MycoReal® Kit Fungi

Instruction for Use

For Research Use Only

REF **DHUF00453**

Σ **50 reactions**

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Annex – symbols

LOT	Batch code		Use by date
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Temperature limit (Store at)
	Consult instructions for use	UDI	Unique device identifier
	Keep away from sunlight	QTY	Contents

1 Intended purpose

MycoReal® Kit Fungi is a non-automated real-time PCR test for the qualitative universal (broad-range) detection and identification of DNA of fungi (ITS2-region). For phylogenetic identification of the pathogen, the PCR amplicon must be sequenced and analysed by BLAST.

MycoReal® Kit Fungi does not displace culture but offers an improvement in the detection of fungi in cases where the presence of fungi is suspected but culture remains negative, or the fungus is difficult to cultivate.

This kit is suitable for specimens taken from normally sterile sites. Proper specimens are DNA extracts isolated from samples of bronchoalveolar lavages (BAL), aspirates, cerebrospinal fluid, tissue, paraffin embedded tissue, fungal colonies as well as of environmental samples. The kit is suitable to a limited extent for the detection of fungi in blood samples. In case of a mixed fungal infection, the pathogen cannot be phylogenetically identified.

The test is intended for research use only.

2 Product description

MycoReal® Kit Fungi is a real-time PCR test and detects the ITS2-region of fungi. The ITS2-region can be universally found in fungi and is therefore a proper target for the broad-range (panfungal) detection of fungal DNA.

Probe-specific amplification curves in the fluorescence channel for FAM detect the ITS2-region of fungi. The PCR amplicon is about 370 bp. For phylogenetic identification of the pathogen, the PCR amplicon must be sequenced and analysed by BLAST in the NCBI database after real-time PCR.

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 DNA IPC (artificial target DNA) is added during sample extraction.

Generally, panfungal PCR is prone to contamination with fungal DNA. Therefore, a high degree of critical evaluation is required to circumvent false-positive interpretation of results (see 10. Interpretation of data, and 12. Specifications). Contaminations are mainly caused by fungi found in the environment. Contamination might occur during sample taking, DNA extraction and preparation of the PCR-reaction.

This test has been validated with the ABI® 7500 Real-time PCR System (Thermo Fisher Scientific, fast cycle parameters are not supported) and with the cobas z 480 Analyzer (Roche). However, it is also suitable for other real-time PCR instruments that can measure and differentiate fluorescence in the FAM 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), LightCycler® 480 II (Roche Diagnostocs)).

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.

PanReal, BactoReal®, MycoReal®, ParoReal® 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

Fungal diseases are mostly opportunistic infections with a great variety of common and uncommon pathogens. Up to 150 fungal species have been shown as potential human pathogens involving all body sites. Invasive mycoses are increasingly recognized as a primary cause of morbidity and mortality especially in immunocompromised patients. These infections are mainly caused by yeasts (such as *Candida* spp., *Cryptococcus neoformans*, *Saccharomyces cerevisiae, Trichosporon* spp.) and moulds (such as *Aspergillus* spp., *Scedosporium* spp., *Fusarium* spp., *Natrassia mangiferae, Curvularia* spp., *Schizophyllum commune, Paecilomyces variotii, Bipolaris* spp., *Cladophialophora bantiana,* and zygomycetes such as *Rhizopus* spp., *Absidia* spp., *Rhizomucor* spp., *Mucor* spp., *Cunninghamella bertholletiae, Saksenaea vasiformis, Apophysomyces elegans, Basidiobolus ranarum, Conidiobolus* spp.). The mortality rate of invasive fungal infections is 40-100%. A rapid diagnosis improves the outcome.
Non-invasive fungal infections (such as infections of the urogenitary tract, of the eye, of the skin, etc.) are mainly caused by *Candida, Aspergillus, Acremonium, Fusarium* and dermatophytes (such as *Trichophyton* spp., *Microsporum* spp.).

4 Principle of real-time PCR

The test is based on multiplex real-time PCR technology by 5´-nuclease-assay technology. Specific DNA sequences in the pathogen genome 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 Contents of the kit, stability and storage

Table 1

Component	**Content**	**Quantity**	**Storage**
Fungi + IPC3 Assay Mix (green cap)	Primers and probes for detection of - ITS2-region (FAM) - 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
Sequencing Primer F Fungi (transparent cap)	Forward primer for sequencing of the ITS2 region of fungi	1 x 100 µl	-25 to -15 °C
Sequencing Primer R Fungi (transparent cap)	Reverse primer for sequencing of the ITS2 region of fungi	1 x 100 µl	-25 to -15 °C
Fungi Positive Control (red cap)	DNA positive control for *Rhizopus oryzae* (approx. 10,000 target copies/µl)	1 x 300 µl	-25 to -15 °C
DNA Reaction Mix, no UNG (white cap)	PCR reaction mix for DNA amplification (without UNG (Uracil-DNA glycosylase)	1 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, ROX™ dye (passive reference) and buffer components – additives optimized to handle RT-PCR inhibitors. It does not contain Uracil-N glycosylase (UNG) to facilitate sequencing of the PCR amplicon after real-time PCR.

Delivery and Storage
Shipment is with coolpacks or on dry ice. 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)
- Reagents and devices for DNA-sequencing & phylogenetic BLAST-analyses (NCBI database)
- 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, and 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

- The use of this kit is limited to qualified personnel instructed in real-time PCR.
- Transportation of 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.
- A negative test result does not exclude the possibility of a fungal infection, because test results may be affected by improper specimen collection, technical error, and specimen mix-up or pathogen quantities below the assay sensitivity. PCR inhibitors may lead to invalid results.
- For this kit universal primers and probes have been selected. However, false-negative or less sensitive results might be obtained due to sequence heterogeneity within the target region of not yet described clinical subtypes.
- This kit is only suitable for specimens taken from normally sterile sites.
- Due to contamination, MycoReal® Kit Fungi is less sensitive compared to species-specific tests. Therefore, this kit is suitable only to a limited extent for the detection of fungi in blood samples.
- Results should be interpreted in context with other laboratory findings.

9 Preparation of samples and real-time PCR

MycoReal® Kit Fungi is suitable for analysis of DNA extracted from bronchoalveolar lavages (BAL), aspirates, cerebrospinal fluid, tissue, paraffin embedded tissue and fungal colonies
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 Fungi. 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.

10.1 Pipetting scheme

		Per sample
Preparation of Master Mix (mix well by pipetting)	DNA Reaction Mix, no UNG Fungi + IPC3 Assay Mix	10.0 µl 1.0 µl
	Total volume Master Mix	**11.0 µl**
Preparation of PCR	Master Mix Sample*	11.0 µl 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

Further information on programming the real-time PCR instrument can be found in the respective operator's manual. Keep in mind that some PCR-platforms have to 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, 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 60 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.

<u>Select detection channels:</u>

FAM channel: Detection of fungi
Cy5 channel: Detection of IPC

For ABI® 7500 Instrument, QuantStudio™ 5/6/7 (Thermo Fisher Scientific), Mx3005P® (Agilent)
FAM-NONE
Cy5-NONE
Passive refence dye: ROX

For MIC Instrument (bio molecular systems)
FAM Green
Cy5 Red
Passive refence dye: no ROX needed

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

11 Interpretation of data

For the analysis of PCR results, select the fluorescence display options FAM 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 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 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 fungi are environmental organisms, contamination with DNA thereof is possible during sampling, extraction, and PCR.
Cq values of the samples in the FAM channel must be interpreted in comparison to Cq values of the negative control of DNA extraction also. The cut-off of Cq values of the negative extraction control is Cq=35.
Samples with Cq values lower than the Cq values of the negative extraction control (difference at least 1.5 cycles!) can be interpreted as potentially positive and should be sequenced.

Table 2 shows the criteria for valid positive and negative controls. Table 3 shows interpretation of data with samples.

11.1 Controls

Table 2: Criteria for valid controls, IPC-Target (DNA) was added during extraction

	Cq FAM channel Fungi	Cq Cy5 channel IPC[1]	Interpretation	Action
Positive control	<28	Negative	Valid	-
Positive control	Negative	Negative	Invalid	See 13.1
Extraction negative control	Negative	27-30[2]	Valid	-
Extraction negative control	Negative	Negative	Invalid	See 13.1
Extraction negative control	>35	27-30[2]	Invalid	See 13.3
NTC PCR (optional)	Negative	Negative	Valid	-
NTC PCR (optional)	Negative	Negative	Invalid	See 13.2

[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).

11.2 Samples

Table 3 Interpretation of data with samples

	Cq FAM channel Fungi	Cq Cy5 channel IPC	Interpretation	Action
Sample	Negative	27-30[1]	Negative	-
Sample	Positive	Positive/Negative[2]	Positive	-
Sample	Negative	Negative	Invalid	See 13.5

[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 load in the sample can lead to a reduced or absent fluorescence signal of the IPC.

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

12 Sequencing and BLAST-analyses

- Samples with Cq values lower than the Cq values of the negative extraction control (difference at least 1.5 cycles!) should be sequenced. Before sequencing, the PCR-product must be purified.
- Then, perform two different sequencing reactions with the purified PCR products (according to manufacturer's instructions of sequencing kit, not provided). Use 0.5 µl (= 5 pmol) Sequencing Primer F Fungi or Sequencing Primer R Fungi (provided in the kit) per sequencing reaction.
  For sequencing, proceed according to manufacturer's instructions of the sequencing kit and sequencing instrument.
- For a proper interpretation of data, the organism is identified by phylogenetic BLAST-analyses. Online BLAST-analysis can be done at the NCBI homepage (http://www.ncbi.nlm.nih.gov). Results must be interpreted in context with clinical data. Only samples revealing BLAST results matching to the overall picture and other test results should be regarded as positive. If the PCR-product cannot be sequenced (sequencing shows low raw data or overlapping sequences), the sample does not contain sufficient amount of fungal or bacterial DNA or it might be a mixed infection or contamination, check Cq values.
- BLAST results shall then be checked for plausibility in context with other data, especially for semisterile samples such as BAL, punch biopsies and for non-sterile samples such as paraffin embedded tissue.

13 Troubleshooting

13.1 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.

13.2 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.

13.3 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.

13.4 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.

13.5 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).

14 Specifications and performance evaluation

14.1 Kit performance

Performance of MycoReal® Kit Fungi with an ABI® 7500 instrument is shown in Figure 1.

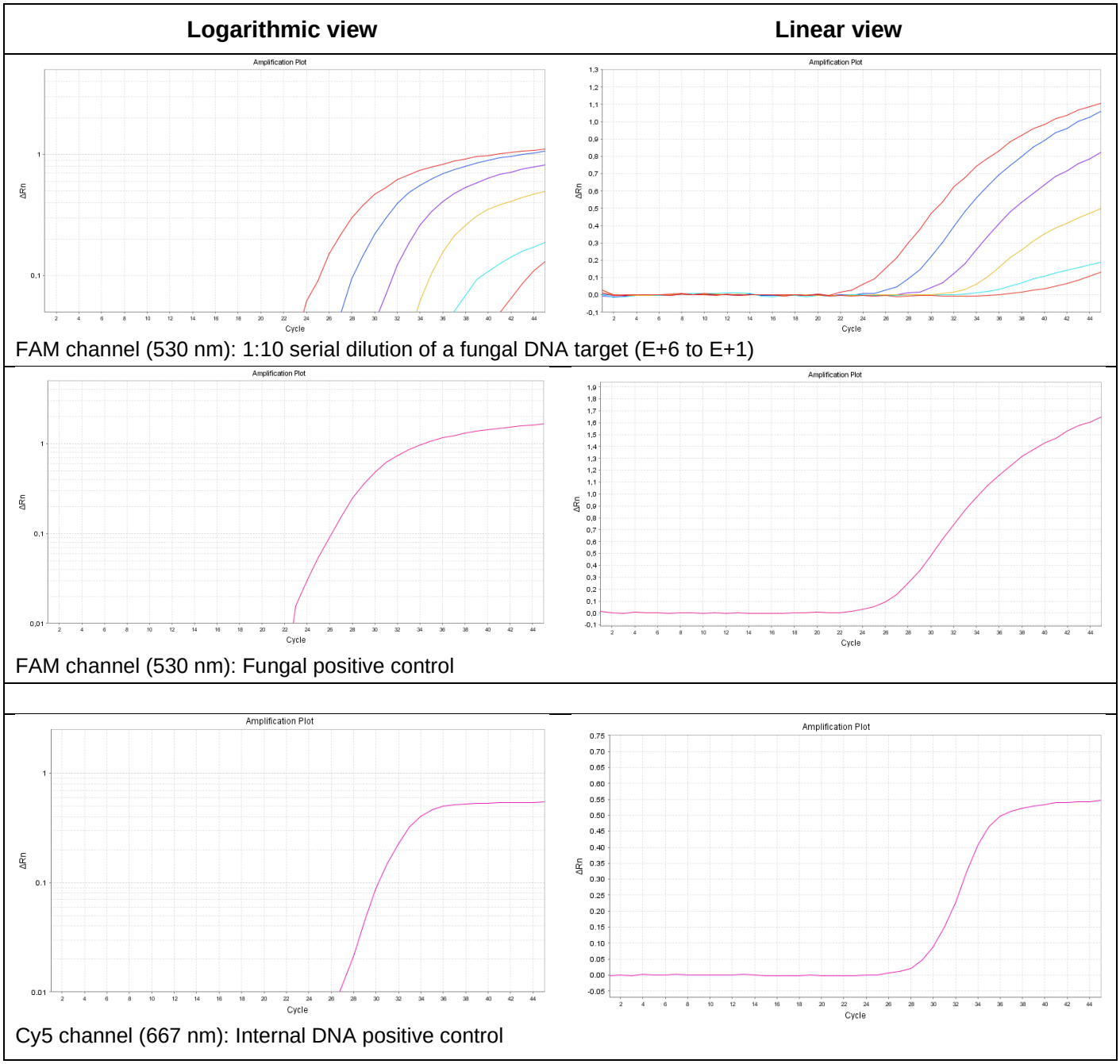

Figure 1 Performance of MycoReal® Kit Fungi

14.2 Limit of detection, LoD95%

Method: MycoReal® Kit Fungi was tested with a 10-fold dilution series of a plasmid containing a fragment of fungal ITS 2.

Result: The limit of detection (LoD95% = smallest number of copies of target DNA which can be detected in 95% of cases) is 40 target copies/reaction.

14.3 Linearity and dynamic range

Method: Linearity was determined using 10-fold dilution series (10^6 – 10^1 target copies/reaction) of the plasmids.

Result: The assay shows linearity over the range of 1,000,000 to 1,000 target copies/reaction with a slope of -3,47 ± and a correlation coefficient R^2 of > 0.9517 as shown in Figure 2.

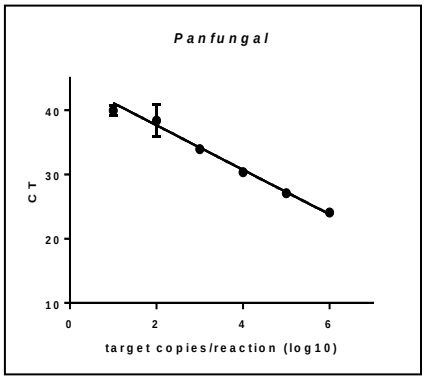

Figure 2 Ten-fold dilution series of a *Candida parapsilosis* DNA plotted against Ct

14.4 Analytical specificity

Method: The panfungal character of this test is ensured by the selection of universal primers and probes. *In silico* validation of primers and probes was performed by examining their sequences for potential homologies to currently published sequences in the NCBI database (BLAST analysis). This database analysis validated the detection of so far known fungal species.

Also, the kit was tested on a great variety of different *Dematiaceae*, dermatophytes, moulds and yeasts and bacteria.

Results: See Table 4 and Table 5.

Table 4 Tested fungal species

Organism	Number of tested strains	Number of positive strains
Moulds		
Aspergillus flavus	1	1
Aspergillus niger	1	1
Aspergillus fumigatus	1	1
Aspergillus terreus	1	1
Aspergillus nidulans	1	1
Aspergillus versicolor	1	1
Aspergillus unguis	1	1
Aspergillus sydowii	1	1
Aspergillus ustus	1	1
Aspergillus ochraceus	1	1
Aspergillus niveus	1	1
Aspergillus clavatus	1	1
Aspergillus candidus	1	1
Aspergillus glaucus	1	1
Penicillium marneffei	1	1
Penicillium olsonii	1	1
Penicillium chrysogenum	1	1
Rhizopus oryzae	1	1
Mucor circinelloides/racemosus	1	1
Rhizomucor pusillus	1	1
Rhizomucor miehei	1	1
Absidia corymbifera	1	1
Cunninghamella elegans	1	1
Syncephalastrum sp.	1	1
Scedosporium apiospermum	1	1
Fusarium oxysporum	1	1
Fusarium verticilloides	1	1
Fusarium solani	1	1
Beauveria bassiana	1	1
Natrassia mangiferae	1	1
Alternaria alternata	1	1
Curvularia lunata var. lunata	1	1
Schizophyllum commune	1	1
Acremonium strictum	1	1
Paecilomyces variotii	1	1
Dematiaceae		
Bipolaris australiensis	1	1
Cladosporium herbarum	1	1
Phialophora richardsiae	1	1
Sporothrix schenkii	1	1
Aureobasidium pullulans	1	1
Cladophialophora sp.	1	1
Dermatophytes		
Microsporum canis	1	1
Trichophyton tonsurans	1	1
Yeasts		
Candida glabrata	1	1
Candida tropicalis	1	1
Candida albicans	1	1
Candida parapsilosis	1	1
Candida krusei	1	1
Candida dubliniensis	1	1
Candida guillermondii	1	1
Candida kefyr	1	1
Candida valida/Pichia membranifacies	1	1
Debaromyces hansenii	1	1
Yarrowia lipolytica	1	1
Rhodotorula rubra	1	1
Pichia fermentans	1	1
Cryptococcus neoformans	1	1
Malassezia furfur	1	1
Malassezia pachydermatis	1	1
Saccharomyces cerevisiae	1	1
Trichosporon cutaneum	1	1

Table 5 Tested bacterial species

Sample Name	PCR result
Aerococcus viridans	Negative
Bartonella henselae	Negative
Bordetella pertussis	Negative
Borrelia garinii	Negative
Campylobacter jejunii	Negative
Clostridium difficile	Negative
Enterococcus faecalis	Negative
Enterococcus faecalis	Negative
Enterococcus faecalis	Negative
Enterococcus faecalis	Negative
Enterococcus faecium	Negative
Enterococcus faecium	Negative
Enterococcus faecium	Negative
Enterococcus faecium	Negative
Escherichia coli O157	Negative
Haemophilus influenzae	Negative
Legionella pneumophila	Negative
Leptospira canicola	Negative
Listeria monocytogenes	Negative
Mycoplasma pneumoniae	Negative
Neisseria gonorrhoeae	Negative
Neisseria meningitidis	Negative
Rickettsia conorii	Negative
Salmonella enteritidis	Negative
Salmonella typhimurium	Negative
Staphylococcus aureus	Negative
Streptococcus agalactiae	Negative
Streptococcus agalactiae	Negative
Streptococcus agalactiae	Negative
Streptococcus bovis	Negative
Streptococcus dysgalactiae	Negative
Streptococcus pneumoniae	Negative
Streptococcus pyogenes	Negative
Streptococcus suis	Negative
Streptococcus uberis	Negative

15 References

1. Zeller, I., Schabereiter-Gurtner, C., Mihalits, V., Selitsch, B., Barousch, W., Hirschl, A.M., 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. 66:1383-1392.

16 Revision history

Revision	Date	Description
1.0	02.03.2023	First version