Dynamiker Biotechnology (Tianjin) Co., Ltd.

MycoMDx *Aspergillus* PCR Assay

Catalogue No.: DNK-1416-1

User Manual / 50 tests

CONTENTS

1. INTENDED USE

The MycoMDx *Aspergillus* PCR Assay is a quantitative real-time multiplex PCR test used for detection of specific nucleic acid fragments of *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger,* and *Aspergillus terreus* in serum and bronchoalveolar lavage fluid (BALF) samples, offering a diagnostic reference for Aspergillus infections.The kit is intended for professional use only.

2. INTRODUCTION

The product is used for the qualitative detection of specific nucleic acid fragments of *A. fumigatus*, *A. flavus*, *A. niger,* and *A. terreus* in human serum and BALF samples. It is mainly used for auxiliary diagnosis of deep Aspergillus infections.

Deep Aspergillus infections refers to an infectious disease in which *Aspergillus* invades the human body, grows and reproduces in tissues, organs or blood, and causes inflammation and tissue damage. *Aspergillus* is widely distributed in natural soil, plants and air, and is an important conditional pathogenic fungus. Severe immune-compromised host (hematological diseases/malignant tumors, solid organ transplantation, cancer, intensive ICU, severe influenza, neutropenia and diabetes, etc.), long-term associated pulmonary underlying diseases (pulmonary tuberculosis, Chronic Obstructive Pulmonary Disease (COPD), asthma, bronchiectasis and *Nontuberculous mycobacteria*, etc.) patients are all high-risk groups for *Aspergillus* infection. At present, the laboratory diagnosis methods of deep *Aspergillus* infection mainly include direct examination (cytology, microscopy or culture), serology and molecular biology detection methods. Among them, PCR detection can detect *Aspergillus* nucleic acid fragments in serum, BALF and tissue samples of patients, and further determine the pathogen (different *Aspergillus* species), which is of great significance for early clinical and accurate initiation of antifungal therapy[1-4]. This product uses the real-time fluorescence quantitative PCR in combination with other methods to realize the auxiliary diagnosis of deep *Aspergillus* infection.

3. PRINCIPLE

This kit uses fluorescent PCR technology. The product is designed with specific primers and probes for *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*. The 3' end of each detection probe is labeled with a quencher group, and the 5' end is labeled with a different reporter group. During PCR amplification, when the probe is intact, the fluorescence energy emitted by the reporter group is absorbed by the quencher group, and the instrument cannot detect the signal. When the primer is

extended, the fluorescent probe bound to the template is cleaved by Taq enzyme (5'→3' exonuclease activity), the reporter group is far away from the quencher group, and its energy cannot be absorbed, that is, a fluorescent signal is generated. After PCR amplification, the probe melting curve analysis was carried out on the system. Under normal conditions, the probe does not emit light. When the probe binds to the product, the distance between the two ends increases, so it emits fluorescence. Using the difference in melting point (Tm) of double-stranded DNA formed after the probe hybridizes with different single-stranded target sequences, different target sequences can be distinguished in a single channel. The fluorescence quantitative PCR instrument can automatically draw real-time amplification curves and melting curves according to the detected fluorescent signals, and realize the detection of *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus* nucleic acids in samples according to the amplification curves and/or melting curves.

4. COMPONENTS

The following materials are included in the kit for 50 reactions.

Components	Volume (μL)	Color of screw cap
PCR Master Mix	660	Orange
PCR PP	440	Brown
Internal Control	280	Pink
Positive Control	1200	Red
Negative Control	1200	Blue

5. STORAGE AND STABILITY

5.1 Inspection on Arrival

Check the MycoMDx *Aspergillus* PCR Assay on arrival. If the packaging is damaged the kit must not be used. In addition, the reagents should be transported at 2-8 °C or lower temperature.

5.2 Storage Space and Temperature

The kit should be stored in an amplicon free laboratory. Positive Control (PC), Negative Control (NC) and Internal Control (IC) in the kit should be stored in the area where the DNA template/sample template is stored. Should be kept in -25 °C to -15 °C, valid for one year.

5.3 Stability

The MycoMDx *Aspergillus* PCR Assay showed that under the condition of more than 10 freeze thaw cycles, its performance is not affected. All reagents contained within the kit have been marked on the kit label and must be used within this effective period.

6. MATERIALS REQUIRED BUT NOT PROVIDED

6.1 General Equipment

Real-time quantitative PCR instrument (over four channels)
Vortex mixer
Mini centrifuge
Microplate processing centrifuge
Refrigerator (2-8 °C) and freezer (-25 °C to -15 °C)
Micropipettes for volumes of 1 to 1000 µL

6.2 Reagents

Recommended nucleic acid extraction reagent

6.3 Consumables

DNase/RNase free plastics were used in the preparation of PCR
DNase/RNase free 1 to 1000 µL pipette tips
Disposable gloves, powderless
Disposable face mask

7. SAMPLE COLLECTION AND DNA EXTRACTION

The quality of extracted DNA is essentially related to the sensitivity of real-time PCR detection, so samples must be collected and stored according to the following guidelines.

According to *Expert Consensus on Diagnosis and Control of Adult Aspergillus Infection*, blood infection is the main infection type in the reported spectrum of *Aspergillus*, accounting for 40% - 100% of *Aspergillus* infections. According to the above suggestions, DNA can be extracted from EDTA whole blood, EDTA plasma and serum samples.

Precise experimental practice recommends including at least one PC and NC extraction in

each analysis.

Extracted samples should be stored at -80°C for long-term storage, in -25 °C to -15 °C for temporary storage.

Roche's High Pure PCR Template Preparation Kit. bioMeriere's NucliSENS easyMAG Extraction Reagents. Qiagen's Fully Automatic Assay Setup System are recommended for DNA extraction.

8. TEST PROCEDURE

8.1 PCR Instrument Setting

It is recommended that a clean laboratory to set up all PCR reactions. To avoid any risk of contamination, users should always follow standard laboratory practices and isolate operational processes properly.

Before starting the PCR setting, the user should prepare the required consumables such as 96-well plates, the sample will be precisely assigned to the appropriate wells in advance. The information should be recorded at any time.

8.1.1 Nucleic Acid Extraction

According to the instructions of nucleic acid extraction kit, 5 μL Internal Control(IC) was added into the sample to be tested, PC and NC, and the nucleic acid was extracted by oscillating eddy current after shaking.

The nucleic acid extraction methods of PC and NC were the same as those of samples.

The extracted nucleic acid samples can be stored at 2-8 ℃ for 7 days and - 20 ℃ for 3 months.

8.1.2 PCR Reaction Procedure

Remove the MycoMDx *Aspergillus* PCR Assay from the refrigerator and allow the reagents to thaw.

Prepare PCR reaction Mix by referring to the reagents and volumes shown in table 1. The volume of each reaction well should be multiplied by the number of reaction wells. At the same time taking into account the number of samples in the analysis. The prepared PCR reaction Mix

should be fully mixed and centrifuged for 10 seconds.

Table 1 Preparation of PCR reaction volume

Reagent component	PCR addition volume (μL)
PCR Master Mix	12
PCR PP	8
Total volume(per well)	20

Take the PCR reaction tube, add 20 μL of PCR premix into each reaction well (see Table 1), and then add 5 μL nucleic acid extracted from samples or PC and NC (see Table 2).

Table 2 Final PCR reaction volume after adding clinical samples

Reagent component	PCR addition volume (μL)
Total volume(per well)	20
Sample	5
Final volume(per well)	25

Then the entire 96-well plate is placed in the centrifuge to ensure that the sample is centrifuged rapidly. The 96-well plate is placed into a PCR instrument to initiate the PCR process.

The centrifuged reaction tube was placed in the centrifuge for instant centrifugation, and the reaction tube was placed at room temperature for 10 min.

8.2 Procedure setting

8.2.1 LightCycler 480 II PCR protocol

Please refer to the instruction on how to operate LightCycler 480 II Real-Time PCR instrument and data analysis.

A detailed list about different detection channels corresponding to the detection targets on Table 3. Set these dyes to report dyes.

Table 3 Detector channels used to detect the corresponding of the *Aspergillus* targets using the LightCycler 480 II

Fluorescence Channel	FAM	HEX	ROX	Cy5
Targets	IC	*A. fumigatus*	*A. flavus/A. niger*	*A. terreus*

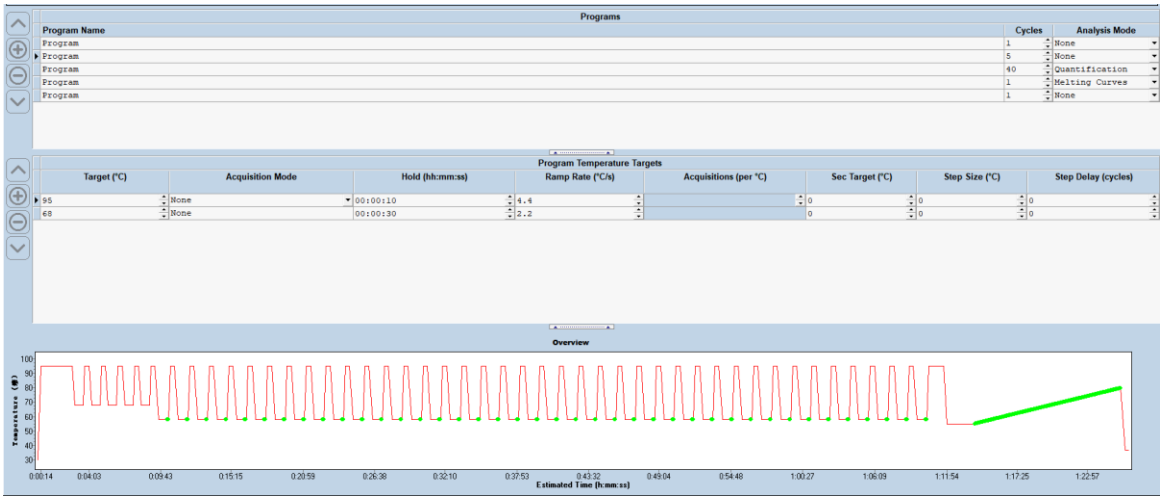
Set the detection format and use LightCycler 480 II to set the following settings:

Reaction volume: 25 μL. The test procedure is shown in table 4. Data of 40 cycles should be collected.

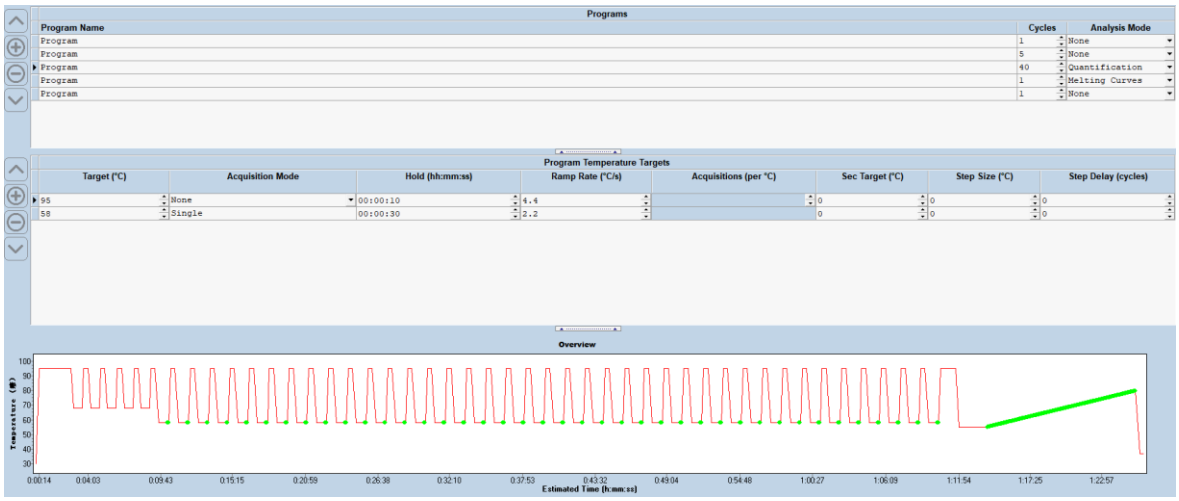
Table 4 PCR parameters for the MycoMDx *Aspergillus* PCR Assay

Step	Temperature (℃)	Data collection	Time	Number of cycles	Analysis Mode
Denaturation	95	None	2 min	1	None
Cycling1	95	None	10 sec	5	None
	68	None	30 sec		
Cycling2	95	None	10 sec	40	Quantification
	58	Single	30 sec		
Melt	95	None	1 min	1	None
	55	Hold	2 min		Melting
	55-80	Melting			
Cooling	37	None	10 sec	1	None

A



B



C

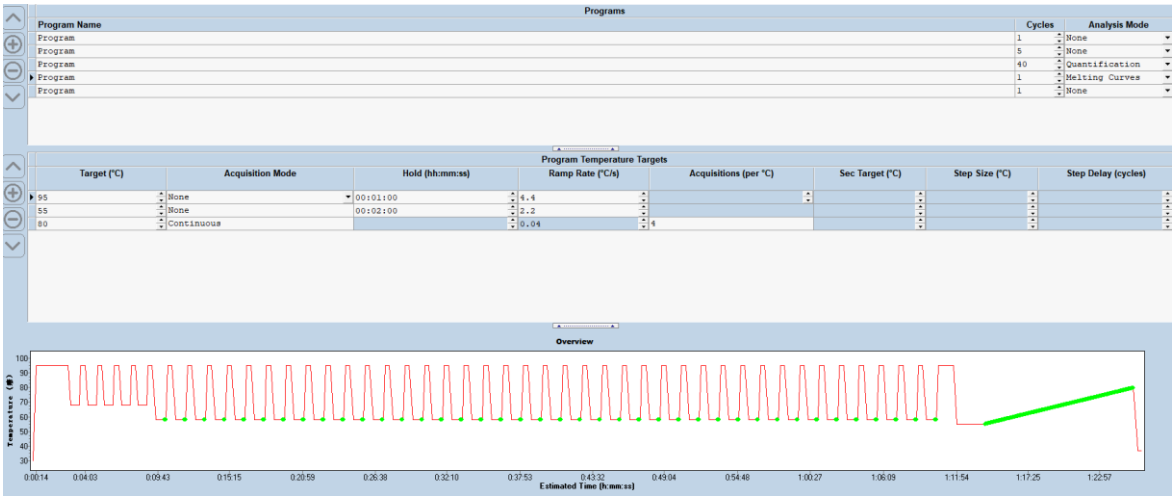


Figure X. Screen for the amplification and melt in the LC480 software (Version: 1.5.1.62) as an example. (A) Select none in the analysis mode and none in the acquisition mode for detection during cycling1. **(B)** Select quantification in the analysis mode and single in the acquisition mode for detection during cycling2. **(C)** Select melting curves in the analysis mode and continuous in the acquisition mode for detection during melting.

8.2.2 ABI7500 PCR protocol

Please refer to the instruction on how to operate ABI 7500 Real-Time PCR instrument and data analysis.

A detailed list about different detection channels corresponding to the detection targets on Table 5. Set these dyes to report dyes.

Table 5 Detector channels used to detect the corresponding of the *Aspergillus* targets using the ABI 7500

Fluorescence Channel	FAM	HEX	ROX	Cy5
Targets	IC	*A. fumigatus*	*A. flavus/A. niger*	*A. terreus*

Set the detection format and use ABI 7500 to set the following Settings:

Reaction volume: 25 μL. The test procedure is shown in table 6. Data of 40 cycles should be collected.

Table 6 PCR parameters for the MycoMDx Aspergillus PCR Assay

Step	Temperature (℃)	Data collection	Time	Number of cycles	Analysis Mode
Denaturation	95	None	2 min	1	None

Cycling1	95	None	10 sec	5	None
	68	None	30 sec		
Cycling2	95	None	10 sec	40	Quantification
	58	Single	30 sec		
Melt	95	None	1 min	1	None
	55	Hold	2 min		Melting
	55-80	1%			
Cooling	60	None	15 sec	1	None

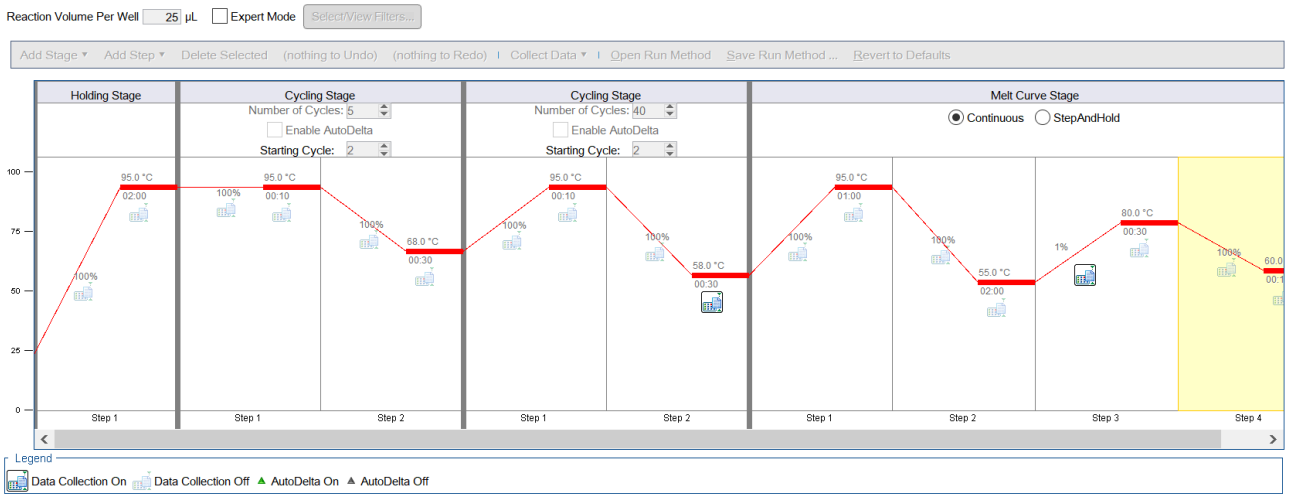


Figure X. Screen for the procedure in the 7500 software(Version: 2.3) as an example.

9. INTERPRETATION OF RESULTS

9.1 Internal Control

If all samples report as negative and internal controls are negative, the same extract should be used for repeated testing. If the internal control is still negative after repeated test, the sample should be tested again from the extraction step.

9.2 Positive and Negative Controls

At least one positive and negative control provided in the kit should be included in each analysis. Each negative or positive control should be prepared and tested in the same way as the patient samples.

Negative control that produce positive test result indicating samples contamination problem. A new "negative control material" should be repeated to ensure proper decontamination of the operating area and equipment.

Positive control that produce negative result indicating problems with the reagent or a wrong

sample addition. Ensure that all reagents are stored correctly and tested within their expiry dates.

Samples sent for testing via the MycoMDx *Aspergillus* PCR Assay may have significantly varied loads of material present and much of this may be very close to the limit of detection of the biomarker, therefore any Ct value less than 38 for the corresponding *Aspergillus* species may indicate a positive sample. Table 7 summarizes the results possible with the MycoMDx *Aspergillus* PCR Assay.

Table 7 Analysis of detection results for the MycoMDx *Aspergillus* PCR Assay

Channel	Target	C_t-values	Tm-values	Result interpretation
FAM	Internal Control	10≤Ct<32	-	Valid
		N/A or < 10 or≥32	-	Invalid
HEX	*A. fumigatus*	≤40	-	*A. fumigatus*
		N/A	-	Negative
ROX	*A. flavus/A. niger*	≤40	64≤Tm≤67	*A. flavus*
			71≤Tm≤74	*A. niger*
			64≤Tm1≤67、71≤Tm2≤74	*A. flavus* & *A. niger*
		N/A	N/A	Negative
Cy5	*A. terreus*	≤40	-	*A. terreus*
		N/A	-	Negative

Note: 1. When the results of the three detection channels (HEX, ROX, Cy5) of the sample are all negative, the internal standard (FAM channel) must be valid to be interpreted as a negative result; when any one of the detection channels of the sample is interpreted as a positive result, the internal standard is not required.

2. "N/A" means that the machine does not have a value; "-" means that there is no requirement for this data result.

3. Please note that the C_t-values and Tm-values were obtained with LC480 Ⅱ and ABI 7500.Other instrument can differ from the Ct-values and Tm-values provided. Nevertheless, the IC must always be detected in negative samples.

10. TROUBLESHOOTING

This troubleshooting section may be helpful in solving any problems that may arise.

Problem	Possible cause	Recommendations
The positive control is not within the normal range	One of the components was not added The kit has expired Incorrect PCR profile/programming Positive control was added to the wrong reaction tube	Ensure that all components have been added. Check the expiry date of the kit box. Check programming of real-time cycler Check your work steps/pipetting scheme and check calibration of real-time PCR machine and pipettes. Ensure that positive control was added to the correct reaction tube.
The negative control generates a positive signal in one or more channels.	Contamination occurred during the adding process	Taking extra care when adding the templates, in particular the Positive Control. Make sure that the work area and instruments are properly decontaminated before and after use.
IC remains negative in *Aspergillus*-negative samples	Nucleic acid degradation. DNA extraction procedure is used which is not validated and results in less efficient IC extraction yield. Incorrect PCR mixture PCR conditions do not comply with the protocol Incorrect elution volume/insufficient IC added	Repeat the extraction according to the protocol. Using the particular DNA extraction platform. Check if sufficient Taq polymerase,mastermix and sample are added Check PCR conditions and repeat the PCR with correct settings if necessary In case the elution is performed in 25 µl，check if 5 µl IC is added to the starting material
Very weak fluorescence signals	Incorrect instrument settings Incorrect real-time PCR mix	Check channel settings. Check if the PCR mixtures are prepared according to the protocol. Check expiry date and storage conditions.
Melting peaks at	To high concentration	Dilute the DNA obtained from the *Aspergillus*-culture

different Tm-values	resulting in saturation of the fluorescence signal	

11. PRODUCT PERFORMANCE

11.1 Limit of detection (LoD)

The LoD or Analytical specificity was determined using a series of quantitative spore suspensions with gradient dilution. The diluted suspension was subjected to DNA-extraction using the recommended method. The final LoD was confirmed by testing 20 replicates with a positivity rate of≥95%. Quantification was performed with cell count technique. An overview of the LoD values for both serum and BALF is shown in table 8.

Table 8. LoD Estimates and Verification of MycoMDx Aspergillus PCR Assay

Sample type	Target Organism	LoD Estimates (copies/μL)	Verification (Positives/20)
serum	*A. fumigatus*	1	20/20
	A. terreus	1	20/20
	A. flavus	2	20/20
	A. niger	2	20/20
BALF	*A. fumigatus*	1	20/20
	A. terreus	1	19/20
	A. flavus	2	20/20
	A. niger	1	20/20

11.2 Analytical specificity

The analytical specificity of the MycoMDx Aspergillus PCR Assay was determined by testing DNA of various relevant respiratory pathogens including Aspergillus species, other fungal strains and bacteria.All DNA samples tested are listed in table 9.In addition, primer and probes were checked for possible homologous to all sequences published in gene banks by sequence comparison analysis to ensure specificity of the MycoMDx Aspergillus PCR Assay.

Table 9. Testing of the specificity of relevant strains. + = positive, - = negative

Pathogen	*A. fumigatus*	*A. terreus*	*A. flavus*	*A. niger*

A. fumigatus	+	-	-	-
A. terreus	-	+	-	-
A. flavus	-	-	+	-
A. niger	-	-	-	+
Penicillium marneffei	-	-	-	-
Penicillium chrysogenum	-	-	-	-
Candida albicans	-	-	-	-
Candida glabrata	-	-	-	-
Candida krusei	-	-	-	-
Candida dubliensis	-	-	-	-
Candid tropicalis	-	-	-	-
Candida parapsilosis	-	-	-	-
Escherichia coli	-	-	-	-
Scedosporium prolificans	-	-	-	-
Rhizopus oryzae	-	-	-	-
Saccharomyces cerevisae	-	-	-	-
Cryptococcus neoformans	-	-	-	-
Haemophilus influenzae	-	-	-	-
Haemophilus influenzae	-	-	-	-
Staphylococcus aureus	-	-	-	-
Streptococcus pneumoniae	-	-	-	-
Klebsiella pneumoniae	-	-	-	-

11.3 Clinical Performance

Using clinically confirmed and excluded samples, the diagnostic sensitivity and diagnostic specificity of the MycoMDx Aspergillus PCR Assay were analyzed.From the clinical evaluation results, the diagnostic sensitivity for serum is 75.20%, the diagnostic specificity is 95.35%, and the total coincidence rate is 95%. The diagnostic sensitivity of BALF was 96.48%, the diagnostic specificity was 85.40%, and the overall coincidence rate was 96%. After preliminary evaluation, it is basically confirmed that the clinical performance of this product can meet the needs of t clinical diagnosis.

Table 10. Clinical performance characteristics of the MycoMDx *Aspergillus* PCR Assay

Sample type	Characteristics (%)	Result
serum	Sensitivity (%)	75.20
	Specificity (%)	95.35
BALF	Sensitivity (%)	85.40
	Specificity (%)	96.90

11.4 Interfering substances

The MycoMDx Aspergillus PCR Assay was tested for the interference by high loads of human DNA (250 ng/µl). Detection of *Aspergillus* by all probes was comparable to Aspergillus samples without the presence of human DNA and therefore detection of the MycoMDx Aspergillus PCR Assay was not compromised by high loads of human DNA.

11.5 Equivalence real-time PCR instruments

Two other real-time cyclers were used in an equivalence study to test and compare the performance of the MycoMDx Aspergillus PCR Assay with the validated LC480 II and ABI 7500.

- CFX96 from Bio-Rad

- ABI QuantStudio Dx from Thermofisher

Ct and Tm-values of the four kinds of Aspergillus genomic DNA samples differed between PCR-instruments. In general, Ct-values are the highest on the LC480 II when using the 2nd derivative function and are comparable on the 7500, CFX96 and QS Dx (less than 1 Ct-difference). The Ct-values change by applying different threshold settings.

The lowest Tm-values are observed on the CFX and are 2,5-3°C lower than the Tmvalues of LC480 II、ABI 7500 and QS Dx.

The amplification curves and melting peaks are clearly visible on all real-time PCR instruments. Therefore, all PCR instruments are considered suitable for use with the MycoMDx Aspergillus PCR Assay.

12. RISK AND SAFETY INFORMATION

The kit contains no harmful substances. The composition of all reagents in the kit pose no specific risk to the user or his property. Other chemicals and materials may be required for the procedures used in this instruction. Read carefully any warnings, instructions or material safety data sheets provided by the supplier and comply with general safety regulations when handling chemicals,

biological hazards or other materials.

12.1 General Precautions

1. The product is ONLY for IVD use.

2. Do not use the kit if the label seal is broken upon receipt of the product. Before beginning the test, the packaging and sealing of the product as well as its shelf life must be checked. The product cannot be used after the specified period of validity.

3. Reagents from different test tubes or kits should not be mixed at will even if they come from the same batch. Also do not replace reagents from different manufacturers.

4. Long-term storage of low-concentration DNA is unstable, so the storage time of samples should be shortened as much as possible.

5. The samples tested by the kit are whole blood, plasma and serum.

6. The testing procedure should be carried out in accordance with the requirements of this instruction.

7. Ensure all required consumables are DNase/RNase free.

12.2 Biological risks

The MycoMDx *Aspergillus* PCR Assay involves potentially dangerous and transmissible biomaterials. People who using the kit must read and follow all necessary health and safety precautions.

It is important to wear appropriate personal protective equipment during operation. At a minimum, laboratory clothing, protective gloves, respirator and safety glasses are required.

13. REFERENCES

[1] Patterson TF, Thompson GR, Denning DW, et al. Executive summary: practice guidelines for the diagnosis and management of Aspergillosis: 2016 update by the infectious diseases society of America[J]. Clin Infect Dis, 2016, 63: 433-442.

[2] Ullmann AJ, Aguado JM, Arikan-Akdagli S, et al. Diagnosis and management of Aspergillus diseases: executive summary of the 2017 ESCMID-ECMM-ERS guideline[J]. Clin Microbiol Infect, 2018, 24 Suppl 1: e1-e38.

[3] Cadena J, Thompson GR, Patterson TF. Aspergillosis: epidemiology, diagnosis, and treatment[J]. Infect Dis Clin North Am, 2021, 35(2):415-434.

[4] Barrs VR, van Doorn TM, Houbraken J, et al. . Aspergillus felis sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. 2013; 8 (6): 1-11.

14. DISPOSAL

Proper handling this production will help to conserve natural resources. Be sure to recycle it in a way that prevents potential negative impacts on the environment and human health.

General disposal

Please dispose of unused reagents, wastes and transport materials in accordance with national and local regulations.

Packaging disposal

Please dispose of all packaging according to local recycling regulations.

15. MANUFACTURER

Company: Dynamiker Biotechnology (Tianjin) Co., Ltd

Address: No.2 Building, Rongzhi Industry Park, No. 3667, Zhongbin Avenue, Sino-Singapore Eco-city, TEDA, Tianjin 300467, China

Post code: 300467

Tel: +86-022-25264212

Fax: +86-022-25264212

Website: www.dynamiker.com

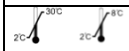
[EC REP]

Company Name: Lotus NL B.V.

Address: Koningin Julianaplein 10, 1e Verd, 2595AA, The Hague, Netherlands.

[**SYMBOLS USED**]

The following symbols may appear on the label of the MycoMDx *Aspergillus* PCR Assay or in the instructions for use.

Symbol	Description
	Use-by date
	Lot Number
	Manufacture Date
	Manufacturer
	Keep Away from Sunlight
	Temperature Limitation
	In Vitro Diagnostic Medical Device
	Authorized Representative in the European Community
	CE Mark

REVISED: 2/2022