MycoReal Kit *Aspergillus*

Instructions for Use

CE

IVD ***In vitro*** **Diagnostikum**

REF **DHUF00253**

Σ **50 reactions**

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Explanation of symbols

LOT	Batch code		Use by
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Store at
CE	This product fulfils the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices	IVD	For *in vitro* diagnostic use
	Corrosion, GHS05		Exclamation mark, GHS07

1. Intended use

MycoReal Kit *Aspergillus* is an *in vitro* diagnostic real-time PCR test for the detection of *Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus, Aspergillus niger* and *Aspergillus nidulans* from samples purified from bronchoalveolar lavage (BAL), blood, aspirate, cerebrospinal fluid and tissue. Furthermore, it detects some other closely related *Aspergillus/Emericella* species belonging to *Aspergillus* section *Fumigati, Flavi, Terrei, Nigri, Nidulantes, Usti* and *Versicolori*. The kit supports the detection of an *Aspergillus* infection of patients with a suspected fungal infection. Results have to be interpreted in context with the overall picture and other clinical parameters.

2. Product description

MycoReal Kit *Aspergillus* 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.* It allows the detection and to some extent also the distinction of medically important *Aspergillus* species, including *A. fumigatus, A. flavus, A. terreus, A. niger* and *A. nidulans*. This test detects also other *Aspergillus* and *Emericella* species belonging to *Aspergillus* section *Aspergillus* section *Fumigati, Flavi, Terrei, Nigri, Nidulantes, Usti* and *Versicolori*. It does not cross react with other fungi (see 11.4. Analytical Specificity). Analysis is done with Assay Mix 1 and Assay Mix 2 in two parallel PCR-reactions.

An internal DNA positive control (DNA IPC) is used as DNA extraction and real-time PCR inhibition control. The target for the DNA IPC is extracted with the sample and is detected in Cy5 channel.

Table 1: Detection channels

	Target 1 FAM (530 nm)	**Target 2 VIC (554 nm)**	**Target 3 CY5 (670 nm)**
Aspergillus Assay Mix 1	*A. flavus*	*A. fumigatus*	IPC
Aspergillus Assay Mix 2	*A. niger* and *A. nidulans*	*A. terreus*	IPC

This test has been validated with the ABI® 7500 instrument (Thermo Fisher Scientific) and tested with a cobas z 480 Analyzer (Roche), but is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM, VIC and Cy5 channel.

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

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. Contents of the kit, stability and storage

Labelling	Content	Amount	Storage
Aspergillus Assay Mix 1 (green cap)	Primer and probe for *A. flavus* (FAM), *A. fumigatus* (VIC) and IPC (Cy5) detection	1 x 50 µl	-15 °C to -25 °C
Aspergillus Assay Mix 2 (green cap)	Primer und probe for *A. nidulans* and *A. niger* (FAM), *A. terreus* (VIC) and IPC (Cy5) detection	1 x 50 µl	-15 °C to -25 °C
DNA IPC Target (orange cap)	Target for DNA IPC (internal DNA positive control system)	1 x 100 µl	-15 °C to -25 °C
Aspergillus Positive Control (red cap)	DNA positive control for *A. fumigatus* + *A. flavus* + *A. nidulans* + *A. terreus* (approx. 1000 target copies/µl)	1 x 200 µl	-15 °C to -25 °C
DNA Reaction Mix (white cap)	DNA reaction mix	2 x 500 µl	**-15 °C to -25 °C, until first use, then at +4 °C**
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	-15 °C bis -25 °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.

The components of MycoReal Kit *Aspergillus* are stable until the expiry date stated on the label.

5. Additionally required materials and devices

- Reagents and devices for DNA-extraction compatible for extraction of fungal cells and clinical specimens
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM, VIC, Cy5 channel
- Optical 96 well reaction plates or optical reaction tubes with optical closing material recommended by the manufacturer of the real-time PCR instrument

6. Precautions and safety information

- Clean benches and devices periodically.
- Ensure the periodical cleaning and maintenance of real-time instruments.
- Use pipette tips with filter.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective disposable powder-free gloves when handling kit reagents and specimens.
- Use separated working areas for specimen preparation, reagent preparation and amplification. Supplies and equipment must be dedicated to each of these separate working areas and ensure workflow in the laboratory from pre- to post-PCR.
- Be careful when handling samples and positive control to avoid cross contamination. Change gloves after handling of samples or positive control. Store positive or potentially positive material separately from reagents
- Prevent contamination of work equipment and reagents with DNA/RNA, nuclease or amplification products by good laboratory practice.
- 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.
- 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 order to exclude false-positive results due to contamination with DNA of pathogen during extraction.
- Please note the expiry date of the kit.
- Do not interchange or mix reagents from kits with different lot numbers.
- Repeated thawing and freezing of kit components should be avoided. Protect kit components from light. **Caution:** DNA IPC Target is stored in RNA/DNA stabilizer which contains DTT/Guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).
- Use established laboratory practices according to your local safety regulations for discarding specimens, reagents and waste disposal.

7. Limitations

- Optimal performance of this test requires 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.
- 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. The presence of 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 the context of clinical and laboratory findings.

8. Preparation of samples and real-time PCR

- Extract samples with a DNA extraction system proper for extraction of fungi and compatible with real-time PCR technology (e.g. use a modified protocol of the High Pure PCR Template Preparation Kit, Roche Diagnostics. See 13. Appendix - Protocol for DNA-extraction). The extraction system must ensure the extraction of fungal cells and shall be free of fungal DNA.
- An extraction negative control shall be included during DNA-extraction (e.g. extraction of water).

Analysis of blood: At least 0.5 - 1 ml of blood should be extracted, the DNA to be analyzed should correspond to at least 50 µl of blood (e.g. 500 µl of blood eluted in 100 µl).
Ingenetix recommends the extraction of one ml of EDTA human blood with beads (e.g. Mag-Bind® Universal Pathogen DNA 96 Kit, M4029-00, Omega). With this extraction method, one ml of EDTA blood is used as starting material and eluted with 100 µl. Ten µl eluate corresponds to 40-60 µl blood.

The **DNA IPC Target** has to be added during extraction. It has a concentration of $6x10^5$ copies/µl. The DNA IPC (Internal Positive Control) is used as a control of DNA extraction, identifies possible PCR inhibition and confirms the integrity of kit reagents.
Caution: The DNA IPC Target must not be added directly to the sample material, but has to be added to the lysis buffer (or added the sample material after the lysis buffer).
→ Add 1 µl of DNA IPC Target per sample

Aspergillus Positive Control is a mixture of four DNA target sequences for *A. fumigatus* + *A. flavus* + *A. nidulans* + *A. terreus* (approx. 1,000 target copies/µl each) and must be stored at -20°C.
→ Add 9 µl Positive Control. Always pipette Positive Control last.

- Include one positive control and one extraction negative control per PCR run and analysis with Assay Mix 1 and 2.
- In general, it is recommended to analyze samples in duplicates to increase the probability of detection and to facilitate the interpretation of the results.
- Thaw DNA samples on ice.
- Thaw kit components at room temperature. When thawed, mix components, centrifuge briefly. Gently Mix the DNA Reaction Mix to ensure homogeneity of solution.

8.3. Pipetting scheme per Assay Mix 1 and Mix 2

Samples must be analyzed with Aspergillus Assay Mix 1 and Aspergillus Assay Mix 2, respectively. For blood and CSF samples it is recommended to use 9 µl DNA sample. When using a sample volume < 9 µl, water must be added to the master mix accordingly, see below.

Analysis Aspergillus Assay Mix 1:

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

Analysis Aspergillus Assay Mix 2:

		Per sample
Preparation of Master Mix 2 (mix well)	DNA Reaction Mix	10,0 µl
	Aspergillus Assay Mix 2	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**

→ **If DNA IPC Target was not added during extraction (not recommended):** Freshly dilute the DNA IPC Target 1:100 with nuclease-free water and add 1 µl per sample directly to the master mix. **Caution:** The use of more than 1 µl diluted (1:100) DNA IPC Target per reaction causes inhibition of the real-time PCR reaction.

For preparation of real-time PCR, dispense 11 µl aliquots of prepared Master Mix into the plate wells and then add 9 µl of DNA sample per well. Always pipet the Positive Control last. Then seal the plate with suitable optical sealing material.

8.4. Programming of temperature profile

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

Sample Volume: 20 µl

Temperature Profile:

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

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

[1]If viral RNA should be also detected in the same PCR run, program 1 has to be prolonged to 15 min at 50°C.

Detection channels:

	Target 1 FAM-NONE (530 nm)	Target 2 VIC-NONE (554 nm)	Target 3 Cy5-BHQ1 (670 nm)
Aspergillus Assay Mix 1	*Aspergillus flavus*	*Aspergillus fumigatus*	IPC
Aspergillus Assay Mix 2	*A. niger* and *A. nidulans*	*A. terreus*	IPC

Passive reference dye, if required (depending on real-time PCR instrument): ROX

Detection channels for cobas z 480 Analyzer (Roche):
FAM: Excitation at 465 nm, Emission at 510 nm
VIC: Excitation at 540 nm, Emission at 580 nm
Cy5: Excitation at 610 nm, Emission at 670 nm
Passive reference dye: None

Detection channels for LightCycler® 480 II (Roche):
FAM: Excitation at 465, Emission at 510 nm
VIC: Excitation at 533, Emission at 580 nm
Cy5: Excitation at 618, Emission at 670 nm
After analysis of Cy5 channel, a color compensation for FAM and VIC has to be selected from the Roche database.
Detection format: 3 Color Hydrolysis Probe
Passive reference dye: None

9. Interpretation of PCR-data

For analysis of PCR results select fluorescence display options 530 nm (FAM channel) and 554 nm (VIC) for the *Aspergillus* Targets and 667 nm (Cy5 channel) for the DNA IPC target.

Important: Samples should be inspected both in logarithmic and linear scale view and compared with the negative control. Besides the Cq-values (Quantification cycle (Cq) = Cycle threshold (Ct) = Crossing point (Cp)) also check the amplification-curves and adjust the threshold (noise band) both in FAM and VIC, if necessary. After you have saved the new settings, export the data. For the cobas z 480 Analyzer, export tables per dye.

Cut-off Cq-values:

- Normally sterile samples such as blood, CSF, punctates, aspirates, tissue: Cut off = Cq ≥45,0
- Semisterile samples such as BAL: Cut off = Cq ≥38,0

Table 2 shows the criteria for valid controls and Tables 3, 4 show interpretation of data with clinical samples.

Table 2: Criteria for valid controls, IPC Target was added during extraction. Applies to the analysis with Assay Mix 1 and Assay Mix 2

	Cq FAM channel Aspergillus	Cq VIC channel Aspergillus	Cq Cy5 channel IPC target[2]	Interpretation	Action
Positive control	<30	<30	Negative	Valid	-
Positive control	Negative	Negative	Negative	Invalid	See 10.1
Positive control	<30	Negative	Negative	Invalid	See 10.1
Positive control	Negative	<30	Negative	Invalid	See 10.1
Positive control	<30	<30	Positive	Invalid	See 10.4
Extraction negative control	Negative	Negative	28-32	Valid	-
Extraction negative control	Negative	Negative	Negative	Invalid	See 10.1
Extraction negative control	Positive	Positive	28-32	Invalid	See 10.3
Extraction negative control	Positive	Negative	28-32	Invalid	See 10.3
Extraction negative control	Negative	Positive	28-32	Invalid	See 10.3
Negative control[1]	Negative	Negative	Negative	Valid	-
Negative control[1]	Positive	Positive	Negative	Invalid	See 10.2
Negative control[1]	Negative	Positive	Negative	Invalid	See 10.2
Negative control[1]	Positive	Negative	Negative	Invalid	See 10.2
Negative control[1]	Negative	Negative	Positive	Invalid	See 10.4

[1] Optional
[2] If the IPC Target has been added directly to the Master Mix, all samples must be positive in Cy5 channel

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, the patient results cannot be interpreted.

Table 3: Interpretation of clinical samples with Assay Mix 1

	Cq FAM channel *Aspergillus*	Cq VIC channel *Aspergillus*	Cq Cy5 channel DNA IPC Target	Interpretation[2]	Action
Clinical sample	Negative	Negative	28-32[1]	Negative	-
Clinical sample	Positive	Positive	Positive/Negative	Positive for *A. flavus* *(+Aspergillus* section *Flavi)* and *A. fumigatus*	-
Clinical sample	Negative	Positive	Positive/Negative	Positive for *A. fumigatus*	-
Clinical sample	Positive	Negative	Positive/Negative	Positive for *A. flavus* *(+Aspergillus* section *Flavi)*	-
Clinical sample	Negative	Negative	Negative	Invalid	See 10.5

[1] A positive signal excludes PCR inhibition. However, IPC Cq values should show comparable results among samples. A shift of Cq values can indicate a partial inhibition of PCR.
[2]See also Table 6

Table 4: Interpretation of clinical samples with Assay Mix 2

	Cq FAM channel *Aspergillus*	Cq VIC channel *Aspergillus*	Cq Cy5 channel DNA IPC Target	Interpretation[2]	Action
Clinical sample	Negative	Negative	28-32[1]	Negative	-
Clinical sample	Positive	Positive	Positive/Negative	Positive for *A.niger/A. nidulans (+Aspergillus section Nidulantes, Usti, Versicolores)* and *A. terreus/citrinoterreus/ floccosus/hortai*	-
Clinical sample	Negative	Positive	Positive/Negative	Positive for *A. terreus/citrinoterreus/ floccosus/hortai*	-
Clinical sample	Positive	Negative	Positive/Negative	Positive for *A.niger/A. nidulans (+Aspergillus section Nidulantes, Usti, Versicolores)*	-
Clinical sample	Negative	Negative	Negative	Invalid	See 10.5

[1] A positive signal excludes PCR inhibition. However, IPC Cq values should show comparable results among samples. A shift of Cq values can indicate a partial inhibition of PCR.
[2]See also Table 6

10. Troubleshooting

10.1. No *Aspergillus* specific signal with positive control and with IPC

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 8. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 8. Preparation of real-time PCR) and repeat the PCR, if necessary.
- The IPC Target was added directly to the master mix, but was not diluted 1:100 before. The PCR reaction is therefore inhibited.
  → Freshly dilute IPC Target 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 has to be added to the master mix. If no IPC Target was added to master mix:
  → Freshly dilute IPC Target and repeat PCR extraction.
- For control of DNA extraction and PCR inhibition, the IPC Target must be added during extraction. If no IPC Target was added to lysis buffer during extraction:
  → Repeat PCR extraction.

10.2. *Aspergillus* specific signal with negative control

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

10.3. *Aspergillus* specific signal with negative control of extraction

- A contamination occurred during extraction.
  → Repeat extraction and PCR using new reagents.
  → Make sure that workspace and instruments are decontaminated at regular intervals.
  → See also 10.2

10.4. IPC specific signal with negative control and positive control

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

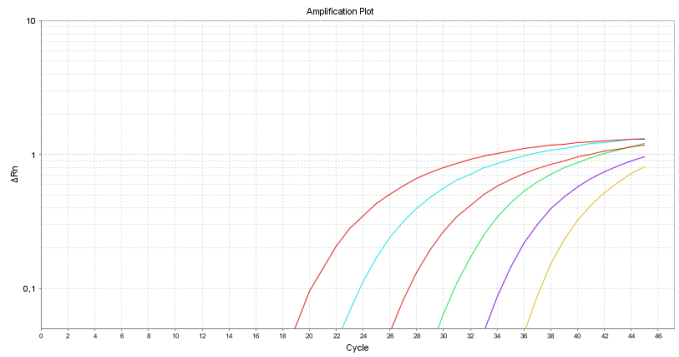
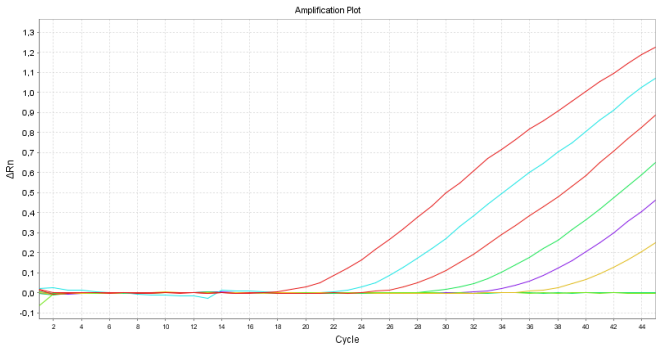
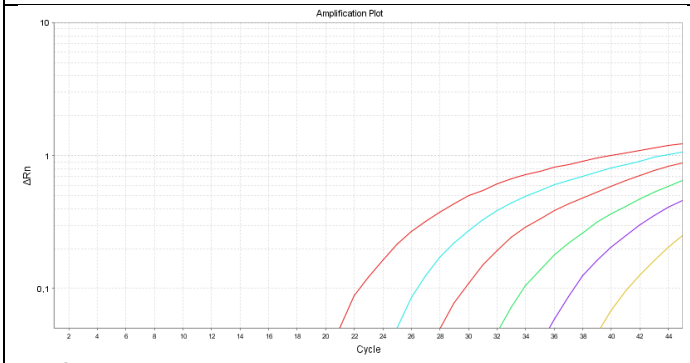
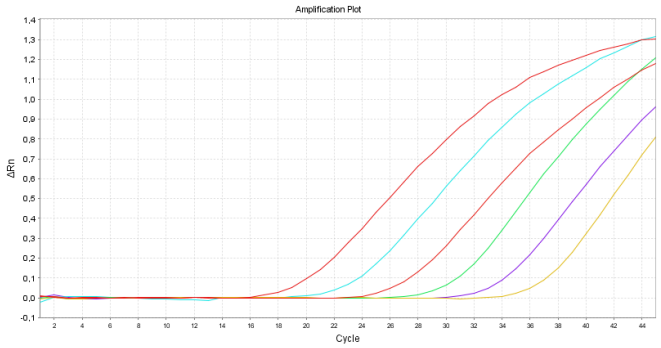
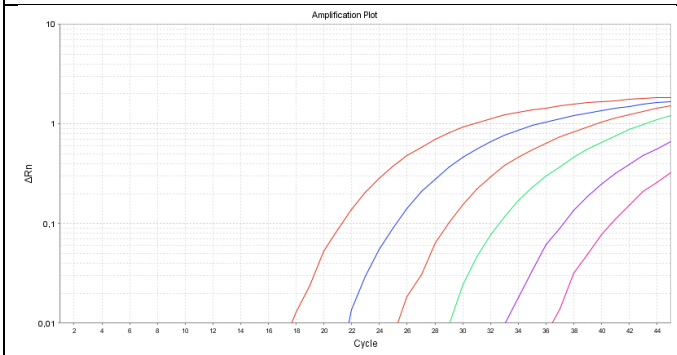
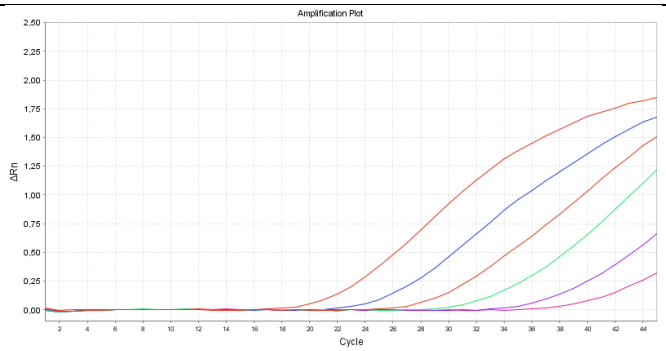
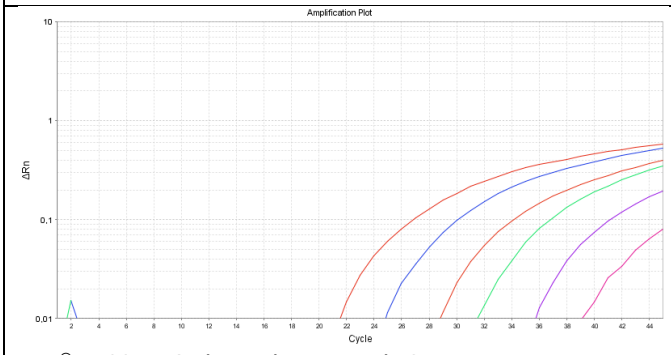
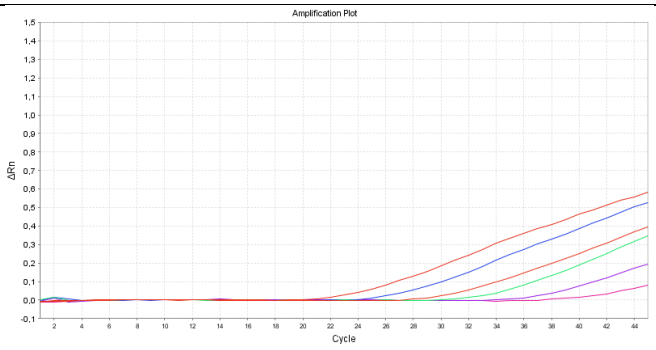
10.5. No signal with IPC and no *Aspergillus* specific signal with sample

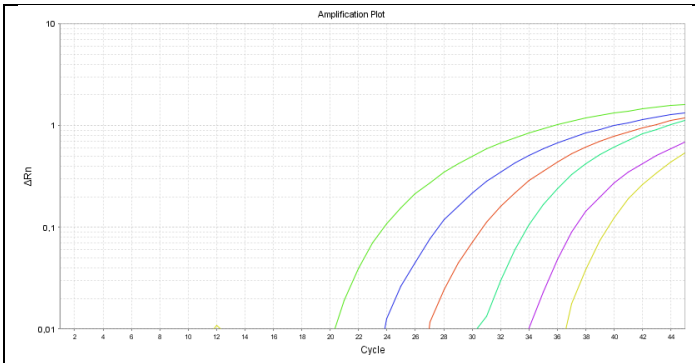
- PCR reaction was inhibited. No interpretation can be made.
  → Make sure that you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during extractions can be retraced, it is recommended to repeat the PCR with lower amounts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

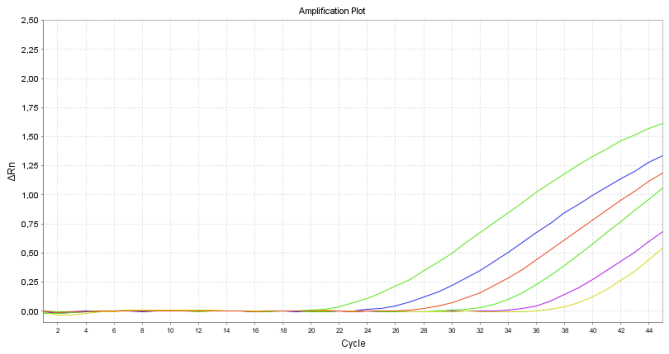
11. Specifications and performance evaluation

11.1. Kit performance

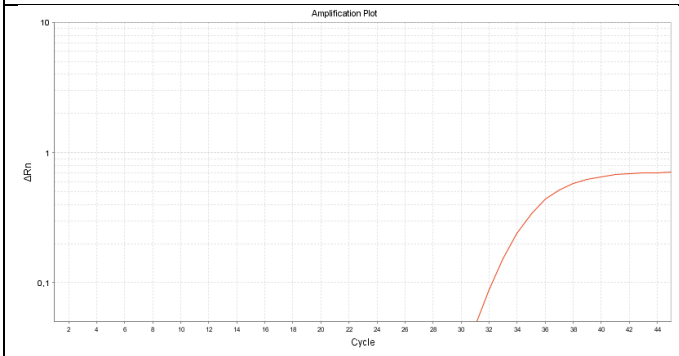
Performance of MycoReal Kit *Aspergillus* with an ABI® 7500 Fast Real-time PCR System (Thermo Fisher Scientific) is shown in Figure 1.

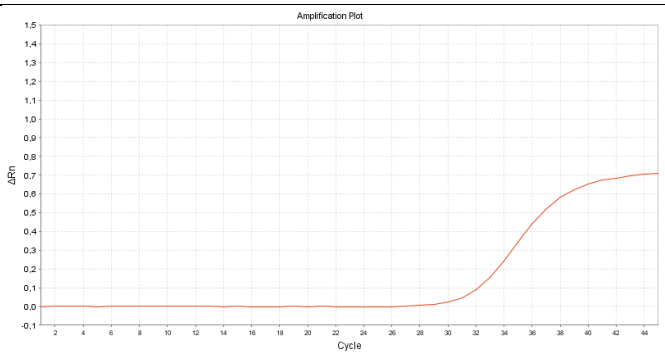
Logarithmic view	Linear view
	
ABI® 7500: FAM channel, Assay Mix 1 1:10 serial dilution (E+6 to E+1) of *A. flavus* DNA target	
	
ABI® 7500: VIC channel, Assay Mix 1 1:10 serial dilution (E+6 to E+1) of *A. fumigatus* DNA target	
	
ABI® 7500: FAM channel, Assay Mix 2 1:10 serial dilution (E+6 to E+1)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





ABI® 7500: FAM channel, Assay Mix 2
1:10 serial dilution (E+6 to E+1) of *A. niger* DNA target





ABI® 7500: Cy5 channel
Internal DNA Positive control

Figure 1 Performance of MycoReal Kit *Aspergillus*

11.2. Limit of detection

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 are shown in Table 5. 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.

11.3. 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 5. The correlation coefficient 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 5 LOD 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

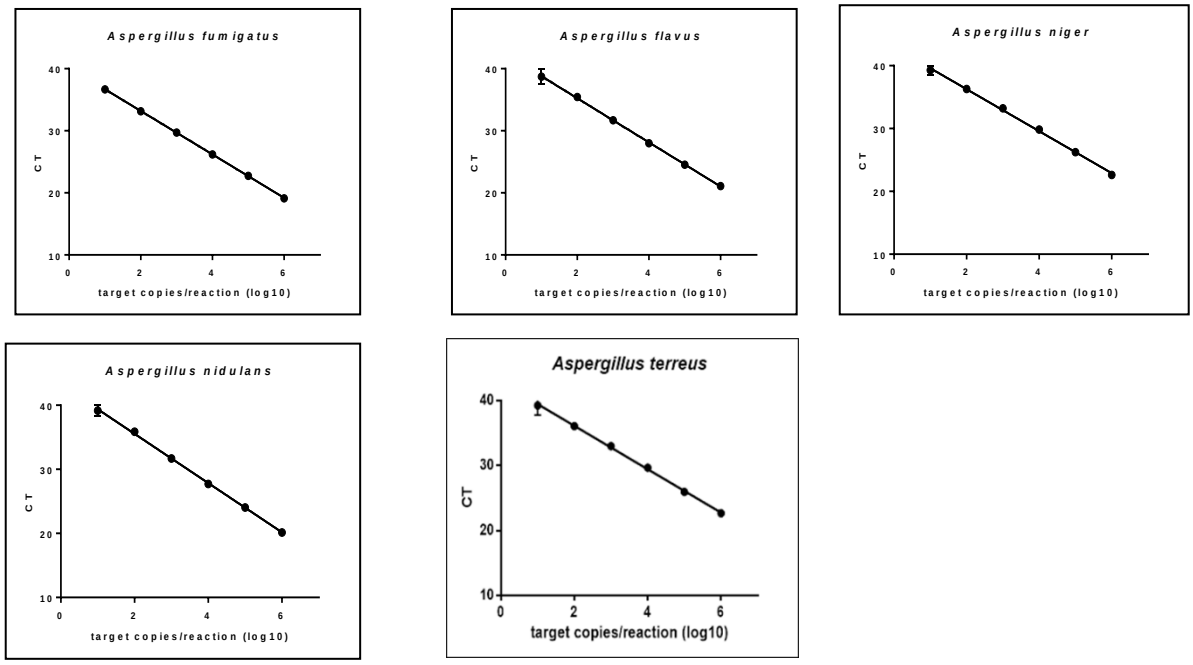


Figure 2 Ten-fold dilution series of DNA standards plotted against Ct

11.4. 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 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 6, Table 7).

Table 6: 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 7 Tested fungal species

	Cq-value Aspergillus Mix 1	Cq-value Aspergillus Mix 2
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

11.5. Precision inter-assay

Inter-assay precision shows the reproducibility between assays done on different days. Inter-assay precision is typically <3%. This ensures the results obtained will be consistent over time.

Method: The inter-assay precision of MycoReal Kit *Aspergillus* was determined by testing 10-fold plasmid DNA dilutions in three independent experiments performed on 3 different days in triplicates. Arithmetic mean ($\bar{x}$), standard deviation (σ) and coefficient of variation (CV %) between the replicate runs were calculated.

Results:
CV's for detection of *A. fumigatus* ranged from 0.28% to 0.61%, with mean overall Inter-assay precision equals 0.42%.
CV's for detection of *A. flavus* ranged from 0.40% to 0,95%, with mean overall Inter-assay precision equals 0.60%.
CV's for detection of *A. nidulans* ranged from 0.51% to 1.48%, with mean overall Inter-assay precision equals 0.93%.
CV's for detection of *A. niger* ranged from 0.32% to 1.25%, with mean overall Inter-assay precision equals 0.68%.
CV's for detection of *A. terreus* ranged from 0.25% to 3.38%, with mean overall Inter-assay precision equals 1.11%.

11.6. Precision intra-assay

Method: Intra-assay precision of MycoReal Kit *Aspergillus* was determined from the replicate runs above. Arithmetic mean ($\bar{x}$), standard deviation (σ) and coefficient of variation (CV %) of the replicates were calculated.

Results:
CV's for detection of *A. fumigatus* ranged from 0.15% to 1.51%, with mean overall Intra-assay precision equals 0.65%.
CV's for detection of *A. flavus* ranged from 0.22% to 4.84%, with mean overall Intra-assay precision equals 1.45%.
CV's for detection of *A. nidulans* ranged from 0.22% to 4.84%, with mean overall Intra-assay precision equals 1.45%.
CV's for detection of *A. niger* ranged from 0.18% to 3.22%, with mean overall Intra-assay precision equals 1.09%.
CV's for detection of *A. terreus* ranged from 0.16% to 4.46%, with mean overall Intra-assay precision equals 0.96%.

11.7. Precision inter-Lot

Method: The inter-lot precision describes the conformity of performance between different manufactured lots. The lot-to-lot equivalence is presented as a percentage conformity between results.
Inter-Lot variability was determined from two different assay mix lots by testing 10-fold plasmid DNA dilutions. Arithmetic mean ($\bar{x}$), standard deviation (σ) and coefficient of variation (CV %) of the replicates were calculated

Results:
CV's for detection of *A. fumigatus* ranged from 0.04% to 1.12%, with mean overall Inter-lot precision equals 0.57%.
CV's for detection of *A. flavus* ranged from 0.28% to 1.53%, with mean overall Inter-lot precision equals 0.65%.
CV's for detection of *A. nidulans* ranged from 0.35% to 1.62%, with mean overall Inter-lot precision equals 0.94%.
CV's for detection of *A. niger* ranged from 0.04% to 1.45%, with mean overall Inter-lot precision equals 0.46%.
CV's for detection of *A. terreus* ranged from 0.15% to 2.84%, with mean overall Inter-lot precision equals 0.99%.

11.8. Clinical evaluation

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)). Results see Table 8 - Table 10.

- 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 8 Results of clinical evaluation

Material	Results culture	Rsults routine PCR	MycoReal Kit *Aspergillus*
EDTA-blood	Not done	*A. fumigatus*[4]	*A. fumigatus* (Cp=37.8) (1/2 reactions positive)
EDTA-blood	Not done	*A. fumigatus* + *Candida albicans*[4]	*A. fumigatus* (Cp=33.0)
EDTA-blood	Not done	*A. fumigatus*[4]	Negative
EDTA-blood	Not done	*A. fumigatus*[4]	Negative
EDTA-blood	Not done	*A. fumigatus* + *Koagulase negative Staphylokokken*[4]	*A. fumigatus* (Cp=40.0)
EDTA-blood	Not done	*A. flavus*[1]	*A. flavus* complex (Cp=37.7)
EDTA-blood	Not done	*C. parapsilosis*[3]	Negative
BAL	*A. fumigatus*	*A. fumigatus*[1] (Cp=37.4)	*A. fumigatus* (Cp=28.5)
BAL	*A. fumigatus*	*A. fumigatus*[1] (Cp=26.3)	*A. fumigatus* (Cp=25.2)
BAL	*A. candidus* Gruppe	*A. persii=A. sclerotiorium*[1] (Cp=28.9)	Negative
BAL	*A. fumigatus*	*Schizophyllum commune*[1] (Cp=30.5)	*A. fumigatus* (Cp=36.3)
BAL	*C. krusei* + *C. glabrata* + *A. fumigatus*	*A. fumigatus*[1] (Cp=32.0)	*A. fumigatus* (Cp=20.9)
BAL	*Geotrichum capitatum*	*Geotrichum capitatum*[1] (Cp=20.03)	Negative
BAL	*A. niger*	*A. niger*[1] (Cp=31.0)	*A. niger/A. nidulans* (Cp=36.9)
BAL	*A. niger* + *C. tropicalis*	*C. tropicalis*[1] (Cp=22.3)	*A. niger/A. nidulans* (Cp=31.7)
BAL	*A. nidulans* + *Saccharomyces cerevisiae* + *C. albicans*	*Saccharomyces cerevisiae*[1] (Cp=29.0)	*A. niger/A. nidulans* (Cp=33.8)
BAL	*A. terreus*	*Cladosporium cladosporioides*[1] (Cp=38.0)	*A. flavus* complex (Cp=35.0) + *A. fumigatus* (Cp=32.8) + *A. terreus* (Cp=36.0)
Bronchial secretion	*A. fumigatus*	*A. fumigatus*[1] (Cp=25.5)	*A. fumigatus* (Cp=19.2)
Bronchial secretion	*A. fumigatus*	*A. fumigatus*[1] (Cp=17.0)	*A. fumigatus* (Cp=15.6)
Pleural puncture	*A. fumigatus*	*A. fumigatus*[1] (Cp=33.0)	*A. fumigatus* (Cp=34.5)
Eye fluid	*Exophiala dermatitidis*	*Exophiala dermatitidis*[1] (Cp=15.4)	Negative
Vitreous fluid	*Fusarium oxysporum*	*Fusarium oxysporum*[1] (Cp=18.3)	Negative
Puncture cyst (kidney)	Not done	Negative[1]	Negative

Ascites fluid	Not done	Negative[1]	Negative
Punctate knee	Not done	Negative[1]	Negative
Punctate knee	Not done	Negative[1]	Negative
CSF	Not done	Negative[1]	Negative
Tissue middle ear	*A. fumigatus*	*A. fumigatus*[1] (Cp=29.7)	*A. fumigatus* (Cp=27.9)
Tissue nasal polyp	*Bipolaris australiensis*	*Bipolaris australiensis*[1] (Cp=28.0)	Negative
Tissue	*A. fumigatus*	*A. fumigatus*[1] (Cp=32.7)	*A. fumigatus* (Cp=25.2)
Tissue wound (sternum)	*Staphylococcus epidemidis* + *Corynebacterium* spp. + *C. albicans*	*C. albicans*[1]	Negative
Paraffin cut middle nasal passage	Not done	*A. fumigatus*[1]	*A. fumigatus* (Cp=31.5)
Swab gluteal abscess	Not done	Not done	*A. flavus* complex (Cp=29.9)
Swab	Not done	*Malassezia restricta*[1]	Negative

[1] Result of in-house panfungal broad-range real-time PCR
[2] Result of in-house *Aspergillus* specific real-time PCR
[3] Result of in-house *Candida* specific real-time PCR
[4] Result of SeptiFast test (Roche)

Table 9 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 10 Statistical evaluation

Statistic	**Wert**	**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%

12. 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.

13. Appendix – protocol for DNA-extraction

Kits for DNA-extraction are provided by different manufacturers. Please use the sample volume as recommended by the respective manufacturer and follow the respective manual. It has to be ensured that all reagents are free of fungal DNA. It is recommended to exclude contamination of new reagent and kit lots by extraction of water instead of sample material before use with clinical samples.

Appropriate extraction methods depending on sample material:
Culture Material: PrepMan Ultra (Applied Biosystems)
Sample Material: Serum, plasma, sputum, BAL, fresh and frozen tissue with MycoGENIE (Ademtech) or High Pure PCR Template Preparation Kit (Roche)
Sample volume 50 to 100 µl: liquid sample with MagNA Pure LC DNA Isolation Kit III (bacteria, fungi) (Roche Diagnostics, Mannheim, Germany). The sensitivity of extraction of fungal DNA can be significantly increased by 3 to 5 "freeze/boil cycles" using liquid nitrogen and a heating block, prior to transfer to the MagNA Pure LC instrument.
Extraction of 1 ml blood: Ingenetix recommends the extraction of 1 ml EDTA-blood with beads (e.g. Mag-Bind® Universal Pathogen DNA 96 Kit, M4029-00, Omega).

13.1 Modified protocol of the High Pure PCR Template Preparation Kit

See also instructions of the manufacturer.

Additionally required materials

- High Pure PCR Template Preparation Kit (100 extractions) (Roche Diagnostics order no. 1796828)
- Molecular Biology Grade Water (10 x 50 ml) (Eppendorf order no. 32006302)
- 1.5 ml reaction tubes (PCR-clean)
- Liquid nitrogen (or -80°C freezer)
- Isopropanol, has to be sterile filtered (e.g. Merck order no. 1009951000)
- Sterile pipette tips with filters
- Vortex-Mixer
- Thermomixer
- Desktop centrifuge with rotor for 2 ml reaction tubes

13.1.1. Extraction from CSF, aspirates, BAL and 200 µl blood

1. Transfer CSF, BAL or aspirates (1 ml or less) in a 1.5 ml reaction tube and centrifuge for 5 min at 13.000 x g. Discard supernatant except for 200 µl. Blood: do not centrifuge, but use 200 µl directly for extraction. However, it is recommended to extract 1 ml blood, if possible.
2. Add 200 µl Binding Buffer (green cap) + 40 µl reconstituted Proteinase K, vortex.
3. Incubate tube for 10 min at 70°C.
4. Freeze-and-thaw step (modification, not mentioned in protocol of extraction kit): 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. (Blood from blood culture bottles: centrifuge for 1 min at 13.000 x g, then use supernatant).
5. Add 100 µl sterile filtered isopropanol.
6. Continue as described in manual of High Pure PCR Template Preparation Kit. Recommended: Elute in 100 µl Elution Buffer

13.1.2. Extraction from tissue and paraffin-embedded tissue

1. Transfer tissue (ca. 0.02 mg) into a sterile petri dish and cut the sample into small pieces with a sterile scalpel. Paraffin-embedded tissue: mind to use only tissue-containing paraffin sections
2. Add tissue to 200 µl Tissue Lysis Buffer in a 1.5 ml reaction tube and mix. Add 40 µl reconstituted Proteinase K, vortex.
3. Incubate at 55°C until tissue is mainly digested.
4. Freeze-and-thaw step (modification, not mentioned in protocol of extraction kit): 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.
5. Add 200 µl Binding Buffer, mix well.
6. Incubate tube for 10 min. at 70°C.
7. Optional: centrifuge for 1 min. at 13.000 x g in the presence of insoluble tissue segments, use supernatant.
8. Add 100 µl sterile filtered isopropanol, mix well.
9. Continue as described in manual of High Pure PCR Template Preparation Kit. Recommended: Elute in 100 µl Elution Buffer.