PanReal Kit Fungi & Bacteria

Kit version 1.1

Instructions for Use

For Research Use Only

REF **DHUFB0153**

Σ **50 reactions**

ingenetix GmbH
Haidingergasse 1
1030 Vienna, Austria
T +43(0)1 36 198 01
office@ingenetix.com
www.ingenetix.com

Index

NOTICE TO PURCHASER: LIMITED LICENSE

The MGB Probes contained in this product is covered by one or more of the following US patents and corresponding patents outside the US: 5801155 and 6084102 and is sold under a license from the ELITech Group. The purchase of this product includes a license to use only this amount of product solely for the purchaser´s own use solely in the human in vitro diagnostic field (in accordance with applicable FDA and other regulatory requirements) and may not be used for any other commercial use, including without limitation repacking or resale in any form.

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

PanReal Kit Fungi & Bacteria is a multiplex-PCR real-time PCR kit for universal detection of fungi and bacteria. For phylogenetic identification of the pathogen, the PCR product must be sequenced and analysed by BLAST.

2 Product description

PanReal Kit Fungi & Bacteria is a real-time PCR test and detects the ITS2-region of fungi as well as a part of the 16S rDNA gene of bacteria.

This test is performed in a multiplex real-time PCR format. For phylogenetic identification of the pathogen, the PCR product has to be sequenced and analysed by BLAST.

- In FAM channel the ITS2-region of fungi is detected. The PCR product is about 370 bp.
- In VIC channel the V3 region of the 16S rDNA gene of bacteria is detected. The PCR product is about 550 bp.
- The internal DNA positive control (DNA IPC) is detected in Cy5 channel and is used as DNA extraction as well as real-time PCR inhibition control. The target for the DNA IPC (artificial target DNA) is added during sample extraction.

PanReal Kit Fungi & Bacteria does not displace culture, but offers an improvement in the detection of fungal and bacterial infections in cases where an infection is suspected but culture remains negative or the pathogen is difficult to cultivate.
The kit allows rapid detection of DNA of fungi and bacteria in samples purified from bronchoalveolar lavages (BAL), aspirates, cerebrospinal fluid, tissue, paraffin embedded tissue and fungal/bacterial colonies. The kit is suitable only to a limited extent for the detection of fungi in blood samples. This test is not suitable for the detection of bacteria in blood samples.

Generally, panfungal and panbacterial PCR is prone to contamination with fungal or bacterial DNA. Therefore, a high degree of critical evaluation is required, to circumvent false-positive interpretation of results (see 11. Interpretation of PCR and sequence data, and 13. Specifications). Contaminations are mainly caused by fungi and bacteria found in the environment. Contamination might occur during sample taking, DNA extraction and preparation of the PCR-reaction and is caused by low-level contamination of the kit reagents. Due to contamination, PanReal Kit Fungi & Bacteria is less sensitive compared to species-specific assays.

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, VIC and Cy5 channel (e.g., QuantStudio™ 5, QuantStudio™ 7 real-time PCR system (Thermo Fisher Scientific), qTOWER^{3}G (Analytik Jena), Mic instrument (bio molecular systems), 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.).

Concerning bacterial infections, broad-range PCR targeting the 16S rDNA gene is useful when antimicrobial therapy has already been initiated or when culture remains negative due to infections caused by bacteria with unusual growth requirements.

4 Principle of real-time PCR

The test is based on multiplex real-time PCR by 5´-nuclease-assay technology. Specific DNA sequences are amplified and the generated PCR-products are detected by oligonucleotide-probes labelled with fluorescent dyes. This allows a sequence-specific detection of PCR amplificates.

During PCR, primers are extended by *Taq* polymerase and probes hybridized to the target are cleaved by the 5'-exonuclease activity of *Taq* polymerase. According to the accumulation of PCR product, the fluorescence of the probe increases with each PCR cycle. The change in fluorescence of the different dyes is recorded cycle by cycle in the real-time PCR instrument in the closed reaction tube at different fluorescence wavelengths.
The Cq value (Cq = Quantification cycle, Ct = Cycle threshold, Cp = Crossing point) describes the cycle at which the fluorescence first rises significantly above the background fluorescence.

5 Contents of the kit, stability and storage

Table 1

Component	Content	Quantity	Storage
Fungi & Bacteria + IPC3 Assay Mix (green cap)	Primers and probes for detection of • ITS2 (FAM) • 16S rDNA (VIC) • IPC (Cy5)	1 x 50 µl	-25 to -15 °C
IPC-Target (DNA) (orange cap)	Target for DNA IPC (internal DNA positive control system)	1 x 200 µl	-25 to -15 °C
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
Sequencing Primer F Bacteria (transparent cap)	Forward primer for sequencing of the 16S rDNA gene of bacteria	1 x 100 µl	-25 to -15 °C
Sequencing Primer R Bacteria (transparent cap)	Reverse primer for sequencing of the 16S rDNA gene of bacteria	1 x 100 µl	-25 to -15 °C
Fungi & Bacteria Positive Control (red cap)	DNA positive control for *Rhizopus* and Glaesserella parasuis (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 until first use, then at +4 °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, VIC 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

- 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.

Recommended measures to avoid DNA contamination:

- 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

- Optimal performance of this test requires 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 or bacterial infection, because test results may be affected by improper specimen collection, technical error, and specimen mix-up or pathogen quantities below the assay sensitivity. The presence of 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, PanReal Kit Fungi & Bacteria 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. This test is not suitable for the detection of bacteria in blood samples.
- Contamination with bacterial DNA is caused by low-level contamination of the kit reagents.
- Results should be interpreted in context with clinical and laboratory findings.

9 Preparation of samples and real-time PCR

PanReal Kit Fungi & Bacteria is suitable for analysis of DNA extracted from bronchoalveolar lavages (BAL), aspirates, cerebrospinal fluid, tissue, paraffin embedded tissue and fungal or bacterial 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 must 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 PanReal Kit Fungi & Bacteria. 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 and bacterial 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)

9.3 Extraction of EDTA blood

The extracted DNA analyzed 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.

9.4 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.

9.4.1 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.

9.4.2 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	10.0 µl
	Fungi & Bacteria + IPC3 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**

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

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

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

10.2 Programming of temperature profile

Please find further information on programming 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
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.

Select detection channels:

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

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

For MIC Instrument (bio molecular systems)
FAM Green
VIC Yellow
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
VIC: Excitation at 540 / 533 nm, Emission at 580 nm
Cy5: Excitation at 610 / 618 nm, Emission at 670 / 660 nm
Passive reference dye: no ROX needed

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

11 Interpretation of PCR-data and BLAST-analyses

For analysis of PCR results gained with PanReal Kit Fungi & Bacteria, select fluorescence display options 530 nm (FAM channel) for the fungal ITS2 target, 554 nm (VIC channel) for the bacterial 16S rDNA target and 667 nm (Cy5 channel) for the IPC. Please, check amplification curves and adjust the threshold manually, if necessary. Samples should be inspected both in logarithmic and linear scale view and must be compared with the negative control of extraction.

Cy5 channel, detection of the IPC:
IPC Cq-values of positive control, negative control and samples should show comparable results. A shift of Cq-values can indicate a partial inhibition of PCR. No signal in Cy5 channel indicates inhibition of PCR.

FAM channel and VIC channel - detection of fungal and bacterial DNA, respectively:

Panfungal and panbacterial PCR is prone to contamination with fungal or bacterial DNA. Therefore, Cq values must be interpreted in context with Cq values of the negative extraction control. Samples with Cq values lower than the Cq values of the negative extraction control (difference at least 1.5 cycles!) can be interpreted potentially positive and should be sequenced for genus or species identification.

FAM channel (fungi): For samples with Cq values lower than the Cq values of the negative extraction control (difference at least 1.5 cycles!): sequence the PCR-amplicon.
Perform two different sequencing reactions with the purified PCR products (according to manufacturer's instructions of sequencing kit, not provided). Use 0.5 µl (10 pmol/µl) Sequencing Primer F Fungi or Sequencing Primer R Fungi (provided in the PanReal Kit).

VIC channel (bacteria): For samples with Cq values lower than the Cq values of the negative extraction control (difference at least 1.5 cycles!): sequence the PCR-amplicon.
But: contamination in the negative control is mainly detected at Cq ~31-32. Cq-values > 31.0 are either generated by pathogenic DNA in a concentration less than 5,000 copies, or by bacterial DNA contamination. 16S rDNA PCR-products with Cq-values > 31.0 often cannot be sequenced due to mixed sequences resulting in overlapping sequences.
Perform two different sequencing reactions with the purified PCR products (according to manufacturer's instructions of sequencing kit, not provided). Use 0.5 µl (10 pmol/µl) Sequencing Primer F Bacteria **or** Sequencing Primer R Bacteria (provided in the PanReal Kit). Samples that contain bacterial DNA in a concentration less than 5,000 copies (DNA of pathogen or contamination) show Cq-values in VIC channel similar to the negative extraction control. 16S rDNA PCR-products of such samples often cannot be sequenced due to overlapping sequences.

Before sequencing, the PCR-product must be purified. Important: to avoid contamination of facilities used for DNA-extraction and preparation of the master mix with the PCR-product, always use disposable gloves and work in a facility spatially separated from other working steps. Use a separate desktop centrifuge.

For sequencing, proceed according to manufacturer's instructions of the sequencing kit and sequencing instrument.

For a proper interpretation of data, the PCR-product has to be sequenced and the organism 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 other 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 Ct values.

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

12 Troubleshooting

12.1 No pathogen specific signal with positive control and with IPC

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

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

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

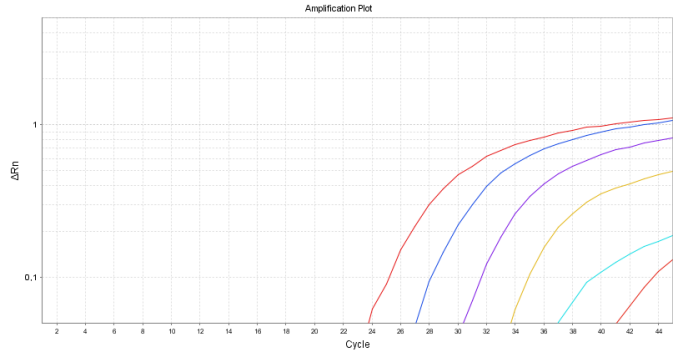
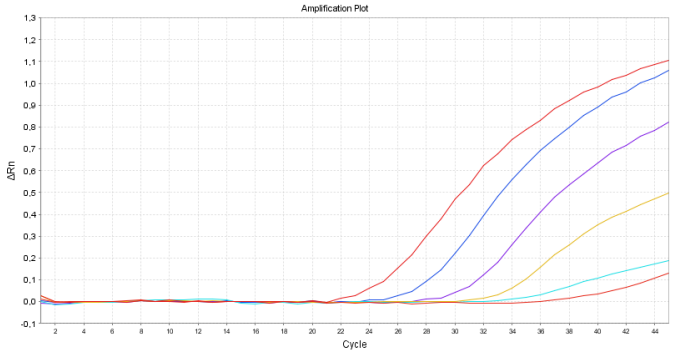
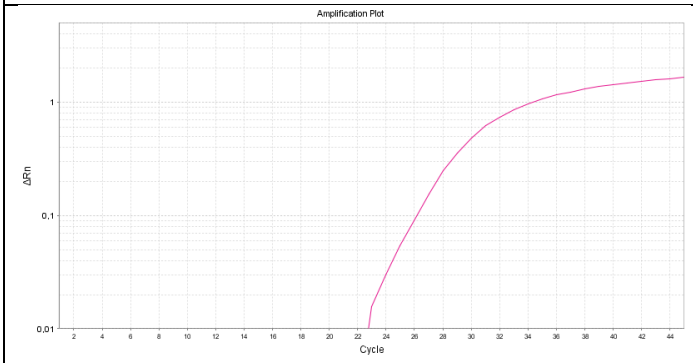
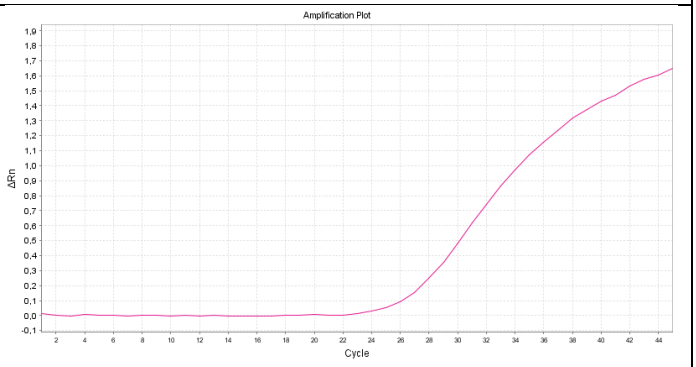
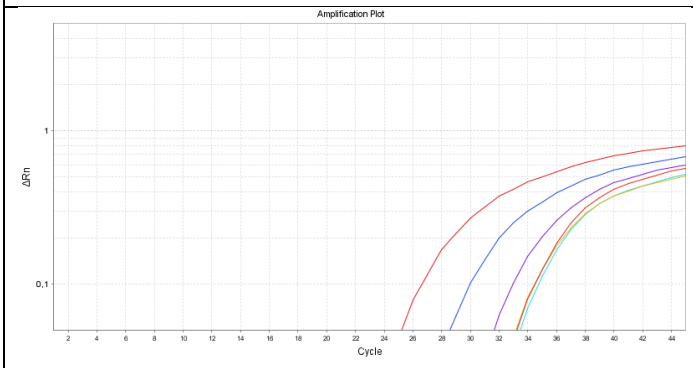
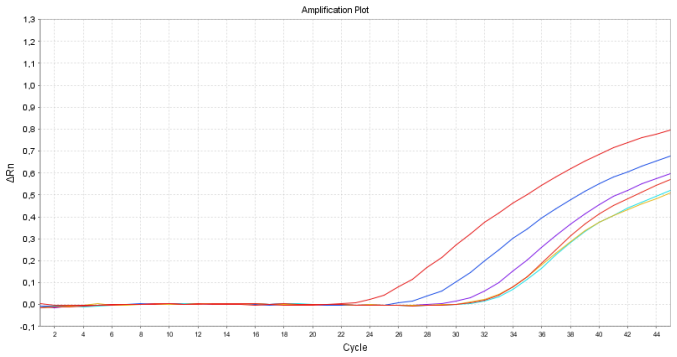
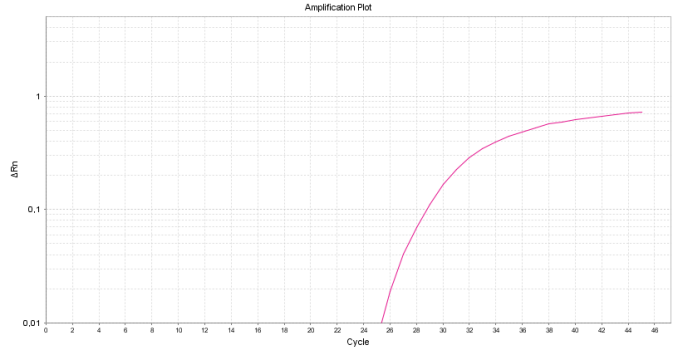
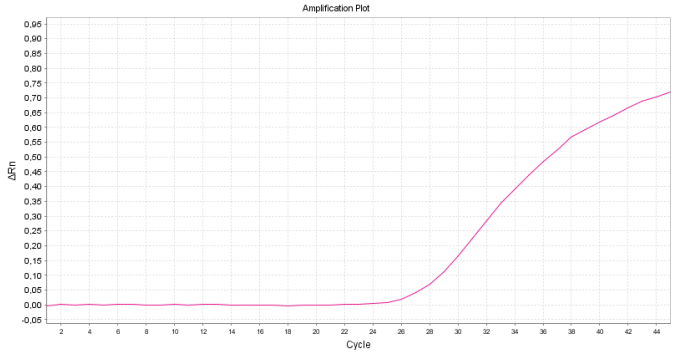
  → No statement is possible. Verify you use a recommended method for DNA isolation and re-examine the single steps of the DNA extraction.

→ If no operating mistakes during DNA extractions can be retraced, it is recommended to repeat the PCR with lower amounts of DNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of nuclease-free water).

13 Specifications and performance evaluation

13.1 Kit performance

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

Logarithmic view	Linear view
	
FAM channel: 1:10 serial dilution of a fungal DNA target (E+6 to E+1)	
	
FAM channel: Fungal positive control	
	
VIC channel: 1:10 serial dilution of a bacterial DNA target (E+6 to E+1). Contamination in NTC is detected at Cq ~31-32	
	
VIC channel: Bacterial positive control	

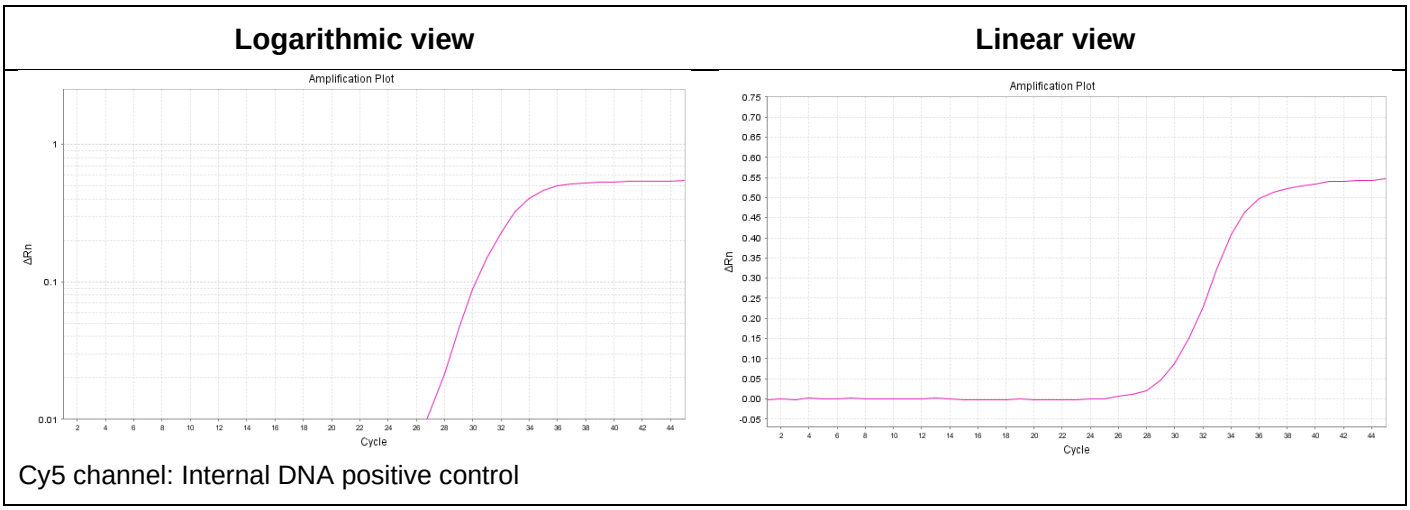


Figure 1 Performance of PanReal Kit Fungi & Bacteria

13.2 Limit of detection and linearity

PanReal Kit Fungi & Bacteria was tested with a 10-fold dilution series of plasmids containing a fragment of fungal ITS 2 and bacterial 16S rDNA.

13.2.1 Fungal ITS region

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

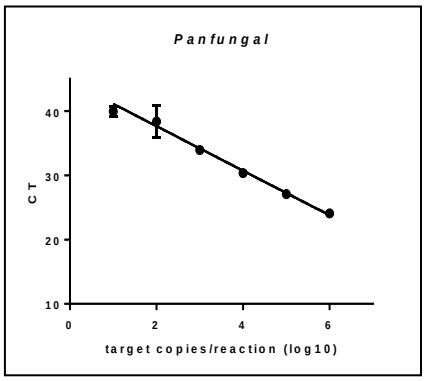


Figure 2 Ten-fold dilution series of a *Candida parapsilosis* DNA plotted against Cq

11.2.1. Bacterial 16S rDNA gene

Due to contamination, the relationship of Cq-values plotted against template DNA becomes nonlinear at DNA levels below 10,000 target copies/reaction.
Cq-values > 31.0 are either generated by pathogenic DNA in a concentration less than 5,000 copies, or by bacterial DNA contamination. 16S rDNA PCR-products with Cq-values > 31.0 often cannot be sequenced due to mixed sequences resulting in overlapping sequences.
Since no cut-off Cq-value can be determined to distinguish between infection and baseline level of contaminating DNA, the limit of detection (LoD95% = smallest number of copies of target DNA which can be detected and sequenced in 95% of cases) is approximately 5,000 target copies/reaction.

13.3 Analytical specificity

The selection of universal primers and probes ensures the broad-range detection of fungi and bacteria. Primers and probes were validated *in silico* by carrying out the basic local alignment search tool (BLAST) against the NCBI database. Extensive sequence comparison analysis were done. This validates the detection of so far known fungi and bacteria species and ensures the panfungal and panbacterial character of this test. The amplification of members of the *Bacteroidetes*, *Mollicutes*, *Rickettsiella* sp., and *Borrelia* spp. might be slightly less efficient due to mismatches in primers or probe.

The kit was tested on a great variety of different *Dematiaceae*, dermatophytes, moulds and yeasts as well as bacteria (Table 2, Table 3).

Table 2 Tested of fungal strains

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

Organism	Number of tested strains	Number of positive strains
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 3 Tested bacterial strains

Sample Name	Cq VIC channel Target Bacteria	Cq FAM channel Target Fungi
Aerococcus viridans	32.6	Negative
Bartonella henselae	32.4	Negative
Bordetella pertussis	25.2	Negative
Borrelia garinii	32.5	Negative
Campylobacter jejunii	15.3	Negative
Clostridium difficile	31.4	Negative
Enterococcus faecalis	32.3	Negative
Enterococcus faecalis	32.4	Negative
Enterococcus faecalis	32.2	Negative
Enterococcus faecalis	32.3	Negative
Enterococcus faecium	32	Negative
Enterococcus faecium	32.5	Negative
Enterococcus faecium	32.4	Negative
Enterococcus faecium	32.3	Negative
Escherichia coli O157	18.1	Negative
Haemophilus influenzae	26.1	Negative
Legionella pneumophila	31.2	Negative
Leptospira canicola	24.7	Negative
Listeria monocytogenes	31.2	Negative
Mycoplasma pneumoniae	30.9	Negative
Neisseria gonorrhoeae	21.6	Negative
Neisseria meningitidis	28.6	Negative
Rickettsia conorii	21.7	Negative
Salmonella enteritidis	17.7	Negative
Salmonella typhimurium	18.3	Negative
Staphylococcus aureus	19.2	Negative
Streptococcus agalactiae	32.3	Negative
Streptococcus agalactiae	32.3	Negative
Streptococcus agalactiae	26.8	Negative
Streptococcus bovis	32.6	Negative
Streptococcus dysgalactiae	32.2	Negative
Streptococcus pneumoniae	27.4	Negative
Streptococcus pyogenes	31.1	Negative
Streptococcus suis	32.6	Negative
Streptococcus uberis	32.2	Negative
Positive control	25.1	24.2

14 References

1. Schabereiter-Gurtner, C., M. Nehr, P. Apfalter, A. Makristathis, M. L. Rotter, and A. M. Hirschl. 2008. Evaluation of a protocol for molecular broad-range diagnosis of culture-negative bacterial infections in clinical routine diagnosis. J. Appl. Microb. 104:1228-37.
2. 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.

15 Revision history

Revision	Date	Description
1.3	02.06.2023	**Change of the company address** Update
1.4	14.06.2023	**5. Contents, stability, and storage** Kit version 1.1: The DNA IPC Target is not stored in RNA/DNA Stabilizer anymore and was renamed to IPC-Target (DNA).