LightMix® Kit Aspergillus fumigatus

Cat.-No. 40-0592-32

Kit with reagents for the detection of *Aspergillus fumigatus* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 / 480II Instruments.

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each. **Store protected from light at room temperature (18-25°C), do N OT freeze!**

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 4-5
Instructions for use with the LightCycler® 480 / 480II Instruments see pages 6-7

1. Introduction

Fungi are ubiquitous microorganisms best known from moulded food. Invasive fungal infections are important for morbidity and mortality of immunocompromised individual, particularly for leukemic patients, solid organ recipients and HIV-1 patients. An early diagnosis is crucial to start antifungal therapy successfully. *Aspergillus fumigatus* is one of the most common fungal species which can be found everywhere in the environment[1] and is a clinical important opportunistic pathogen.
Conventional diagnostic tests by blood culture are slow and have been reported to lack sufficient sensitivity and specificity while PCR was reported to yield better results[2] for an early diagnosis.

This LightMix® Kit contains primers and hybridization probes targeting the 18S RNA gene as published by Löffler et al. and others [2-4].

[1] John Mullins: Aspergillus and Aerobiology. The Genus Aspergillus. Plenum Press, New York 1994 351-359

[2] Quantification of Fungal DNA Using Fluorescence Resonance Energy Transfer and the LightCycler System., Löffler J, Henke N, Hebart H, Schmidt D, Hagmeyer L, Schumacher U, Einsele H. JCM 38 (2000) 586-590

[3] Polymerase chain reaction detection of aspergillus DNA in experimental models of invasive aspergillosis. Loeffler J, Kloepfer K, Hebart H, Najvar L, Graybill JR, Kirkpatrick WR, Patterson TF, Dietz K, Bialek R, Einsele H. J Infect Dis. 2002 Apr 15;185(8):1203-6.

[4] Molecular detection and identification of Candida and Aspergillus sp p. from clinical samples using real-time PCR. Klingspor L. and Jalal S. Clin Microbiol Infect 2006; 12: 745–753

The LightMix® Kit *Aspergillus fumigatus* provides a fast, easy and accurate system to identify this target in a nucleic acid extract.

This LightMix® Kit is tested on the LightCycler® 1.x / 2.0 / 480 Instruments (96 well and 384 well formats) with Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'.

2. Description

The LightMix® Kit detects a region of the fungal 18S RNA indicating the presence of fungal DNA in a nucleic acid extract. As fungi are found widespread in the environment[1] this kit might yield false positive results due to laboratory contaminations.

A 504 bp long fragment of the *Aspergillus fumigatus* genome is amplified with primers and detected with specific probes labeled with LightCycler® Red 640 (detected in channel 640).

For use in LightCycler® 1.2 and 1.5 Instruments with software version 3.5.3 read channel F2 instead of channel 640. We recommend upgrading LightCycler® 1.x instruments to software version 4.1.

3. Set contents

3 Vials with green caps containing premixed lyophilized primers and probes for 32 PCR reactions each of *Aspergillus fumigatus*
1 Vial with colorless cap containing control DNA *Aspergillus fumigatus*, 10^5 target equivalents per reaction

4. Additional reagents and items required

Roche Diagnostics:

LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 692 001

5. Product characteristics

PCR results are obtained within 60 minutes (45 cycles and melting curve) with the LightCycler® 1.x / 2.0 Instruments and within 75 minutes (50 cycles and melting curve) with the LightCycler® 480 Instruments.

Sensitivity
These reagents detect 10 copies of *Aspergillus fumigatus* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 Instruments (in an exemplary system, using cloned targets as reference).

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *Aspergillus fumigatus* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 Instruments.

Storage and Stability
- Lyophilized reagents are stable for at least 3 months after shipment when stored protected from light at room temperature (18-25°C).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days when stored protected from light and refrigerated (4°C).

6. Experimental protocol

The following procedure was developed for use with the LightCycler® 1.x / 2.0 / 480 Instruments. Start programming before preparing the solutions. See the LightCycler® Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. Roche Diagnostics 'High Pure PCR Template Preparation Kit').
Negative control: Always run at least one no-template control (NTC) - replace the template DNA with water.

Positive control: Run a positive control - replace the template DNA with the provided control DNA.

6.1. Preparation of parameter-specific reagents (32 reactions):

One reagent vial with a **green** cap contains primers and probes to run 32 LightCycler® reactions for *Aspergillus fumigatus*

Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►**Use 2 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C. Avoid prolonged exposure to light.

6.2. Preparation of the control DNA

Add 40 µl PCR-grade water to the vial (8 x 10^5 target molecules) with a colorless cap. Mix the target DNA by pipetting the solution up and down 10 times (final concentration: 10^5 target molecules in 5 µl).

►**Use 5 µl** control DNA for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C, for long term storage freeze at -20°C. Avoid repeated freezing thawing cycles. For the control DNA provided with the kit please note that the relative amounts of DNA may change during time.
Please note that opening this vial may cause contaminations of the work-space (aerosol).

6.3. Preparation of the LightCycler® reaction mix

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

For use with the Roche FastStart Master	
Single reaction	**Component**
9.4 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
1.6 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)
2.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)
2.0 µl	Roche Master (red cap, for preparation see Roche manual)
15.0 µl	Volume of reaction mix

Mix gently, spin down and **transfer 15 µl** each of the reaction mix to a LightCycler® capillary (LightCycler® 1.x / 2.0 Instrument) or to a multiwell plate (LightCycler® 480 Instruments).

Add 5 µl of sample or control DNA to each capillary or well for a final reaction volume of 20 µl.

Start run.

7. LightCycler® 1.x / 2.0 Instruments

7.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	45			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:15	00:00:25	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [°C/s]	20	20	20	20	20	20	0.2	20
Acquisition Mode	None	None	Single	None	None	None	Continuous	None

(Melting not relevant for detection)

7.2. Data Analysis

For use in LightCycler® 1.x Instruments select channel F2 instead of channel 640 and channel F1 instead of channel 530 for detection.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.
We recommend using the Second Derivative Maximum method (Automated (F" max)). The cycle number of the Crossing Point (CP) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *Aspergillus fumigatus* data in channel 640, Quantification mode (LightCycler® 2.0 Instrument). The negative control (NTC) must show no signal.

7.3. Sample Data – typical results

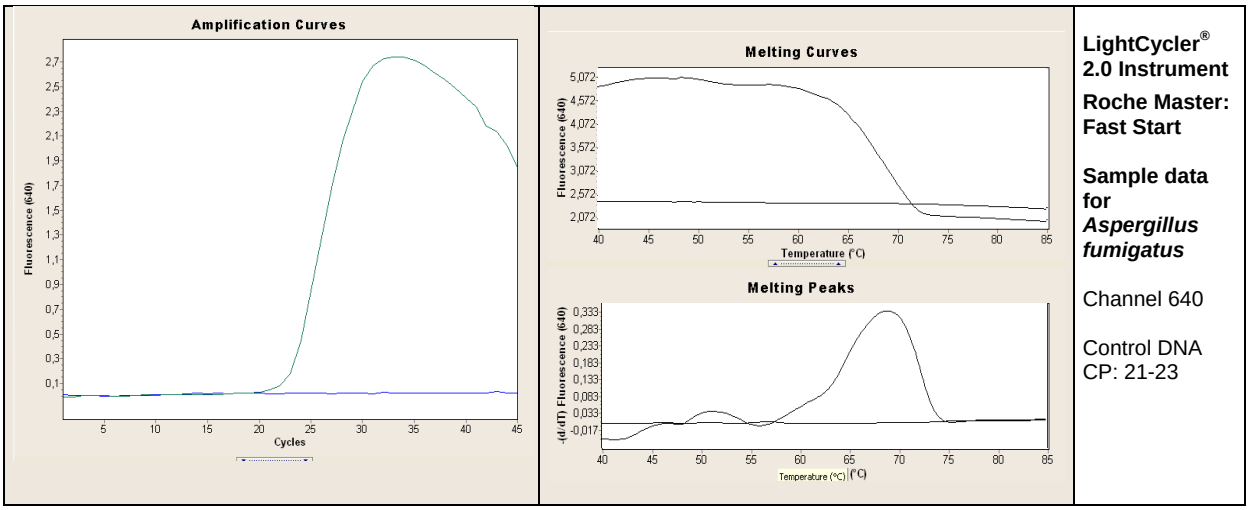

Fig.1. Sample data for the *Aspergillus fumigatus* detection system.

Upper panels: Data from LightCycler® 2.0 Instrument.
Left panel channel 640 quantification mode (Second Derivative Maximum) with amplification curves for *Aspergillus fumigatus*.
Right panel channel 640 melting analysis for *Aspergillus fumigatus* (not relevant for detection).

* **Note:** Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The CP values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta CP). The CP values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

7.4. Interpretation of data

Aspergillus fumigatus (sample)	NTC	Result
no amplification	negative	Negative
amplification signal	negative	Positive
amplification signal	positive	Contamination, repeat experiment

Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start)

***Notes**:* The values of the respective melting temperatures (T_M) may vary ±2.5°C between different experiments. Th e ΔT between the melting peaks for heterozygote genotypes may vary ±1.5°C. Samples with deviating melting curves sh ould be subject to further investigations; sequence analysis can be provided by TIB MOLBIOL Berlin (contact service@tib-molbiol.de).

Warning:

Fungi are found pervasive in the environment. Mullins[1] reported in average 100 and more spores per cubic meter of 'clean' air, possibly resulting in false positives outcomes even in a very clean diagnostic laboratory environment. Repeat the analysis for low-positive samples reporting 10 or less copies per reaction.

8. LightCycler® 480 Instrument

8.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Detection Format:
LightCycler® 480 Instrument: 483-640
LightCycler® 480 II Instrument: 498-640

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:15	00:00:25	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per °C]	-	-	-	-	-	-	**3**	-

(Melting not relevant for detection)

8.2. Data Analysis

Note: For use on LightCycler® 480 II Instruments select Filter Combination 498-640 instead of Filter Combination 483-640 for detection.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.
We recommend using the Second Derivative Maximum method (Automated (F" max)). The cycle number of the Crossing Point (CP) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *Aspergillus fumigatus* data with Filter Combination 483-640, Quantification mode. The negative control (NTC) must show no signal.

8.3. Sample Data – typical results

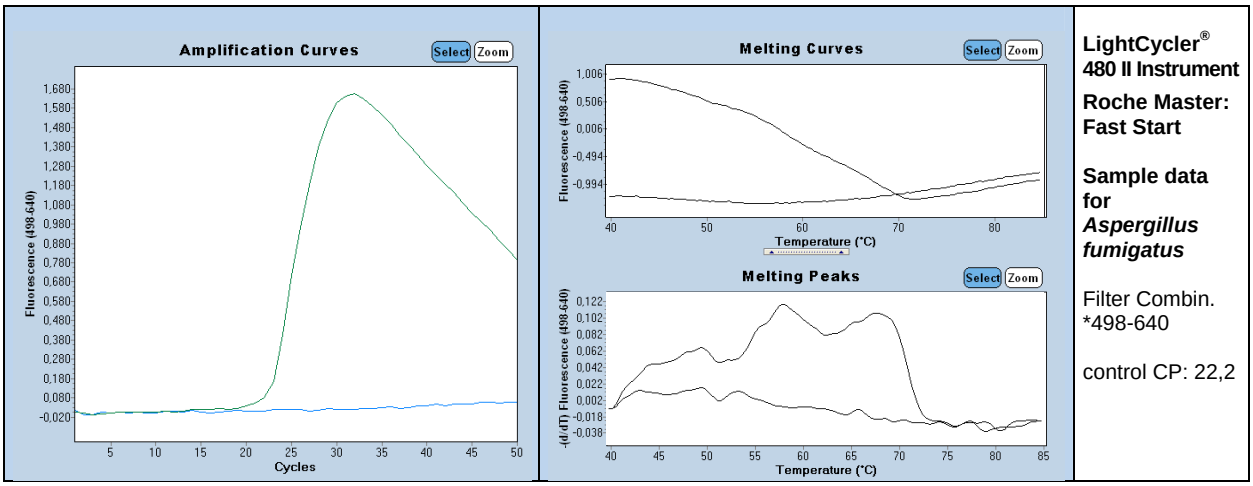

Fig.1. Sample data for the *Aspergillus fumigatus* detection system.

Upper panels: Data from LightCycler® 480 Instrument.
Left panel Filter Combination 483-640 quantification mode (Second Derivative Maximum) with amplification curves for *Aspergillus fumigatus*.
Right panel Filter Combination 483-640 melting analysis/peaks *Aspergillus fumigatus* (not relevant for detection).

* **Note:** Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The CP values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta CP). The CP values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

8.4. Interpretation of data

***Aspergillus fumigatus* (sample)**	**NTC**	**Result**
no amplification	negative	Negative
amplification signal	negative	Positive
amplification signal	positive	Contamination, repeat

Typical analysis results (LightCycler® 480 II Instrument, Roche Master: Fast Start)

***Notes**:* The values of the respective melting temperatures (T_M) may vary ±2.5°C between different experiments. Th e ΔT between the melting peaks for heterozygote genotypes may vary ±1.5°C. Samples with deviating melting curves sh ould be subject to further investigations; sequence analysis can be provided by TIB MOLBIOL Berlin (contact service@tib-molbiol.de).

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Fungi are found pervasive in the environment. Mullins[1] reported in average 100 and more spores per cubic meter of 'clean' air, possibly resulting in false positives outcomes even in a very clean diagnostic laboratory environment. Repeat the analysis for low-positive samples reporting 10 or less copies per reaction.

9. Version history

V_100804	**First version**
V_111017	**Editorial changes and minor corrections**

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