PanReal Kit Fungi & Bacteria

Manual

For Research Use Only

REF **DHUFB0153**

Σ **50 reactions**

ingenetix GmbH
Arsenalstr. 11
1030 Vienna, Austria
T +43(0)1 36 198 0 198
F +43(0)1 36 198 0 199
office@ingenetix.com
www.ingenetix.com

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Annex – symbols

LOT	Batch code		Use by
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Store at
	Corrosion, GHS05		Exclamation mark, GHS07

1. Intended use

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 has to be sequenced and analysed by BLAST.

2. Product description

PanReal Kit Fungi & Bacteria contains primers and probes for the amplification and detection of the internal transcribed spacer 2 (ITS2-region) of fungi as well as the 16S rDNA gene of bacteria, further a positive control, the amplification mix and an internal positive control. This test has been developed for use with the ABI® 7500 instrument (Thermo Fisher Scientific) and LightCycler® 480 II (Roche), but is also compatible with most real-time PCR instruments that detect and differentiate fluorescence in FAM, VIC and Cy5 channel, except capillary instruments.

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/HEX channel 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 (667 nm) and is used as DNA extraction as well as real-time PCR inhibition control. The target for the DNA IPC is extracted with the sample.

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, in order to circumvent false-positive interpretation of results (see 10. Interpretation of PCR and sequence data, and 12. 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.

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.

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. Contents of the kit, stability and storage

Component	Content	Quantity	Storage
Fungi & Bacteria Assay Mix (green cap)	Primers and probes for ITS2 (FAM), 16S rDNA (VIC) and IPC (Cy5) detection	1 x 50 µl	-15 °C to -25 °C
DNA IPC Target (orange cap)	Target for DNA IPC (internal DNA positive control system)	1 x 100 µl	-15 °C to -25 °C
Sequencing Primer F Fungi	Forward primer for sequencing of the ITS2 region of fungi	1 x 100 µl	-15 °C to -25 °C
Sequencing Primer R Fungi	Reverse primer for sequencing of the ITS2 region of fungi	1 x 100 µl	-15 °C to -25 °C
Sequencing Primer F Bacteria	Forward primer for sequencing of the 16S rDNA gene of bacteria	1 x 100 µl	-15 °C to -25 °C
Sequencing Primer R Bacteria	Reverse primer for sequencing of the 16S rDNA gene of bacteria	1 x 100 µl	-15 °C to -25 °C
Fungi & Bacteria Positive Control (red cap)	DNA positive control for *Rhizopus* and *Haemophilus parasuis* (approx. 2,000 target copies/µl)	1 x 200 µl	-15 °C to -25 °C
DNA Reaction Mix, no UNG* (white cap)	DNA reaction mix without UNG (Uracil-DNA glycosylase)	1 x 500 µl	-15 °C to -25 °C until first use, then at +4 °C
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	-15 °C to -25 °C

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

The components of PanReal Kit Fungi & Bacteria are stable until the expiry date stated on the label. Repeated freeze/thaw cycles should be avoided. Protect kit components from light.

5. Additionally required materials and devices

- Reagents and devices for DNA-extraction (see 14. Appendix - protocol for DNA-extraction)
- Reagents and devices for DNA-sequencing & phylogenetic BLAST-analyses
- Aerosol barrier pipette tips
- Appropriate optical 96-well reaction plates or reaction tubes with optical closing material recommended by the manufacturer of the real-time PCR instrument
- Real-time PCR instrument which is able to detect and differentiate fluorescence in the FAM, VIC and Cy5 channel. Not for use with LightCycler 1.0 or 2.0 (Roche).

Examples of real-time PCR instruments with required FAM (510 nm), VIC (554 nm) and Cy5 (667 nm) channels:

e.g. ABI® 7500, QuantStudio™ 5 or 6 with the correct color calibration (Thermo Fisher Scientific), Mx3005P® (Agilent), LightCycler® 480 II or cobas z 480 (Roche), CFX96 (BioRad)

6. Precautions and safety information

- Clean benches and devices periodically.
- Ensure the periodical cleaning and maintenance of real-time 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.
- Do not interchange or mix reagents from kits with different lot numbers.
- Repeated thawing and freezing of kit components should be avoided. Protect kit components from light.
- **Caution:** DNA IPC Target is stored in stabilizer which contains 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.
- 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.

8. Preparation of samples and real-time PCR

- Extract samples with a DNA extraction system 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).
- An extraction negative control must be included during DNA-extraction (e.g. extraction of water instead of sample material).
- For analysis of blood at least one ml blood should be extracted. This means that the extracted DNA examined should correspond to 50 µl of blood (e.g. 500 µl of blood eluted in 100 µl). Only suitable for fungi.
- Include one positive control and one extraction negative control per PCR run.
- 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. Internal DNA Positive Control (IPC)

The **DNA IPC Target** has to be added during extraction. The DNA IPC is used as a control of DNA extraction, identifies possible PCR inhibition and confirms the integrity of kit reagents.

→ Spike 1 µl of undiluted DNA IPC Target into the sample material after the lysis buffer was added, then continue the extraction procedure. **Caution:** The DNA IPC Target must not be added directly to the sample material.

8.2. Positive Control

The Positive Control consists of a DNA fragment of *Rhizopus oryzae* and *Haemophilus parasuis*.
Use 9 µl of Fungi & Bacteria Positive Control. Pipette positive control at last.

8.3. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well by pipetting)	DNA Reaction Mix, no UNG	10.0 µl
	Fungi & Bacteria 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:** Freshly dilute the DNA IPC Target 1:100 with nuclease-free water and add 1 µl per sample directly to the master mix.

Caution: The use of more than 1 µl diluted (1:100) DNA IPC Target per reaction causes inhibition of the real-time PCR reaction.

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

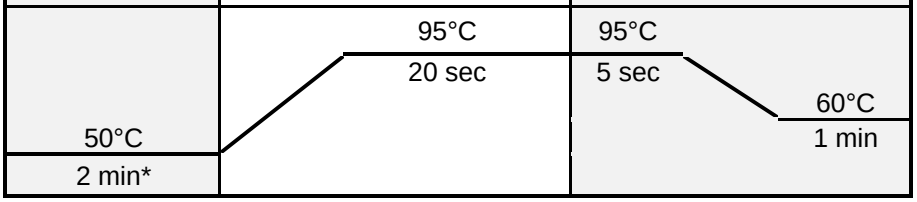
8.4. Programming of temperature profile

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

Temperature Profile:

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



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

For LightCycler® 480 II instrument
Detection format: 3 Color Hydrolysis Probe
(dyes see above)

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

Detection channels:

FAM-NONE, 530 nm for detection of fungal ITS2
VIC-NONE for detection of bacterial 16S rDNA
Cy5-NONE, 667 nm for detection of internal positive control (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 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 DNA IPC target. 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 Ct-values of positive control, negative control and samples should show comparable results. A shift of Ct-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:
NOTE: Ct values have to be interpreted in context with Ct values of the negative extraction control and results have always to be checked for plausibility in context with clinical data and BLAST results.
Panfungal and panbacterial PCR is prone to contamination with fungal or bacterial DNA. Contamination might occur during sample taking, DNA extraction, preparation of the PCR-reaction and is caused by low-level contamination of the kit reagents. Therefore, results have always to be interpreted with respect to the amount of contamination in the negative extraction control: **Samples with Ct values lower than the Ct values of the negative extraction control (difference at least 1.5 cycles!) can be interpreted potentially positive and should be sequenced.**
The positive control contains approx. 10,000 target copies/PCR.

Before sequencing, the PCR-product has to 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 samples with Ct values lower than the Ct values of the negative extraction control (difference at least 1.5 cycles!) in FAM channel: 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).

For samples with Ct values lower than the Ct values of the negative extraction control (difference at least 1.5 cycles!) in VIC channel: 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 Ct-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.

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 have to 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 Ct values.

In case of invalid data, the analysis has to be repeated with the remaining or newly extracted DNA sample (see 10. Troubleshooting).

10. Troubleshooting

10.1. No signal in FAM, VIC and Cy5 channel with controls and sample

Incorrect programming of the temperature profile or detection channels of the real-time PCR instrument.
→ Compare temperature profile and programming of detection channels with the protocol.

Incorrect configuration of PCR reaction.
→ Check your work steps (see pipetting scheme) and repeat PCR, if necessary.

10.2. Valid results for controls, but no signal in FAM, VIC and Cy5 channel with sample

Incorrect programming of detection channels with the sample.
→ Compare programming of detection channels with protocol.

If the DNA IPC Target was added during extraction:

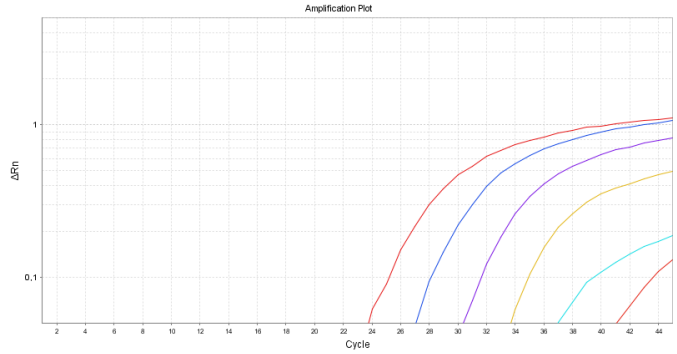
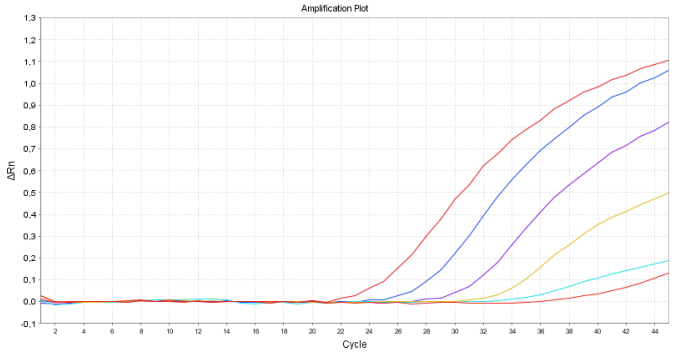
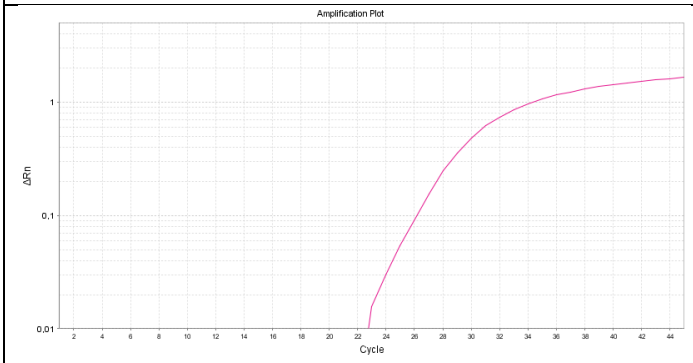
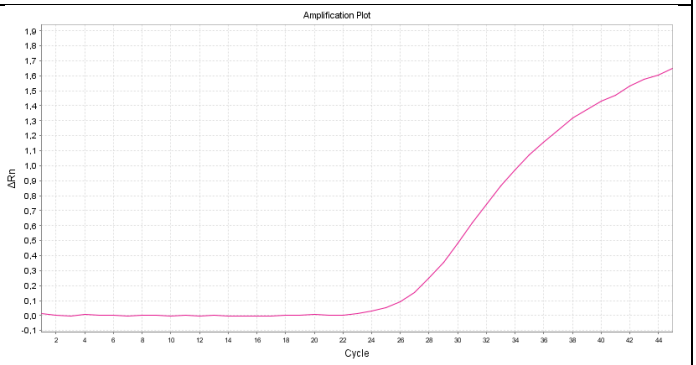
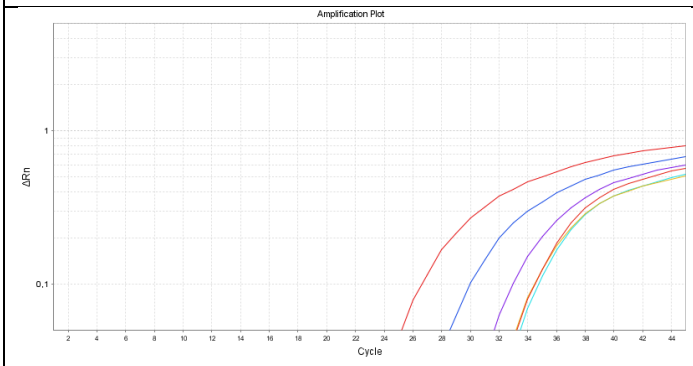
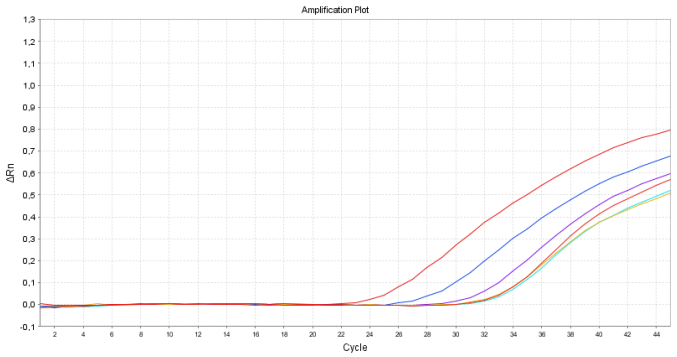
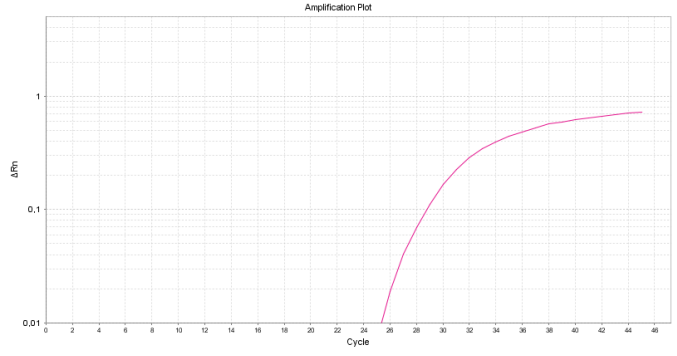
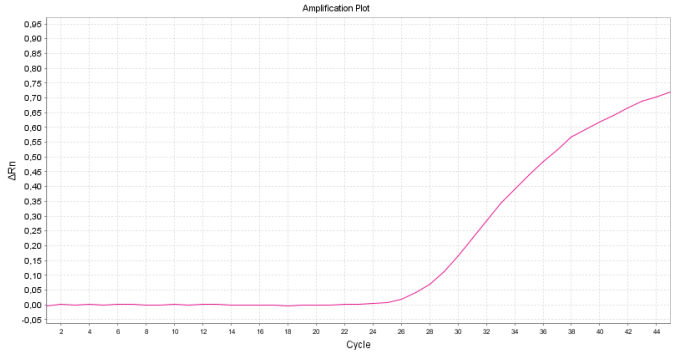
- PCR reaction has been inhibited.
- DNA extraction has failed.
- The undiluted DNA IPC Target has not been added to lysis buffer of sample.
- The extracted sample has not been added to PCR reaction.

→ No interpretation can be made. Make sure you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions. Check your work steps.

11. Specifications and performance evaluation

11.1. Kit performance

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

Logarithmic view	Linear view
	
FAM channel (530 nm): 1:10 serial dilution of a fungal DNA target (E+6 to E+1)	
	
FAM channel (530 nm): Fungal positive control	
	
VIC channel (554 nm): 1:10 serial dilution of a bacterial DNA target (E+6 to E+1). Contamination in NTC is detected at Ct~31-32	
	
VIC channel (554 nm): Bacterial positive control	

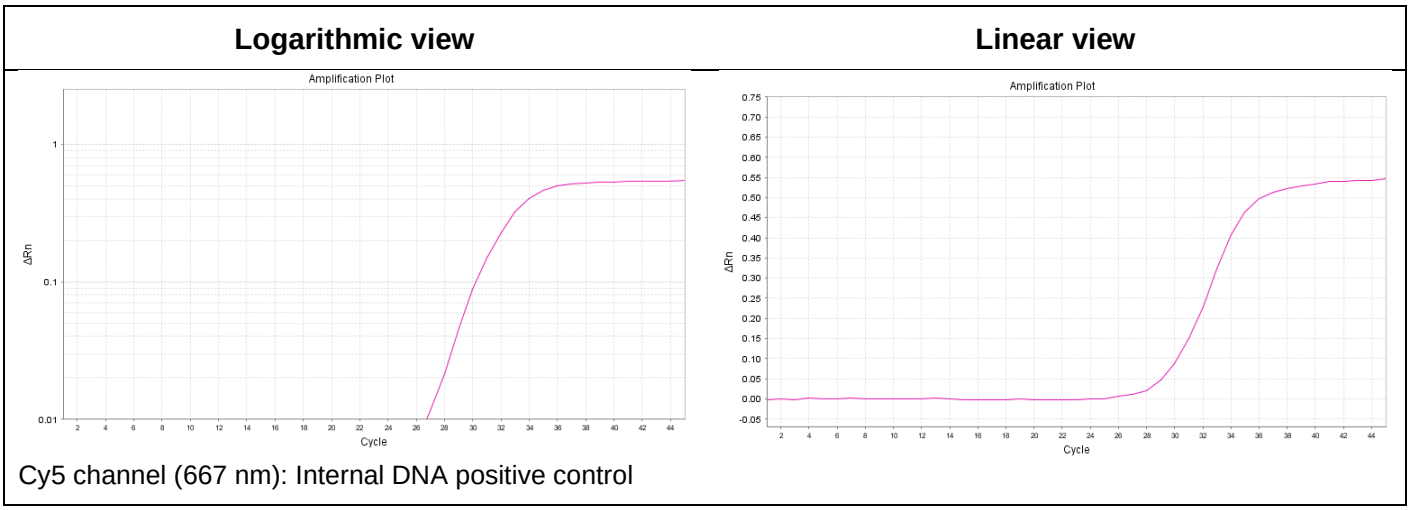


Figure 1 Performance of PanReal Kit Fungi & Bacteria

11.2. 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.

11.2.1. 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 a R^2 of > 0.9517 as shown in Figure 2.

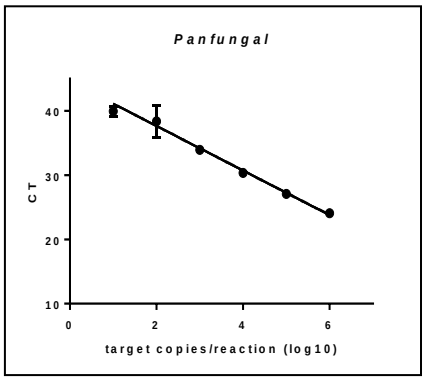


Figure 2 Ten-fold dilution series of a *Candida parapsilosis* DNA plotted against Ct

11.2.1. Bacterial 16S rDNA gene

Due to contamination, the relationship of Cp-values plotted against template DNA becomes nonlinear at DNA levels below 10,000 target copies/reaction.
Ct-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 Ct-values >31.0 often cannot be sequenced due to mixed sequences resulting in overlapping sequences.
Since no cut-off Ct-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.

11.3. 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 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 and bacteria (Tables 2, 3).

Table 2: Test of fungal strains with PanReal Kit Fungi & Bacteria

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: Test of bacterial strains with PanReal Kit Fungi & Bacteria

Sample Name	Ct VIC channel PanReal Kit Fungi & Bacteria Target Bacteria	Ct FAM channel PanReal Kit Fungi & Bacteria 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

Sample Name	Cт VIC channel PanReal Kit Fungi & Bacteria Target Bacteria	Cт FAM channel PanReal Kit Fungi & Bacteria Target Fungi
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

12. 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.

13. Appendix – protocol for DNA-extraction

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

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

13.1 Modified protocol of the High Pure PCR Template Preparation Kit

See also instructions of the manufacturer.

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

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

- 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.