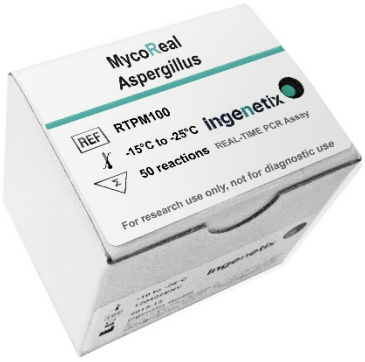
MycoReal *Aspergillus*

Manual

For use with the

- **LightCycler® 2.0 instrument**



For research use only

REF RTPM100

50 reactions

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1. Product description

MycoReal *Aspergillus* is based on the amplification and detection of the internal transcribed spacer 2 (ITS2-region) of *Aspergillus* using real-time PCR. It allows the rapid and sensitive detection as well as differentiation of 5 medically important species of *Aspergillus* (*A. fumigatus, A. flavus, A. nidulans, A. niger* and *A. terreus*). This test does not show cross reactivity with other moulds, excepted some strains of other *Aspergillus, Emericella* and *Neosartorya* species (see 10. Specification, Table 2).
This test was developed and validated for the LightCycler® 2.0 instrument (Roche) and is validated for DNA samples purified from bronchoalveolare lavages (BAL), blood, aspirates, cerebrospinal fluid, tissue and paraffin embedded tissue. *Aspergillus* DNA can be recovered efficiently from sample material using a modified protocol of the High Pure PCR Template Preparation Kit (Roche Diagnostics) (see 11. Appendix - Protocol for DNA-extraction).

MycoReal *Aspergillus* contains primers and species-specific probes for the detection and differentiation of 5 *Aspergillus* species. For exclusion of false-negative interpretation of results caused by PCR inhibition, an internal positive control assay containing a probe detected at 610 nm and an internal control target is included. Furthermore, it contains positive control samples for *A. fumigatus* and *A. niger,* a negative control sample and a manual. The amplification mix is not included. A probe-specific amplification curve at 640 nm or 705 nm indicates the amplification of *Aspergillus*. Species can then be differentiated by specific melting curves (see 8. Interpretation of LightCycler® 2.0 PCR data).

Important information:
Results have to be interpreted in context with the overall picture and other parameters.
Paraffin embedded tissue might lead to positive results due to contamination of the native sample material with *Aspergillus* DNA. Here, results have to be interpreted in context with histological data.

Not only the detection, but also the identification of the *Aspergillus* species is important. The species might provide information on a potential contamination, colonisation or infection and might inform about antifungal resistance.

Ingenetix MycoReal assays (MycoReal *Aspergillus*, MycoReal *Candida*, MycoReal *Pneumocystis* and MycoReal Fungi) are optimized to run under the same thermal cycling conditions and with the same amplification mix using the LightCycler® 2.0 instrument, which allows for a comprehensive detection of fungal infections within only a few hours.

2. Pathogen information

Aspergillus is a fungal genus consisting of several hundred species. *Aspergillus* species are ubiquitously found and some species can cause opportunistic infections such as allergic bronchopulmonary aspergillosis, pulmonary aspergilloma and invasive aspergillosis. Invasive aspergillosis is increasingly recognized as a primary cause of morbidity and mortality especially in immunocompromised patients. *Aspergillus fumigatus* is the predominant *Aspergillus* species causing disease. However, other species than *A. fumigatus*, in particular *A. terreus* and *A. flavus* but also *A. niger, A. nidulans* and *A. ustus* have gained greater clinical significance.
Not only the detection, but also the identification of the fungal species is important. The species might provide information on a potential contamination, colonisation or infection and might inform about antifungal resistance.

References:
Schabereiter-Gurtner, C., B. Selitsch, M. Rotter, A. M. Hirschl, and B. Willinger. 2007. Development of novel real-time PCR assays for detection and differentiation of eleven medically important *Aspergillus* and *Candida* species in clinical specimens. J. Clin. Microbiol. 45:906-914.

3. Principle of real-time PCR

A specific DNA sequence of the pathogen genome is amplified and the generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye.
Performing MycoReal *Aspergillus,* PCR amplification is followed by melting curve analysis on the LightCycler®. The species-specific probes allow a sequence-specific detection of PCR amplificates by generation of amplification curves and the differentiation of the species by melting curves.

4. General precautions

Fungi can be found ubiquitously. Therefore, there is high risk of contamination with *Aspergillus* DNA resulting in false-positive results.

The user should always pay attention to the following:

- Always include a negative control per PCR-run (water instead of sample).
- Optional: for valid interpretation of results, a negative control should be included during DNA-extraction (for example extraction of water instead of clinical material), in order to exclude false-positive results due to contamination with *Aspergillus* DNA during extraction.
- Be careful when handling the positive control.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated workspace.
- Periodically decontaminate benches and devices.
- Use sterile pipette tips with filters.
- Thaw all components thoroughly at room temperature before starting an assay. When thawed, mix the components and centrifuge briefly.

5. Contents

Assays

Labelling	Content	Amount
MR Aspergillus Assay Mix (green cap)	Primer and probes for detection of *Aspergillus*-DNA	2 x 50 µl
MR Aspergillus IPC Assay Mix (yellow cap)	Internal positive control assay	2 x 50 µl
H_2O (blue cap)	Water as negative control	1 x 1000 µl

Positive controls

Labelling	Content	Amount
Aspergillus fumigatus Positive Control (red cap)	Control-DNA	1 x 25 µl
Aspergillus niger Positive Control (red cap)	Control-DNA	1 x 25 µl

The components of MycoReal *Aspergillus* should be stored at -20°C and are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction (see 11. Appendix - protocol for DNA-extraction)
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- 1,5 ml reaction tubes
- Desktop centrifuge with rotor for 2 ml reaction tubes
- LC™-FastStart DNA Master Kit Hybridisation Probes (Roche Diagnostics order no. 12239272001: Kit for 480 reactions of 20 µl final reaction volume, *or* order no. 03003248001: Kit for 96 reactions of 20 µl final reaction volume)
- LightCycler® Capillaries (20 µl) (Roche Diagnostics order no. 04929292001: 1 pack containing 5 boxes, each with 96 capillaries and stoppers)
- LightCycler® Cooling Block
- LightCycler® Capping Tool
- LightCycler® 1.2/1.5 or 2.0 Instrument
- LightCycler® Carousel Centrifuge
- LightCycler® Multicolor Demo Set (Roche Diagnostics order no. 03 624 854 001: 1 set 5 color compensation runs and 20 multicolor reactions)

7. Preparation of real-time PCR

Please make sure that at least one negative control (water, blue cap), as well as one positive control (red cap) and one extraction negative control (optional, recommended) are included per PCR run.
Ingenetix highly recommends performing PCR analyses in duplicates, which increases the probability of detection of the pathogen and facilitates interpretation of results.

Please use the following pipetting scheme:

		Per sample
Preparation of master mix (mix well)	H_2O	6.6 µl
	$MgCl_2$ stock solution [25 mM]	2.4 µl
	LC-HYBR*	2.0 µl
	MR Aspergillus Assay Mix	2.0 µl
	MR Aspergillus IPC Assay Mix	2.0 µl
	Total volume	**15.0 µl**
Preparation of PCR assay	Master Mix	15.0 µl
	Sample**	5.0 µl
	Total volume	**20.0 µl**

*LC-HYBR (LC™-FastStart DNA Master Kit Hybridisation Probes, order no. 12239272001 *or* order no. 03003248001, Roche Diagnostics). See instructions of the manufacturer.
**1-5 µl of the sample can be used. Ingenetix recommends the use of 5 µl. When using an amount < 5 µl of the sample, the amount of H_2O has to be changed accordingly.

Positive Control: As positive control please use 1 µl of the *Aspergillus fumigatus* + 1 µl of the *Aspergillus niger* Positive Control + the appropriate amount of H_2O.

Programming of the temperature profile in the LightCycler® 2.0 instrument:
Please find further information on programming the LightCycler® instrument in the respective LightCycler® operator's manual.
For programming the correct temperature profile for MycoReal *Aspergillus,* open the LightCycler® 2.0 software, activate the option *NEW* in the menu strip and choose *LightCycler® Experiment*.
Please create a temperature profile according to the following four steps i) Initial activation of the Hot Start-Enzyme ii) Amplification of the DNA iii) Melting curve analysis iv) Cooling.

This temperature profile has to be saved. It takes 75 minutes and can be used for ingenetix MycoReal assays.

Program Name: Activation **Cycles:** 1 Cycle **Analysis Mode:** None

Target Temp.	Incubation time	Temp. Trans. Rate	Sec. Target Temp.	Step Size	Step Delay	Acquisition Mode
95	00:10:00	20.00	0	0.0	0	NONE

Program Name: Amplification **Cycles:** 50 Cycles **Analysis Mode:** Quantification

Target Temp.	Incubation time	Temp. Trans. Rate	Sec. Target Temp.	Step Size	Step Delay	Acquisition Mode
95	00:00:10	20.00	0	0.0	0	NONE
55	00:00:10	20.00	0	0.0	0	SINGLE
72	00:00:25	20.00	0	0.0	0	NONE

Program Name: Melt **Cycles:** 1 Cycle **Analysis Mode:** Melting Curves

Target Temp.	Incubation time	Temp. Trans. Rate	Sec. Target Temp.	Step Size	Step Delay	Acquisition Mode
95	00:00:30	20.00	0	0.0	0	NONE
48	00:01:00	20.00	0	0.0	0	NONE
90	00:00:00	0.20	0	0.0	0	CONT

Program Name: Cool **Cycles:** 1 Cycle **Analysis Mode:** None

Target Temp.	Incubation time	Temp. Trans. Rate	Sec. Target Temp.	Step Size	Step Delay	Acquisition Mode
40	00:00:10	20.00	0	0.0	0	NONE

8. Interpretation of LightCycler® 2.0 PCR data

Examples for interpretation of positive reactions are shown in Figures 1-5.

Only samples revealing a positive Cp-value and an appropriate melting curve in channel 640 nm or 705 nm can be regarded as positive.

For analyses please use the designated LightCycler® software. See also instructions in the LightCycler® 2.0 Instrument Operator's Manual.

For analysis of PCR data please proceed as follows:

- **Important:** Crosstalk between the channels is observed in the LightCycler® instrument. This crosstalk can be corrected using a color-compensation file. Therefore, make sure that the color-compensation is activated: open the color-compensation file by activating *Color Comp* (*On/Off*) followed by *Select Color Compensation.* If no color-compensation file is installed, please generate this file with the LightCycler® Multicolor Demo Set by following the instructions in the LightCycler® 2.0 Instrument Operator's Manual.
- Activate the function *Analysis* in the menu strip and select the option *Absolute Quantification*.
- After activation of the color-compensation file seperated signals at 610 nm, 640 nm and 705 nm are generated. For analysis of the PCR results gained with MycoReal *Aspergillus* please select fluorescence display options 640 nm for the detection of *A. fumigatus* and *A. terreus,* and 705 nm for the detection of *A. flavus, A. niger* and *A. nidulans* (Table 1). The internal positive control target is detected at 610 nm. Samples with Cp-values set in brackets have to be regarded as negative. Please always check the presence of amplification-curves also manually. For this, click with the left mouse button on the respective sample positions.
- In cases where samples show a positive Cp-value, the species can be identified by melting curve analysis. After analysis of the amplification curves please activate the function *Analysis* and choose the option *Tm Calling*. Select fluorescence display options 640 nm for the detection of *A. fumigatus* and *A. terreus,* and 705 nm for the detection of *A. flavus, A. niger* and *A. nidulans* (Table 1). Always adjust the Tm bar also manually.

Important: The *Color Compensation File* has to be reopened before performing melting curve analysis.

Table 1: Melting curve analysis

Species	Melting temperature (Tm)	Fluorescence channel
Aspergillus terreus	59°C or 63°C ± 1°C**	640 nm
Aspergillus fumigatus group*[1]	67°C ± 1°C	640 nm
Aspergillus flavus group*[2]	67°C ± 1°C	705 nm
Aspergillus niger group*[3]	60°C ± 1°C	705 nm
Aspergillus nidulans group*[4]	56°C ± 1°C	705 nm
Internal positive control (IPC)	57°C ± 1°C	610 nm

*Cross reactivity was checked by sequence homology (BLAST) analyses.
**Detection of *A. terreus* at 59°C or 63°C due to mismatches in the binding site of the probe depending on the strain
[1]*Aspergillus fumigatus* and some strains of *Aspergillus arvii, Aspergillus fumisynnematus, Aspergillus lentulus, Aspergillus novofumigatus, Aspergillus viridinutans, Neosartorya aureola, Neosartorya botucatensis, Neosartorya coreana, Neosartorya glabra, Neosartorya fischeri, Neosartorya laciniosa, Neosartorya spinosa* and *Neosartorya udagawae*
[2]*Aspergillus flavus* and some strains of *Aspergillus oryzae* and *Aspergillus parasiticus*
[3]*Aspergillus niger* and some strains of *Aspergillus awamori, Aspergillus brasiliensis, Aspergillus carbonarius, Aspergillus coreanus, Aspergillus foetidus, Aspergillus ibericus, Aspergillus lacticoffeatus, Aspergillus phoenicis, Aspergillus piperis, Aspergillus sclerotioniger, Aspergillus tubingensis* and *Aspergillus wentii*
[4]*Aspergillus nidulans* and some strains of *Aspergillus caespitosus, Aspergillus granulosus, Aspergillus insuetus, Aspergillus unguis, Aspergillus pseudodeflectus, Emericella acristata, Emericella appendiculata, Emericella astellata, Emericella cleistominuta, Emericella corrugata, Emericella dentata, Emericella echinulata, Emericella falconensis, Emericella fruticulosa, Emericella foveolata, Emericella indica, Emericella miyajii, Emericella omanensis, Emericella parvathecia, Emericella quadrilineata, Emericella qinqixianii, Emericella rugulosa, Emericella similis, Emericella striata, Emericella sublata, Emericella undulata, Emericella violacea, Emericella variecolor* and *Emericella venezuelensis*

For a valid interpretation, the following criteria must be fulfilled:

	Cp (610 nm) IPC target	**Cp (640 nm) *Aspergillus* target**	**Cp (705 nm) *Aspergillus* target**	**Interpretation**
Negative control	34.0-38.0	Negative	Negative	Valid
Positive control	34.0-38.0	30.0-33.0	30.0-33.0	Valid
Negative control of extraction*	34.0-39.0	Negative	Negative	Valid
Negative sample	34.0-39.0	Negative	Negative	Valid
Positive sample	>34.0 or negative	Positive or negative	Positive or negative	Valid

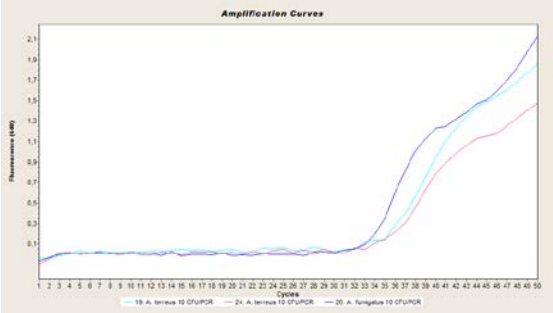
*Optional

Once analyses of PCR are completed, the following results can be observed with the samples:

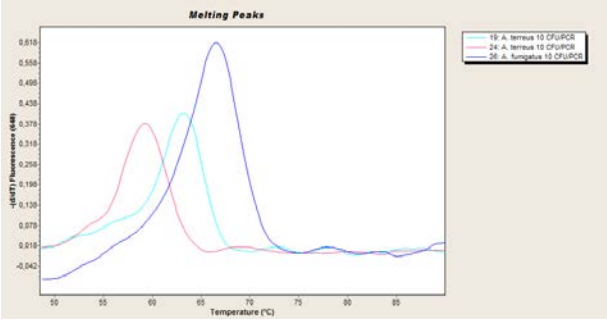
1. Amplification signal and a distinct melting curve at 640 nm and/or 705 nm:
→ *Aspergillus* DNA was amplified. The species can be identified via melting curve analysis.
Only samples revealing a positive Cp-value and a corresponding melting curve at 640 nm or 705 nm can be interpreted as positive.
In this case the detection of the IPC at 610 nm is not essential, since high concentrations of *Aspergillus* DNA can lead to a reduced or absent fluorescence signal of the internal positive control assay at 610 nm (competition of PCR).

2. No signal at 640 nm and 705 nm but signal at 610 nm (signal of the internal positive control):
→ No *Aspergillus* DNA was amplified. The sample has to be interpreted as negative
The positive signal of the internal positive control assay at 610 nm (Cp = 34.0 - 39.0) excludes a putative PCR inhibition.

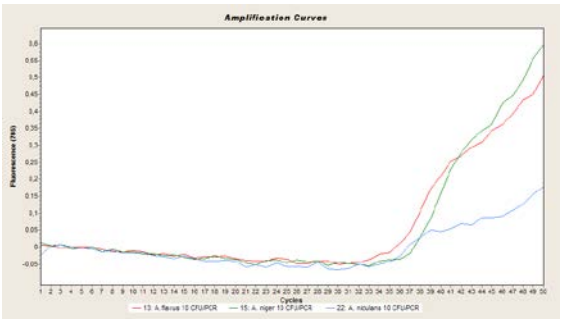
3. No signals at 610 nm, 640 nm and 705 nm:
→ No interpretation can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.



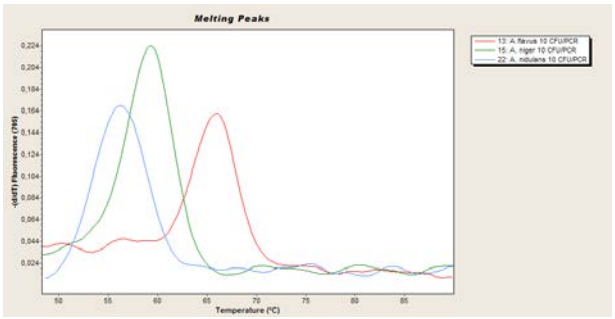
Amplification curves of 10 CFU/PCR of *A. fumigatus* and *A. terreus* (640 nm)



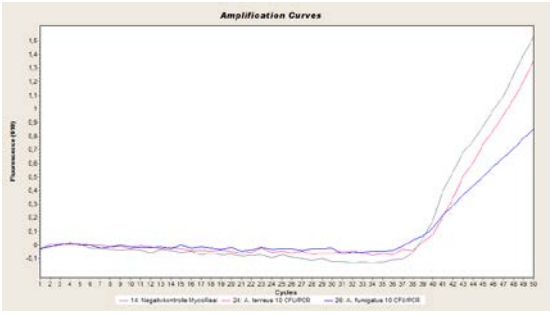
Species identification: Melting curves of 10 CFU/PCR of *A. fumigatus* (67°C) and 2 different strains of *A. terreus* (59°C or 63°C) (640 nm)



Amplification curves of 10 CFU/PCR of *A. flavus, A. niger* and *A. nidulans* (705 nm)



Species identification: Melting curves of 10 CFU/PCR of *A. flavus* (67°C), *A. niger* (60°C) and *A. nidulans* (56°C) (705 nm)



Amplification curves of the internal positive control (610 nm)

9. Troubleshooting

1. No signal with the positive controls at 640 nm and 705 nm:

- Incorrect programming of the temperature profile of the LightCycler® 2.0 instrument.
  → Compare the temperature profile with the protocol (see 7. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps by means of the pipetting scheme (see 7. Preparation of real-time PCR) and repeat the PCR, if necessary.

2. Weak (Cp-value >39.0) or no signal with the internal positive control at 610 nm and no signal with the sample at 640 nm or 705 nm:

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

3. Signal with the negative control at 640 nm or 705 nm:

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

4. Signal with the negative control of DNA-extraction (optional) at 640 nm or 705 nm:

A contamination occurred during extraction.
→ Repeat the extraction and PCR of the sample to be tested using new reagents.
→ Make sure that work space and instruments are decontaminated at regular intervals.

10. Specifications

10.1. Limit of detection (LoD)

The LoD was determined by analysis of a dilution series of DNA of an *A. fumigatus* isolate on 10 different days (in duplicate). Carrier DNA was the extracted DNA of 3 ml negative EDTA-blood.
MycoReal *Aspergillus* has a LoD 95% (defined as the concentration, where 95% of 20 PCR repeats were positive) of 3 CFU/PCR. This means, that 3 CFU/PCR-reaction can be detected with a probability of 95% (CFU = colony forming unit).

10.2. Analytical specificity

The specificity is ensured by the selection of highly specific probes. The primers and probes were checked for possible homologies to other sequences by sequence comparison analyses. This also validated the detection of so far known strains of *A. fumigatus, A. flavus, A. nidulans, A. niger* and *A. terreus*.
BLAST analyses revealed possible cross-reactions with some strains of *Aspergillus arvii, Aspergillus awamori, Aspergillus brasiliensis, Aspergillus caespitosus, Aspergillus carbonarius, Aspergillus coreanus, Aspergillus foetidus, fumisynnematus, Aspergillus granulosus, Aspergillus ibericus, Aspergillus insuetus, Aspergillus lacticoffeatus, Aspergillus lentulus, Aspergillus novofumigatus, Aspergillus oryzae, Aspergillus parasiticus, Aspergillus phoenicis, Aspergillus piperis, Aspergillus pseudodeflectus, Aspergillus sclerotioniger, Aspergillus tubingensis, Aspergillus unguis, Aspergillus viridinutans, Aspergillus wentii, Emericella acristata, Emericella appendiculata, Emericella astellata, Emericella cleistominuta, Emericella corrugata, Emericella dentata, Emericella echinulata, Emericella falconensis, Emericella fruticulosa, Emericella foveolata, Emericella indica, Emericella miyajii, Emericella omanensis, Emericella parvathecia, Emericella quadrilineata, Emericella qinqixianii, Emericella rugulosa, Emericella similis, Emericella striata, Emericella sublata, Emericella undulata, Emericella violacea, Emericella variecolor, Emericella venezuelensis, Neosartorya aureola, Neosartorya botucatensis, Neosartorya coreana, Neosartorya glabra, Neosartorya fischeri, Neosartorya laciniosa, Neosartorya spinosa* and *Neosartorya udagawae*. See 8. Interpretation of LightCycler® 2.0 PCR data, Table 1.

The assay was tested on different *Dematiaceae*, dermatophytes, moulds and yeasts (Table 2).

10.3. Cut-off Value

Only samples revealing a positive Cp-value and a corresponding melting curve at 640 nm or 705 nm can be interpreted as positive. Samples with Cp-values set in brackets have to be interpreted as negative.

10.4. Evaluation with clinical samples

Clinical samples (bronchoalveolare lavages, EDTA-blood, cerebrospinal fluid, tissue and paraffin embedded tissue, aspirates and samples from the maxillary sinus) of patients with a suspected fungal infection were tested with MycoReal *Aspergillus*. Results were compared to culture and additionally whether to histology, KOH test or serology.
Furthermore, EDTA-blood samples of symptomatic and asymptomatic high-risk patients were screened with MycoReal *Aspergillus* and results were compared to results obtained by the Platelia Aspergillus (Bio-Rad Laboratories).

Table 2: Determination of cross reactions with MycoReal *Aspergillus*

Organism	Number of tested strains	Number of strains positive with MycoReal *Aspergillus*
Moulds		
Aspergillus flavus	3	3
Aspergillus niger	9	9
Aspergillus fumigatus	4	4
Aspergillus terreus	7	7
Aspergillus nidulans	5	5
*Aspergillus unguis**	1	1
*Aspergillus versicolor **	1	1
*Aspergillus ustus***	2	0
Aspergillus sydowii	1	0
Aspergillus ochraceus	1	0
Aspergillus niveus	1	0
Aspergillus clavatus	1	0
Aspergillus candidus	1	0
Aspergillus glaucus	1	0
Penicillium sp.	1	0
Penicillium marneffei	1	0
Penicillium olsonii	1	0
Penicillium chrysogenum	1	0
Rhizopus oryzae	1	0
Mucor circinelloides/racemosus	1	0
Rhizomucor pusillus	1	0
Absidia corymbifera	1	0
Cunninghamella elegans	1	0
Syncephalastrum sp.	1	0
Scedosporium apiospermum	1	0
Scedosporium prolificans	1	0
Fusarium oxysporum	1	0
Fusarium verticilloides	1	0
Fusarium solani	1	0
Beauveria bassiana	1	0
Natrassia mangiferae	1	0
Alternaria alternata	1	0
Alternaria tenuissima	1	0
Curvularia lunata var. *lunata*	1	0
Schizo*phyllum commune*	1	0
Acremonium strictum	1	0
Paecilomyces variotii	1	0
Dematiaceae		
Bipolaris australiensis	1	0
Bipolaris hawaiiensis	1	0
Cladosporium herbarum	1	0
Phialophora spp.	2	0
Sporothrix schenkii	1	0
Aureobasidium pullulans	1	0
Cladophialophora sp.	1	0
Dermatophytes		
Microsporum canis	1	0
Trichophyton tonsurans	1	0
Yeasts		
Candida glabrata	1	0
Candida tropicalis	1	0
Candida albicans	1	0
Candida parapsilosis	1	0
Candida krusei	1	0
Candida dubliniensis	1	0
Candida lusitaniae	1	0
Candida guillermondii	1	0
Candida kefyr	1	0
Candida valida	1	0
Debaromyces hansenii	1	0
Yarrowia lipolytica	1	0
Rhodotorula rubra	1	0
Pichia fermentans	1	0
ryptococcus neoformans	2	0
Malassezia furfur	1	0
Malassezia pachydermatis	1	0
Saccharomyces cerevisiae	1	0
Trichosporon sp.	3	0

* Positive Cp-value and Tm 55°C
** Negative Cp-value, but Tm 54°C

11. 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 assured that all reagents are free of fungal DNA. It is recommended to exclude contamination of new lots of reagents and kits by extraction of water instead of sample material before use with clinical samples.

The following DNA-extraction protocols are recommended, since MycoReal *Aspergillus* was evaluated with these:

11.1. Extraction from CSF, aspirates, BAL, 200 µl blood, tissue, paraffin-embedded tissue

DNA-extraction of fungi from CSF, aspirates, BAL, 200 µl EDTA-blood, tissue and paraffin-embedded tissue is performed with a modified protocol of the High Pure PCR Template Preparation Kit (Roche Diagnostics) including additional mechanical lysis by freeze-and-thaw. 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)
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex-Mixer
- Thermomixer
- Desktop centrifuge with rotor for 2 ml reaction tubes

11.1.1. 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.
- 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* (aliquoted à 500 µl to avoid contamination with fungal DNA).
Store DNA at -20°C.

11.1.2. 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 take paraffin without tissue as little as possible.
2. Put the tissue together with 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* (aliquoted à 500 µl to avoid contamination with fungal DNA).
Store DNA at -20°C.

11.2. Extraction from 1.5 ml EDTA-blood

Usually only small amounts of fungal DNA are circulating in blood, therefore the extraction from at least 1.5 ml blood is recommended.
Ingenetix recommends extraction with the SeptiFast Prep Kit + SeptiFast Lys Kit (Roche Diagnostics). With this kit, fungal cells are lysed mechanically. See instructions of the manufacturer.

Required materials and devices:

- SeptiFast Prep Kit, CE, M-grade (10 extractions) (Roche Diagnostics order no. 4404459001)
- SeptiFast Lys Kit, CE, M-grade (50 extractions) (Roche Diagnostics order no. 4404432001)
- 5 ml filter tips, M-Grade (7 x 54 pieces) (Greiner bio-one order no. 940525)
- Pipette for 5000-µl tips
- 1,5 ml reaction tubes (PCR-clean)
- Sterile pipette tips with filters
- Devices for DNA-extraction (e.g. MagNA Lyser instrument, thermo block, centrifuges, etc.). See instructions of the manufacturer

12. Annex – symbols

Batch code

Use by

Catalogue number

Manufactured by

Contains sufficient for <n> tests

Store at