flavi, Terrei, Nigri, Nidulantes, Usti* and *Versicolori*. This test does not show cross reactivity with other fungi. Sequence variabilities in the target-region of some *Aspergillus* subtypes (strains) may lead to false-negative or less sensitive results (Table 7, Table 8).

Table 7 Results of BLAST analyses; species detected with MycoReal® Kit *Aspergillus*

Detection channel	Detected species
Assay Mix 1, FAM	• *Aspergillus flavus* and some *Aspergillus* species belonging to the *Aspergillus* section *Flavi (A. oryzae, A. parasiticus, A. aflatoxiformans, A. nomius, A. sojae, A. arachidicola, A. tamarii, A. transmontanensis, A. mottae, A. microviridicitrinus, A. subflavus, A. pseudonomius, A. pseudotamarii, A. minisclerotigenes)* • The pathogenic species A. *avenaceus* might be detected with less sensitivity. • The pathogenic species *A. alliaceus* is not detected
Assay Mix 1, VIC	• *Aspergillus fumigatus* • *Aspergillus* species belonging to the *Aspergillus* section *Fumigati* such as *Aspergillus lentulus* might be detected, but with less sensitivity.
Assay Mix 2, FAM	• *Aspergillus* species belonging to the *Aspergillus* section *Nigri,* including the medically significant species *A.aculeatus, A. brasiliensis A. tubingensis, A. welwitschiae (awamori*) and A. *foetidus.* • *Aspergillus nidulans* and some species belonging to the *Aspergillus* section *Nidulantes* and *Usti* including the medically significant species *A. delacroxii* (=*A. spinulosporus*), *A. dentatus*, *A. quadrilineatus, A. sublatus, A. stellatus* and *A. ustus.* • The pathogenic species A. *avenaceus (*section *Nidulantes), A. sydowii, A. versicolor* (section *Versicolori*) and *A. granulosus (*section *Usti)* might be detected with less sensitivity. • The pathogenic species *A. unguis, A. protuberus*, *A. unguis*, *A. hongkongensis (*section *Nidulantes),* as well as *A. deflectus (*section *Usti)* and *A. japonicus* (section *Nigri*) are not detected.
Assay Mix 2, VIC	• *Aspergillus* species belonging to the *Aspergillus* section *Terri: A. terreus, A. citrinoterreus, A. floccosus, A. hortai*
Assay Mix 1, 2, Cy5	• Internal positive control (IPC)

Table 8 Tested fungal species

	Cq-value Aspergillus 1 Mix	Cq-value Aspergillus 2 Mix
Moulds		
Aspergillus flavus	Cq=28.4	Negative
Aspergillus niger	Negative	Cq=29.2
Aspergillus fumigatus	Cq=32.4	Negative
Aspergillus terreus	Negative	Cq=34.0
Aspergillus nidulans	Negative	Cq=29.9
Aspergillus versicolor	Negative	Cq=37.6
Aspergillus unguis	Negative	Negative
Aspergillus sydowii	Negative	Cq=39.8
Aspergillus ustus	Negative	Cq=36.0
Aspergillus ochraceus	Negative	Negative
Aspergillus niveus	Negative	Negative
Aspergillus clavatus	Negative	Negative
Aspergillus candidus	Cq=38.6	Negative
Aspergillus glaucus	Negative	Negative
Penicillium marneffei	Negative	Negative
Penicillium olsonii	Negative	Negative
Penicillium chrysogenum	Negative	Negative
Rhizopus oryzae	Negative	Negative
Mucor circinelloides/racemosus	Negative	Negative
Rhizomucor pusillus	Negative	Negative
Rhizomucor miehei	Negative	Negative
Absidia corymbifera	Negative	Negative
Cunninghamella elegans	Negative	Negative
Syncephalastrum sp.	Negative	Negative
Scedosporium apiospermum	Negative	Negative
Fusarium oxysporum	Negative	Negative
Fusarium verticilloides	Negative	Negative
Fusarium solani	Negative	Negative
Beauveria bassiana	Negative	Negative
Natrassia mangiferae	Negative	Negative
Alternaria alternata	Negative	Negative
Curvularia lunata var. lunata	Negative	Negative
Schizophyllum commune	Negative	Negative
Acremonium strictum	Negative	Negative
Paecilomyces variotii	Negative	Negative
Dematiaceae		
Bipolaris australiensis	Negative	Negative
Cladosporium herbarum	Negative	Negative
Phialophora richardsiae	Negative	Negative
Sporothrix schenkii	Negative	Negative
Aureobasidium pullulans	Negative	Negative
Cladophialophora	Negative	Negative
Dermatophytes		
Microsporum canis	Negative	Negative
Trichophyton tonsurans	Negative	Negative
Yeasts		
Candida glabrata	Negative	Negative
Candida albicans	Negative	Negative
Debaromyces hansenii	Negative	Negative
Yarrowia lipolytica	Negative	Negative
Rhodotorula rubra	Negative	Negative
Pichia fermentans	Negative	Negative
Cryptococcus neoformans	Negative	Negative
Malassezia furfur	Negative	Negative
Malassezia pachydermatis	Negative	Negative
Saccharomyces cerevisiae	Negative	Negative

13.5 Precision

Method: Precision within a run (intra-assay), between multiple runs (inter-assay) and between two lots (inter-lot) was determined.

Result: The mean values of the coefficients of variation (CV%) are summarized in Table 9.

Table 9 Summary of mean values of the coefficients of variation

	Mean CV% intra-assay	Mean CV% inter-assay	Mean CV% inter-lot
A. fumigatus	0.65%	0.42%	0.57%
A. flavus	1.45%	0.60%	0.65%
A. nidulans	1.45%	0.93%	0.94%
A. niger	1.09%	0.68%	0.46%
A. terreus	0.96%	1.11%	0.99%

13.6 Clinical evaluation

Method:
MycoReal® Kit *Aspergillus* was tested by an external service provider (Department of Clinical Microbiology, General Hospital Vienna) with 34 clinical samples of patients with a suspected fungal infection. Samples included EDTA-blood (n=7), bronchoalveolar lavage (BAL, n=10), bronchial secretion (n=2), punctates (n=7), cerebrospinal fluid (CSF, n=1), tissue (n=5) and swabs (n=2).
Extraction of EDTA-blood was performed with the SeptiFast LysKit (Roche). The other samples were extracted with a modified protocol of the High Pure PCR Template Preparation Kit (Roche Diagnostics).
Analyses were performed in single reactions on the cobas z 480 analyzer (Roche) and on the ABI® 7500 instrument (Thermo Fisher Scientific), respectively. Results were compared with results of culture or results of routine real-time PCR (SeptiFast test (Roche), in-house real-time PCR based on specific biprobes for *Candida* and *Aspergillus* (Schabereiter-Gurtner et al., 2007), in-house real-time PCR based on HybProbes and species identification by sequencing and BLAST analyses (Zeller et al., 2017)).

Result: see Table 10, Table 11
In 32 cases, results of the reference methods and MycoReal® Kit *Aspergillus* were concordant.

- Two samples positive with SeptiFast (positive for *A. fumigatus)* were false-negative with MycoReal® Kit *Aspergillus*. One possible explanation may be the 5-times higher sample volume used in the LightCycler® SeptiFast Test.

Table 10 Overall results of clinical performance evaluation of MycoReal® Kit *Aspergillus* with 34 clinical samples

	Reference			
		pos	neg	Total
MycoReal® Kit *Aspergillus*	pos	19	0	19
	neg	2	13	15
	Total	21	13	34

Table 11 Statistical evaluation

Statistic	Value	95% CI
Sensitivity	90.48%	69.62% - 98.83%
Specificity	100%	75.29% - 100.00%
Prevalence	61.76%	43.56% – 77.83%
Positive predictive value (PPV)	100%	
Negative predictive value (NPV)	86.67%	63.50% - 96.05%
Accuracy	94.12%	80.32% - 99.28%

Furthermore, MycoReal® Kit *Aspergillus* was tested with 24 paraffin embedded tissues and results were compared to patient history and an in-house real-time PCR test based on specific biprobes for *Candida* and *Aspergillus* (Schabereiter-Gurtner et al., 2007). Based on results, a cut-off with Cq ≥ 34.0 was defined for paraffin embedded tissue samples. For samples with Cq values above the cut-off, real infection cannot be differentiated to contamination (data not shown).

14 References

1. Schabereiter-Gurtner, C., B. Selitsch, M. Rotter, A. M. Hirschl, and B. Willinger. 2007. Development of novel real-time PCR assays for detection and differentiation of eleven medically important *Aspergillus* and *Candida* species in clinical specimens. J. Clin. Microbiol. 45:906-914.
2. Zeller I, Schabereiter-Gurtner C, Mihalits V, Selitsch B, Barousch W, Hirschl AM, Makristathis A, Willinger B. 2017. Detection of fungal pathogens by a new broad range real-time PCR assay targeting the fungal ITS2 region. J Med Microbiol. 66:1383.

15 Revision history

Revision	Date	Description
1.2	April 08 2021	IVD version
1.1	01 May 2023	**Change of the company address** **1. Intended use** Intended use was specified in more detail. **5. Contents, stability and storage** Kit version 1.1: Filling volume of IPC-Target (DNA) and Positive Control was changed. The DNA IPC Target is not stored in RNA/DNA Stabilizer anymore and was renamed to IPC-Target (DNA). Aspergillus 1 Assay Mix was renamed to Aspergillus 1 + IPC3 Assay Mix. Aspergillus 2 Assay Mix was renamed to Aspergillus 2 + IPC3 Assay Mix. **6. Additionally required materials and equipment** Update **7. Precautions and safety instructions** Update of this section Inclusion of additional information on specimen collection and storage, processing of specimens after collection, storage conditions of specimens, transport and storage conditions of purified DNA. Recommended measures to avoid DNA contamination added. **9. Sample preparation** Update Mag-Bind® Universal Pathogen DNA 96 Kit, M4029-00, Omega has been removed innuPREP AniPath DNA RNA - KFFLX Kit was added Note that for Cq values < 27, the IPC-Target (DNA) must be diluted prior to extraction. Annex with information on extraction was removed. The information was added in short version in section 9. **10. Preparation of real-time PCR** The temperature profile has changed - the activation step in program 2 has been extended from 20 sec to 2 min. **11. Interpretation of PCR data** Definition of Cq, Ct and Cp values. Update of tables for results with positive and negative controls and clinical samples. Inclusion of an explanation of how to evaluate clinical specimen test results in relation to controls. **12. Troubleshooting** Update regarding references in the new results tables. **13. Specifications and performance evaluation** **13.8 Clinical evaluation:** Summary of Precision in Table 9 Table with listed clinical samples was removed. Update for paraffin embedded tissue

Note:
Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

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