MycoReal Kit *Candida & A. fumigatus*

Instructions for Use

CE

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

REF **DHUF00153**

Σ **50 reactions**

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Annex – 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 *Candida* & *A. fumigatus* is an *in vitro* diagnostic test based on real-time PCR, for the qualitative detection of specific DNA of *C. albicans, C. dubliniensis, C. glabrata, C. krusei* (= *Pichia kudriavzevii, Issatchenkia orientalis*), *C. parapsilosis* group, *C. tropicalis* and *Aspergillus fumigatus*. Starting material is DNA purified from blood, biopsies, aspirates, punctates, cerebrospinal fluid (CSF) or samples of the respiratory tract. The kit supports the detection of a *Candida* or *A. fumigatus* infection of patients with a suspected sepsis or fungal infection. Results have to be interpreted in context with the overall picture and other clinical parameters.

2. Product description

MycoReal Kit *Candida* & *A. fumigatus* detects the ITS2 region of *C. albicans*, *C. dubliniensis, C. glabrata, C. krusei, C. parapsilosis* group (*C. parapsilosis, C. orthopsilosis, C. metapsilosis), C. tropicalis* and *Aspergillus fumigatus* using real-time PCR. The ITS region is a multi-copy gene and well suited for the specific detection of fungal species.

The species are detected with two different assay mixes (Module 7 and Module 8) which have to be analyzed in parallel. *C. glabrata, C. krusei, C. parapsilosis* and *A. fumigatus* can be differentiated from other *Candida* species, which enables specific antifungal therapy (Table 1).
A probe-specific amplification-curve in FAM and/or VIC channel indicates amplification of *Candida* or *A. fumigatus* specific DNA. An internal DNA positive control (IPC) system in Cy5 channel monitors DNA extraction and excludes false-negative interpretation of results caused by inhibition of real-time PCR. The IPC Target can be extracted with the sample or is added to the master mix.

For analysis of blood at least 500 - 1000 µl blood should be extracted. The kit is suitable for sample volumes up to 9 µl.

Table 1 shows an overview of the detection channels of Module 7 and 8

Table 1 Overview of detection

	FAM	VIC	CY5
MycoReal Kit *Candida* & *A. fumigatus* Module 7 Assay Mix	*C. parapsilosis* group	*C. glabrata, C. krusei*	IPC
MycoReal Kit *Candida* & *A. fumigatus* Module 8 Assay Mix	*C. albicans*, *C. tropicalis*, *C. dubliniensis*	*Aspergillus fumigatus*	

This kit was validated with the ABI® 7500 instrument (Thermo Fisher Scientific) and with 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 for a sequence-specific detection of PCR amplificates.

When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR is recommended. Keep in mind that some PCR-platforms first have to be calibrated with the corresponding dye before performing multiplex-PCR.

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

3. Pathogen information

Candidiasis is a yeast infection caused by different *Candida* species. *Candida* can cause opportunistic infections of the skin and mucosa as well as invasive infections. *Candida albicans* and C. glabrata are the predominant species causing diseases. However, other species such as *C. tropicalis, C. krusei, C. parapsilosis*, *C. dubliniensis*, *C. lusitaniae, C. guillermondii* and *C. kefyr* have also gained clinical significance. Invasive candidiasis is increasingly recognized as a primary cause of morbidity and mortality especially in immunocompromised patients, patients with severe underlying disorders or intensive care patients. The pathogen can be detected in biopsies from the affected organ or in the blood. However, it should be noted that *Candida* can also occur as a component of a transient flora without an infection having to be present. Therefore, positive fungal detection in test materials that do not originate from sterile body areas is difficult to interpret without knowledge of other parameters in patients at risk. Invasive candidiasis remains an important cause of death in vulnerable patients

Aspergilli are ubiquitous molds that can cause allergies as well as infections (aspergilloses). The genus *Aspergillus* includes over 300 species, with the majority of *Aspergillus* infections caused by *A. fumigatus, A. flavus*, and *A. terreus*. Less common are infections caused by *A. nidulans, A. niger, A. ustus*, and other species. Studies showed that other members of the *A. fumigatus* complex can also cause infections. Most invasive aspergilloses affect the respiratory tract. Fungal pneumonias caused by *Aspergillus* are particularly feared infections in severely immunocompromised patients, such as leukemia patients or patients after bone marrow transplantation, and can have a very high lethality. In patients with invasive aspergillosis, the fungus can be detected in biopsies from the affected organ or in the blood.

The rapid and early diagnosis of a fungal infection is of particular importance with regard to survival and cure rates. Not only the detection, but also the exact identification of the fungal species is important. The species can provide information on whether contamination, colonization or infection is more likely to be present and also provides information on possible resistance behavior. For example, *C. krusei* is intrinsically resistant to fluconazole and, to a lesser extent, to amphotericin B. *Candida glabrata* is generally less sensitive to fluconazole than other *Candida* species.

4. Contents of the kit, stability and storage

Labelling	Content	Amount	Storage
Module 7 Assay Mix (green cap)	Primer and probes for detection of - *C. parapsilosis* group (FAM) - *C. glabrata, C. krusei* (VIC) - DNA IPC (Cy5)	1 x 50 µl	-15 °C to -25 °C
Module 8 Assay Mix (green cap)	Primer and probes for detection of - *C. albicans*, *C. tropicalis, C. dubliniensis* (FAM) - *A. fumigatus* (VIC) - DNA IPC (Cy5)	1 x 50 µl	-15 °C to -25 °C
DNA IPC Target (orange cap)	Target for DNA IPC (Internal DNA Positive control)	1 x 100 µl	-15 °C to -25 °C
Module 7-8 Positive Control (red cap)	DNA Positive control (approx. 200 - 1000 target copies/µl of *C. parapsilosis, C. glabrata, C. albicans, A. fumigatus*)	1 x 400 µl	-15 °C to -25 °C
DNA Reaction Mix (white cap)	DNA reaction mix	2 x 500 µl	**-20°C until first use, then at +4°C**
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	-15 °C to -25 °C

DNA Reaction Mix: The reaction 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 *Candida* & *A. fumigatus* are stable until the expiry date stated on the label. Repeated freeze/thaw cycles should be avoided. Protect kit components from light. The kit is delivered at 4°C.

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

- For *in vitro* diagnostic use. The use of this kit is limited to persons instructed in the procedures of real-time PCR and *in vitro* diagnostics.
- Clean benches and devices periodically.
- Ensure the periodical cleaning and maintenance of real-time PCR instruments.
- Use aerosol barrier pipette tips.
- 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 must 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.
- Repeated thawing and freezing of kit components should be avoided. Protect kit components from light.
- **Caution:** DNA IPC Target is stored in 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 has been validated for blood, biopsies, aspirates, punctates, cerebrospinal fluid (CSF) or samples of the respiratory tract. Test performance with other specimen types has not yet been assessed.
- A low concentration of leukocytes in the blood can lead to a loss of pathogen DNA during extraction.
- This kit is only suitable for specimens taken from normally sterile/semi-sterile sites.
- A negative test result does not exclude the possibility of a *Candida* or *A. fumigatus* 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

- 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.

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

Module 7-8 Positive Control is a mixture of four DNA target sequences for *C. parapsilosis, C. glabrata, C. albicans* (approx. 200 target copies/µl each) and *A. fumigatus* (approx. 1,000 target copies/µl) 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 Module 7 and 8.
- 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.

8.1. Pipetting scheme per assay mix

Samples must be analyzed with Module 7 Assay Mix and Module 8 Assay Mix, respectively. For blood and CSF sample, it is recommended to use 9 µl DNA sample. When using a sample volume < 9 µl, water has to be added accordingly to the master mix.

Analysis with Module 7:

		Per sample
Preparation of Master Mix no. 7 (mix well by pipetting)	Nuclease free water	-
	DNA Reaction Mix	10.0 µl
	Module 7 Assay Mix	1.0 µl
	Total volume Master Mix	**11.0 µl**
Preparation of PCR	Master Mix	11.0 µl
	Sample	9.0 µl
	Total volume	**20.0 µl**

Analysis with Module 8:

		Per sample
Preparation of Master Mix no. 8 (mix well by pipetting)	Nuclease free water	-
	DNA Reaction Mix	10.0 µl
	Module 8 Assay Mix	1.0 µl
	Total volume Master Mix	**11.0 µl**
Preparation of PCR	Master Mix	11.0 µl
	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 DNA IPC Target per reaction causes inhibition of real-time PCR.

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.

8.2. 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: Ramp speed: Without "fast cycling" Parameter for ABI® 7500 Instrument, QuantStudio™ 5/7

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[2]

[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.
[2]30 sec in combination with SeptiReal® Kit

Detection channels:

	FAM-NONE (530 nm)	**VIC-NONE (554 nm)**	**CY5-BHQ1 (670 nm)**
Module 7 Assay Mix	*C. parapsilosis* group	*C. glabrata, C. krusei*	IPC
Module 8 Assay Mix	*C. albicans*, *C. tropicalis, C. dubliniensis*	*Aspergillus fumigatus*	IPC

Passive reference dye, if required: ROX

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

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 FAM and VIC channel for the *Candida* and *Aspergillus* targets and 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, punch biopsies: Cut off = Cq ≥38.0

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

Table 2 Criteria for valid controls, IPC Target was added during extraction. Applies to the analysis with Assay Mix Module 7 and Assay Mix Module 8, respectively

	Cq FAM channel Fungus	Cq VIC channel Fungus	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 samples with Module 7

	Cq FAM channel *C. parapsilosis* group[1]	**Cq VIC channel** *C. glabrata* *C. krusei*[2]	**Cq Cy5 channel DNA IPC Target**	**Interpretation[4]**	**Action**
Sample	Negative	Negative	28-32[3]	Negativ	-
Sample	Positive	Positive	Positive/Negative	Positive for members of *C. parapsilosis* group + *C. glabrata/C. krusei*	-
Sample	Negative	Positive	Positive/Negative	Positive for *C. glabrata/C. krusei*	-
Sample	Positive	Negative	Positive/Negative	Positive for members of *C. parapsilosis* group	-
Sample	Negative	Negative	Negative	Invalid	See 10.5

[1]A *Candida parapsilosis* positive sample can show a low background signal with a shift of Cq-value of approx. 7 in FAM channel of Module 8
[2]A *Candida krusei* positive sample can show a low background signal in FAM channel
[3]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.
[4]No differentiation of species between *Candida glabrata* and *Candida krusei*

Table 4 Interpretation of samples with Module 8

	Cq FAM channel[1] *C. albicans* *C. tropicalis* *C. dubliniensis*	**Cq VIC channel** *A. fumigatus*	**Cq Cy5 channel DNA IPC Target**	**Interpretation[3]**	**Action**
Sample	Negative	Negative	28-32[2]	Negative	-
Sample	Positive	Positive	Positive/Negative	Positive for *C. albicans*/*C. tropicalis/C. dubliniensis* and *A. fumigatus*	-
Sample	Negative	Positive	Positive/Negative	Positive for *A. fumigatus*	-
Sample	Positive	Negative	Positive/Negative	Positive for *C. albicans*/*C. tropicalis/C. dubliniensis*	-
Sample	Negative	Negative	Negative	Invalid	See 10.5

[1]A C*andida parapsilosis* positive sample can show a low background signal in FAM channel of Module 8
[2]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.
[3]No differentiation of species between *Candida albicans*, *Candida tropicalis* and *Candida dubliniensis.*

10. Troubleshooting

10.1. No *CandidalAspergillus* 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. *CandidalAspergillus* 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. *CandidalAspergillus* 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 *CandidalAspergillus* 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 *Candida & A. fumigatus* with an ABI® 7500 instrument is shown in Fig. 1-3.

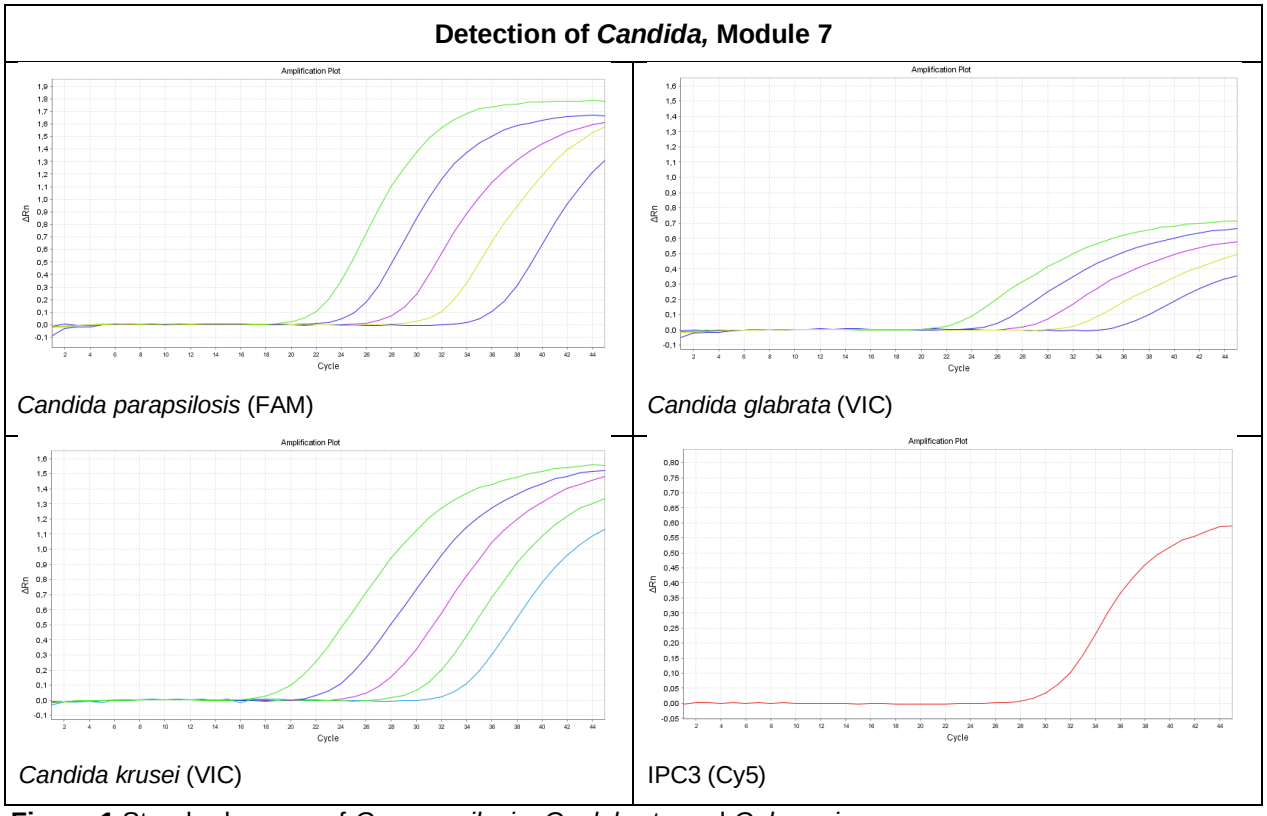


Figure 1 Standard curves of *C. parapsilosis, C. glabrata* and *C. krusei*

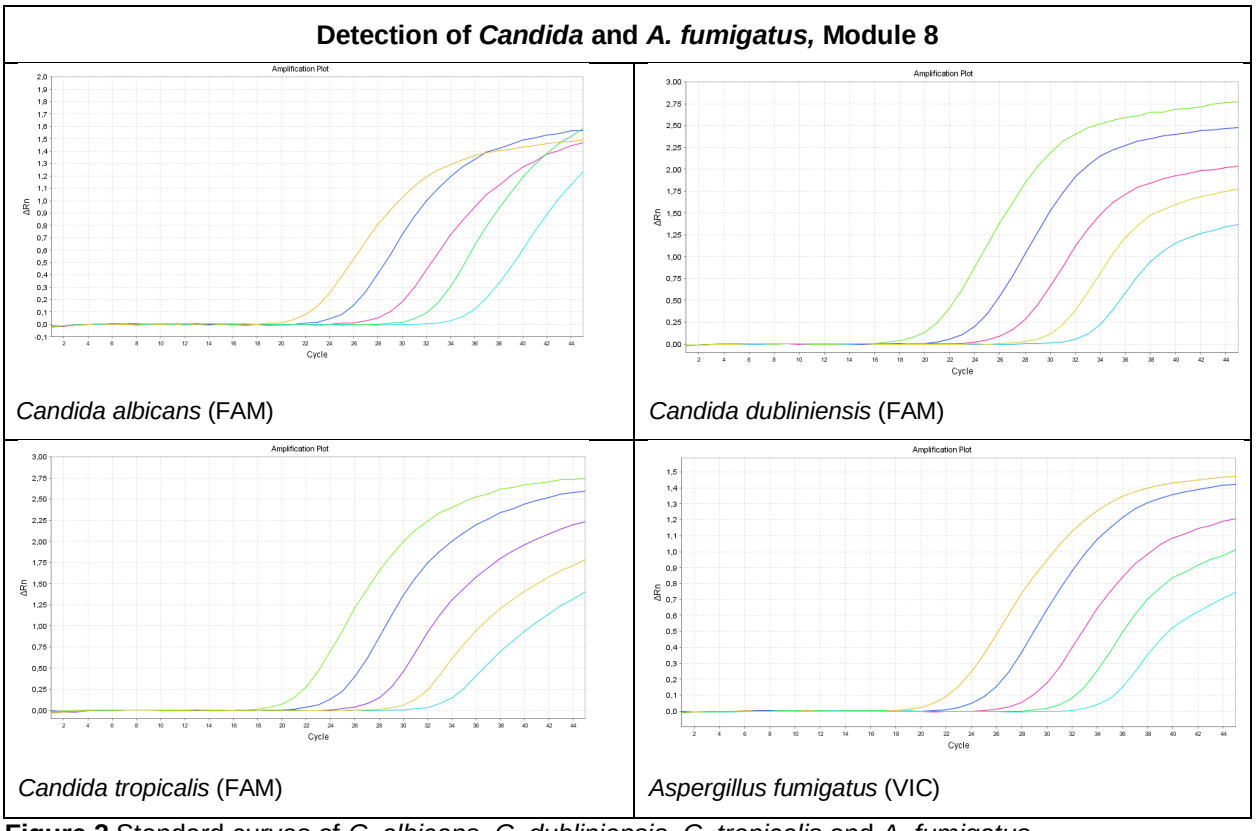
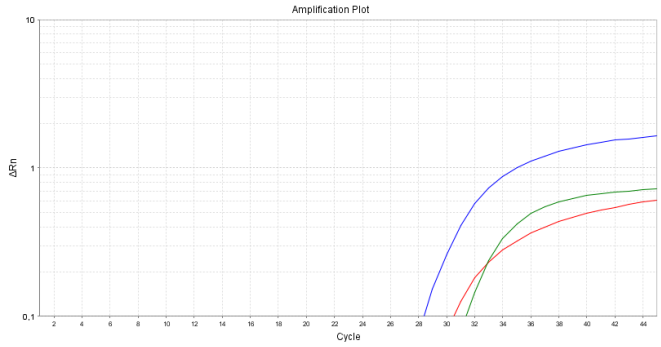
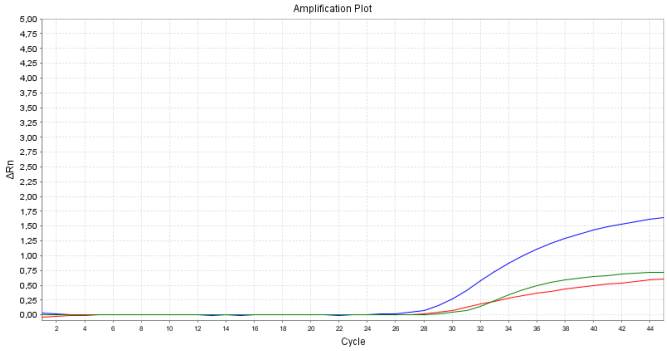
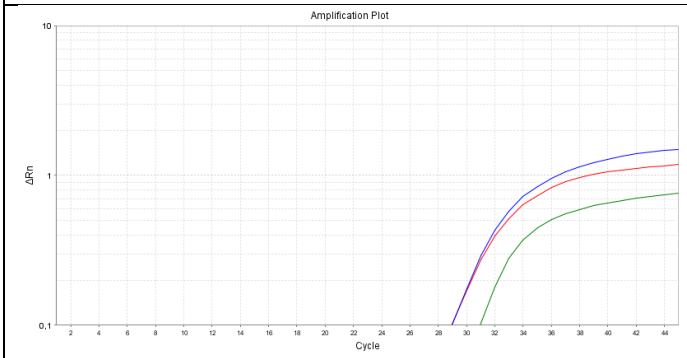


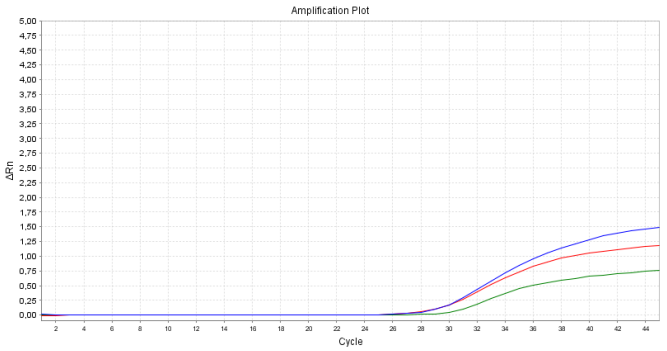
Figure 2 Standard curves of *C. albicans, C. dubliniensis, C. tropicalis* and *A. fumigatus*

Logarithmic view	Linear view
	

ABI® 7500: FAM (*C. parapsilosis* group), VIC (*C. glabrata*) and CY5 (IPC)

Module 7, Positive Control





ABI® 7500: FAM (*C. albicans*), VIC (*A. fumigatus*) and CY5 (IPC) Kanal

Module 8, Positive Control

Figure 3 Amplification curves of Positive Control

11.2. Limit of detection

Method: The **limit of detection** (LoD95 = smallest number of copies of target DNA which can be detected in 95% of cases) was determined with twelve replicates at five different concentrations (1, 2, 4, 10, 20, 100 copies) around the detection limit with plasmid DNA containing a fragment of the ITS2 region of *C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis* and *Aspergillus fumigatus,* respectively. It was then calculated with a non-linear (logistic) curve fit using Graph Pad Prism Software. The fungal ITS2 region is a multiocopy gene.

Results: See Table 5. LoD95 ranges from 3 to 32 copies, depending on the species.

Note: Existing primer or probe region sequence variabilities may lead to false-negative or less sensitive results for some *Candida* and *A. fumigatus* strains.

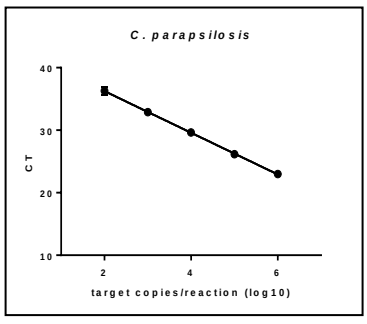
Table 5 Summary of LoD95

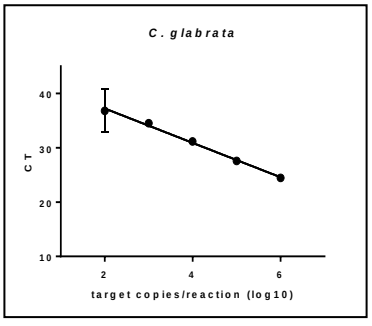
	LOD95	**Linearity – Slope**	**Linearity – Coefficient of correlation R^2**
Modul 7 *C. parapsilosis* group	6	-3.33 ± 0.04	0.9928
Modul 7 *C. glabrata*	5	-3.16 ± 0.18	0.8725
Modul 7 *C. krusei*	32	-3.43 ± 0.02	0.9986
Modul 8 *C. albicans*	9	-3.25 ± 0.04	0.9931
Modul 8 *C. tropicalis*	9	-3.32 ± 0.03	0.9967
Modul 8 *C. dubliniensis*	3	-3.04 ± 0.10	0.9551
Modul 8 *A. fumigatus*	6	-3.32 ± 0.03	0.9970

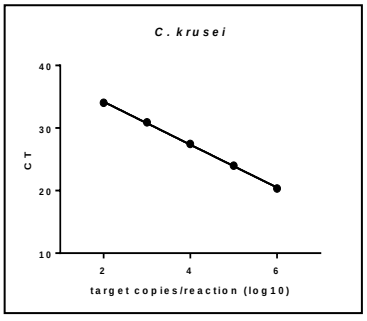
11.3. Linearity and dynamic range

Method: The linearity was determined with serial 10-fold dilutions of plasmid DNA (10^6 to 10^2 target copies/PCR).

Results are shown in Table 5**Fehler! Verweisquelle konnte nicht gefunden werden.** and Figure 4. The correlation coefficient R^2 for all species was between 0.87 and 0.99 and the slope was between -3.04 and -3.43, indicating good test performance. The test shows linearity over the range of 100 to 1,000,000 target copies/reaction.







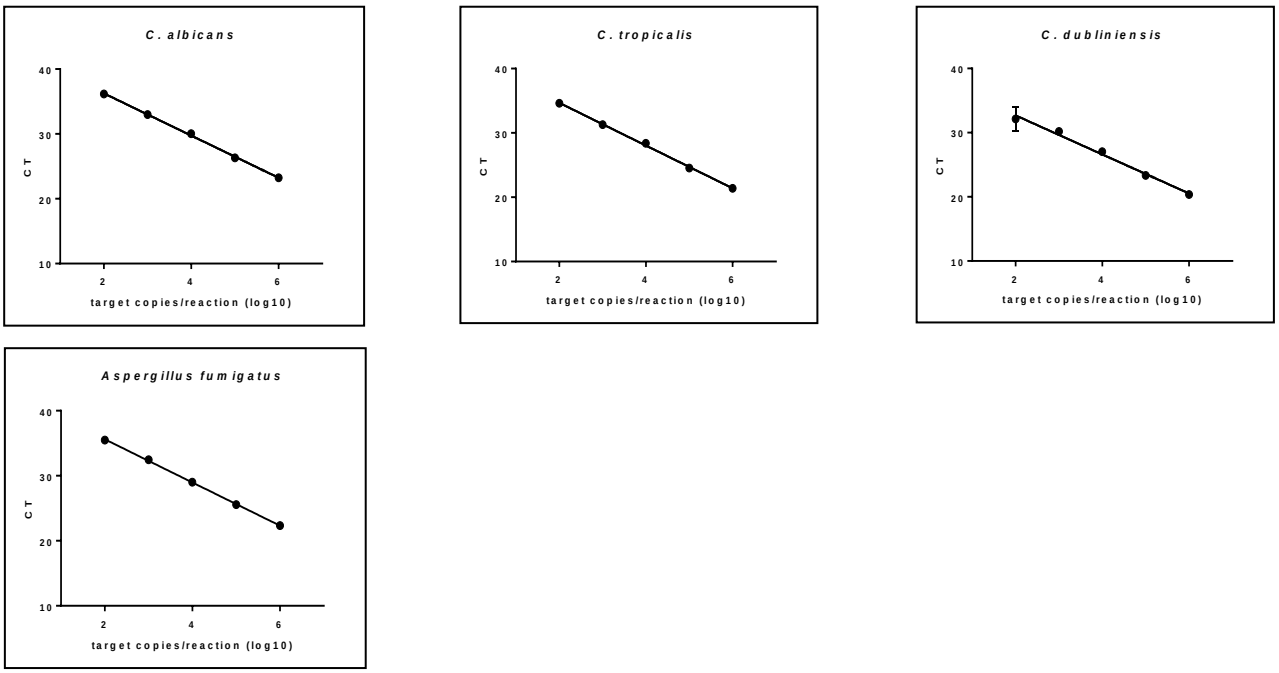


Figure 4

11.4. Analytical specificity

Method: The ITS region is a multi-copy gene and, due to its high species specificity, suited for the differentiation of *Candida* species and *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 eight *Candida* species and *A. fumigatus*. Furthermore, specificity was tested on several fungal strains.

Result: See Table 6 and Table 7. MycoReal Kit *Candida* & *A. fumigatus* detects *C. albicans, C. dubliniensis, C. glabrata, C. krusei* (= *Pichia kudriavzevii, Issatchenkia orientalis*), *C. parapsilosis* group, *C. tropicalis* and *Aspergillus fumigatus* and a few other *Candida* species listed in Table 6. Sequence variabilities in the target-region of some *Candida* and *A. fumigatus* subtypes (strains) may lead to false-negative or less sensitive results. 2.5% of strains classified as *C. albicans* and 6% of strains classified as *C. tropicalis* in the NCBI database might not be detected due to mismatches. However, for some of these entries, the correct phylogenetic species classification of the provided ITS2 sequence is not yet clear.

The kit was tested with 47 fungal strains. Amplification curves with high Cq-values >39 were generated by *Syncephalastrum* sp., *Aureobasidium pullulans* and *Pichia fermentans,* as shown in Table 7. This might be due to contamination or cross-reactivity with low sensitivity. BLAST analyses with these species show sequence mismatches at the primer and probe binding sites.

Table 6 Results of BLAST analyses

	Detected species
Module 7 (FAM) ***Candida parapsilosis* group**	*Candida parapsilosis, Candida metapsilosis, Candida orthopsilosis*
Module 7 (VIC) ***Candida glabrata, C. krusei***	*Candida glabrata, Candida krusei*
Module 8 (FAM) ***Candida albicans, C. tropicalis, C. dubliniensis***	*Candida albicans, Candida africana, Candida tropicalis, Candida sojae, Candida neerlandica, Candida dubliniensis*
Module 8 (VIC) ***Aspergillus fumigatus***	*Aspergillus fumigatus* *Aspergillus* species belonging to the *Aspergillus* section *Fumigati* such as *Aspergillus lentulus* might be detected but with less sensitivity.

Table 7 Fungal isolates tested with MycoReal Kit *Candida & A. fumigatus*

	Module 7	**Module 8**
Moulds		
Aspergillus fumigatus	Negative	Cqt=34 (VIC)
Aspergillus flavus	Negative	Negative
Aspergillus nidulans	Negative	Negative
Penicillium marneffei	Negative	Negative
Penicillium chrysogenum	Negative	Negative
Rhizopus oryzae	Negative	Negative
Mucor circinelloides/racemosus	Negative	Negative
Rhizomucor pusillus	Negative	Negative
Absidia corymbifera	Negative	Negative
Cunninghamella elegans	Negative	Negative
Syncephalastrum sp.	Cq=40 (FAM) (contamination/cross-reaction)	Negative
Scedosporium apiospermum	Negative	Negative
Fusarium oxysporum	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	Cq=39 (contamination/cross-reaction)	Negative
Cladophialophora	Negative	Negative
Dermatophytes		
Microsporum canis	Negative	Negative
Trichophyton tonsurans	Negative	Negative
Yeasts		
Candida glabrata	Cq=25 (VIC)	Negative
Candida tropicalis	Negative	Cq=27 (FAM)
Candida albicans	Negative	Cq=30 (FAM)
Candida parapsilosis	Cq=27 (FAM)	Cq=35 (FAM) (cross-reaction)
Candida krusei	Cq=30 (VIC) + Cq=38 (FAM)	Negative
Candida dubliniensis	Negative	Cq=28 (FAM)
Candida lusitaniae	Negative	Negative
Candida guillermondii	Negative	Negative
Candida kefyr	Negative	Negative
Candida valida/Pichia membranifacies	Negative	Negative
Debaromyces hansenii (Candida famata)	Negative	Negative
Yarrowia lipolytica	Negative	Negative
Rhodotorula rubra	Negative	Negative
Pichia fermentans	Cq=40 (VIC) (contamination/cross-reaction)	Negative
Cryptococcus neoformans	Negative	Negative
Malassezia furfur	Negative	Negative
Malassezia pachydermatis	Negative	Negative
Saccharomyces cerevisiae	Negative	Negative
Trichosporon cutaneum	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 *Candida & A. fumigatus* was determined by testing 10-fold plasmid DNA dilution series (10^6 to 10^2 target copies/PCR) 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.

Result:

CV for detection of *C. albicans* ranged from 0.13% to 1.02%, with mean overall inter-assay precision equals 0.56%.
CV for detection of *C. dubliniensis* ranged from 0.51% to 6.82%, with mean overall inter-assay precision equals 2.00%.
CV for detection of *C. glabrata* ranged from 0.22% to 6.28%, with mean overall inter-assay precision equals 1.46%.
CV for detection of *C. krusei* ranged from 0.16% to 0.51%, with mean overall inter-assay precision equals 0.29%.
CV for detection of *C. parapsilosis* ranged from 0.95% to 1.50%, with mean overall inter-assay precision equals 1.15%.
CV for detection of *C. tropicalis* ranged from 0.10% to 0.55%, with mean overall inter-assay precision equals 0.34%.
CV for detection of *A. fumigatus* ranged from 0.17% to 0.81%, with mean overall inter-assay precision equals 0.45%.

11.6. Precision intra-assay

Intra-assay precision is defined as the reproducibility of the results of a sample within a PCR run.

Method: Intra-assay precision 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 for detection of *C. albicans* ranged from 0.17% to 2.52%, with mean overall intra-assay precision equals 0.96%.
CV for detection of *C. dubliniensis* ranged from 0.12% to 1.75%, with mean overall intra-assay precision equals 0.72%.
CV for detection of *C. glabrata* ranged from 0.06% to 20.06%, with mean overall intra-assay precision equals 1.82%.
CV for detection of *C. krusei* ranged from 0.03% to 0.99%, with mean overall intra-assay precision equals 0.41%.
CV for detection of *C. parapsilosis* ranged from 0.08% to 2.43%, with mean overall intra-assay precision equals 0.77%.
CV for detection of *C. tropicalis* ranged from 0.11% to 1.47%, with mean overall intra-assay precision equals 0.69%.
CV for detection of *A. fumigatus* ranged from 0.14% to 2.13%, with mean overall intra-assay precision equals 0.58%.

11.7. Precision inter-lot

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.
Method: The inter-lot variability of two different kit lots was determined with E+04 target copies/PCR of plasmid DNA in triplicates, with the Positive Control in duplicates and with NTC in quadruplicates. Arithmetic mean ($\bar{x}$) standard deviation (σ) and coefficient of variation (CV %) of the replicates were calculated.

Results:
The CV mean kit inter-lot precision for detection of C. *parapsilosis* equals 1.09%.
The CV mean kit inter-lot precision for detection of C. *glabrata* equals 1.06%.
The CV mean kit inter-lot precision for detection of C. *albicans* equals 1.03%.
The CV mean kit inter-lot precision for detection of C. *krusei* equals 0.86%.
The CV mean kit inter-lot precision for detection of C. *dubliniensis* equals 0.74%.
The CV mean kit inter-lot precision for detection of C. *tropicalis* equals 1.16%.
The CV mean kit inter-lot precision for detection of *A. fumigatus* equals 0.95%.

11.8. Clinical evaluation

Method: MycoReal Kit *Candida & A. fumigatus* was tested by an external service provider on 13 (Module 7) and 32 (Module 8) whole blood samples from 39 patients. Extraction of one ml blood was performed with the SeptiFast LysKit (Roche). Analyses were performed in single reactions on the cobas z 480 analyzer (Roche). Results were compared with results of the LightCycler® SeptiFast Test (Roche).
Furthermore, MycoReal Kit *Candida & A. fumigatus* was tested on 31 different clinical samples from 31 patients. Analyses were performed in single reactions on the cobas z 480 analyzer (Roche). Samples with discrepant results compared to the reference method were repeated on ABI® 7500. Results were compared with results of culture and of a panfungal in-house real-time PCR based on HybProbes. Furthermore, some samples with discrepant results were reanalyzed with a *Candida*-specific real-time PCR test based on HybProbes (MycoReal *Candida)*.

Results: See Table 8

Table 8 Summary of clinical performance with 70 clinical samples

	Blood + other samples (n=70)	**Blood samples (n=39)**	**Other samples (n=31)**
Sensitivity	90.91%	85.29%	100.00%
Specificity	93.33%	100.00%	90.00%
NPV	73.68%	50.00%	100.00%
PPV	98.04%	100.00%	95.45%
Prevalence	78.57%	87.18%	67.74%
Accuracy	91.43%	87.18%	96.77%

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 kits 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

- 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.
- Add 200 µl Binding Buffer (green cap) + 40 µl reconstituted Proteinase K, vortex.
- Incubate tube for 10 min at 70°C.
- 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).
- Add 100 µl sterile filtered isopropanol.
- 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.