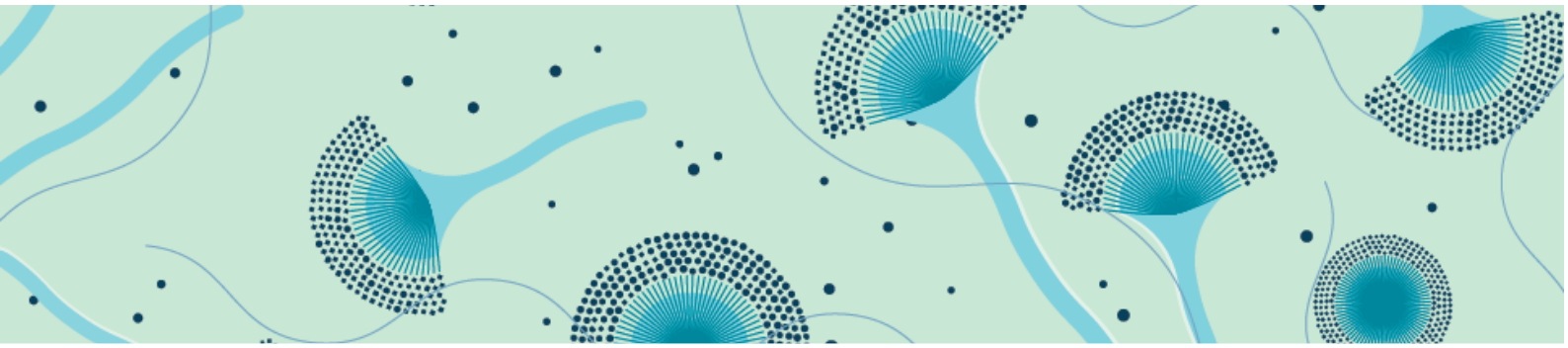

AsperGenius® 2.0 Species and Resistance TR Multiplex real-time PCR kits

PN-101

Basic UDI: 87193267509PN-101E9

Σ **50**

PN-201

Basic UDI: 87193267509PN-201EE

Σ 50

Instructions For Use Version 2.2 EN

CE IVD

Disclaimer

The results obtained from these or any other diagnostic test should be used and interpreted only in the context of the overall clinical picture. PathoNostics BV cannot accept responsibility for any clinical decisions that are made. PathoNostics BV does not represent this guide as a comprehensive summary of all possible outcomes from using the AsperGenius® 2.0 Species and Resistance TR Multiplex real-time PCR Kits. This guide is intended for use solely as an aid to memory. It is not for use in any clinical interpretation of the results of the assay. Laboratories must interpret and report the results of the assay in accordance with their own locally developed procedures.

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THE ASPERGENIUS® 2.0 SPECIES AND RESISTANCE TR MULTIPLEX REAL-TIME PCR KITS ARE SUPPLIED FOR PROFESSIONAL IN VITRO DIAGNOSTIC TESTING. THESE PRODUCTS DO NOT PROVIDE A LICENSE TO PERFORM PCR UNDER PATENTS OWNED BY ANY THIRD-PARTY INCLUDING HOFFMAN-LA ROCHE (HOFFMANN-LA ROCHE LTD, DIAGNOSTICS, CH-4070 BASEL, SWITZERLAND) AND ROCHE MOLECULAR SYSTEMS, INC (ROCHE MOLECULAR SYSTEMS, INC., 1145 ATLANTIC AVENUE, ALAMEDA, CALIFORNIA 94501)

The customer is responsible for validation of assays and compliance with regulatory requirements that pertain to their procedures and uses of the AsperGenius® 2.0 Species and Resistance TR Multiplex real-time PCR kits.

Content

1. Summary and background

Invasive pulmonary aspergillosis (IPA) is the most frequent invasive mould infection in immunocompromised patients and is mainly caused by *Aspergillus fumigatus* (*A. fumigatus*). Spores of the *Aspergillus* species can be inhaled and can cause an infection in the lower lungs during immune suppression.
The AsperGenius® 2.0 Species Multiplex real-time PCR kit (PN-101) can detect all clinical relevant *Aspergillus* species – specifically: *A. fumigatus, A. flavus*, and *A. terreus* (CFX96™ and Rotor-gene® Q only) directly in clinical material, such as: BAL, serum, and plasma.

Resistance of *A. fumigatus* against the first line therapy with voriconazole is increasingly reported. To determine if a resistant strain is present, the AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit (PN-201) can be used to identify the most prevalent environmental resistance mechanisms: TR34 (L98H) and TR46 (Y121F/T289A).
The survival rates of immunocompromised patients with invasive aspergillosis have improved dramatically due to many factors, one of which is the availability of azole antifungal drugs. A number of these azoles have activity against aspergillus infections, including itraconazole, voriconazole, posaconazole, and isavuconazole. Voriconazole is normally the recommended first line therapy for IPA; however, azole resistance in *A. fumigatus* has been increasingly detected over the past decade with prevalence rates varying between 1% and 20% . The main resistance mechanism of these isolates consists of a substitution of leucine for histidine at codon 98 of the *cyp*51A-gene, in combination with a 34-base pair tandem repeat in the promoter region of this gene (TR34/L98H), which can result in a voriconazole MIC_{50} of 8 mg/l. In addition, the emergence of a second combination of mutations (TR46/Y121F/T289A) in *A. fumigatus* has increasingly been reported, which results in a MIC_{50} of >16 mg/l for voriconazole. The *cyp*51A gene with these mutations is illustrated in figure 1. There is strong evidence that environmental exposure to azole fungicides is driving the emergence of TR34/L98H and TR46/Y121F/T289A resistance mechanisms. Isolates harboring these mechanisms of resistance are widespread in the environment and have been recovered from the environment and in hospitals located in several European countries, as well as other parts of the world. Crucially, between 64% and 71% of patients with IA due to an azole-resistant *Aspergillus* never received azole treatment and can be considered as azole-naive. Furthermore, surveillance studies have indicated that the TR34/L98H and TR46/Y121F/T289A resistance mechanisms were responsible for over 80% of aspergillosis due to azole-resistant *Aspergillus*. Diagnosis among patients at risk of invasive pulmonary aspergillosis (IPA) is often based solely on the detection of *Aspergillus* galactomannan antigen detection in clinical specimens, and identification of characteristic abnormalities in the lung by computed tomographic (CT) scans. Conventional culture remains insensitive but is required for in vitro susceptibility testing to detect azole resistance. Direct detection of resistance mutations by polymerase chain reaction (PCR) from clinical specimens can identify resistance. The AsperGenius® 2.0 Multiplex real-time PCR kits provide a solution in this matter as it can be used directly on clinical specimens (BAL or serum) to identify the causative *Aspergillus* species and identifies the most prevalent resistance mechanisms.

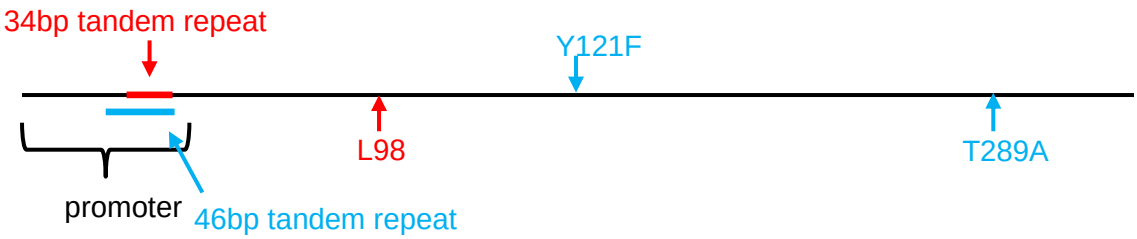


Figure 1. Schematic overview of the *cyp*51A gene of *A. fumigatus*

2. Intended Purpose

The AsperGenius® 2.0 Species Multiplex real-time PCR kit (PN-101) is a non-automated, qualitative real-time multiplex PCR test for detection and differentiation of *Aspergillus fumigatus, Aspergillus terreus, Aspergillus flavus*, and DNA of other *Aspergillus* species which is obtained after nucleic acid extraction with a NucliSENS® easyMAG® instrument (BioMérieux) or QIAamp MinElute virus spin kit (QIAGEN) from bronchoalveolar lavage (BAL). The test aids in the diagnosis of invasive pulmonary aspergillosis (IPA) in 'at risk' patients (humans), such as patients with haematological malignancies , solid organ transplant (SOT) and allogeneic transplant recipients, patients with chronical lung diseases (e.g. CF, COPD) and as a co-infection with other infections, when used in combination with other clinical and laboratory findings. The AsperGenius® 2.0 Species Multiplex real-time PCR kit is for use with LightCycler® 480 II (Roche), Rotor-Gene® Q (QIAGEN), QuantStudio™ 5 (Thermo Fisher Scientific), Mic qPCR instrument (BMS: Biomolecular Systems), and CFX96™ (Bio-Rad).

The AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit (PN-201) is a non-automated, qualitative real-time multiplex PCR test for detection and differentiation of *Aspergillus fumigatus cyp*51A wildtype, tandem repeat 34 mutant, and tandem repeat 46 mutant DNA, which is obtained after nucleic acid extraction with a NucliSENS® easyMAG® instrument (BioMérieux) or QIAamp MinElute virus spin kit (QIAGEN) from bronchoalveolar lavage (BAL) samples. The test aids in the diagnosis of pan-azole resistant invasive pulmonary aspergillosis (IPA) in 'at risk' patients (humans), such as patients with haematological malignancies, solid organ transplant (SOT) and allogeneic transplant recipients, patients with chronical lung diseases (e.g. CF, COPD), and as a co-infection with other infections when used in combination with other clinical and laboratory findings. The AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit is for use with LightCycler® 480 II (Roche), Rotor-Gene® Q (QIAGEN), QuantStudio™ 5 (Thermo Fisher Scientific), Mic qPCR instrument (BMS) and CFX96™ (Bio-Rad).

The products are for use by laboratory professionals only.

3. Principle of the test

The AsperGenius® 2.0 Multiplex real-time PCR kits are based on real-time polymerase chain reaction (PCR) technology using optimized mixtures of target specific primers and different fluorescent probes for the detection and differentiation of *Aspergillus* species and tandem repeat (TR) related resistance mechanisms conferring resistance against azole drugs.
An Internal Control (IC) is included in the kits to discriminate between true negative samples and false negative samples which can be a result of nucleic acid degradation, PCR inhibition or test failure. Positive Controls (PC) are also provided which can be integrated in every run.

4. Products and Targets

Two AsperGenius® 2.0 Multiplex real-time PCR kits are available: the AsperGenius® 2.0 Species Multiplex real-time PCR kit (PN-101) and the AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit (PN-201). The Universal Color Compensation kit (PN-501) is available for LightCycler® 480 II end-users. The PathoNostics Color Calibration kit (PN-507) is available for QuantStudio™ 5 users.

An overview of the AsperGenius® 2.0 Multiplex real-time PCR kits and their corresponding targets can be found in table 1 and 2.

Table 1: Targets of the AsperGenius® 2.0 Species Multiplex real-time PCR kit.

	AsperGenius® 2.0 Species Multiplex	**Target**	**Detection channel**
PN-101	*A. fumigatus*	28S rRNA gene	Green
	A. flavus	28S rRNA gene	Yellow
	A. species	28S rRNA gene	Orange
	Internal Control	M13 phage	Red
	*A. terreus**	28S rRNA gene	Crimson

*****Detection of the *A. terreus* is only possible on real-time PCR cyclers with 5 detection filters (QS5, CFX96, and RGQ 5-plex)

Table 2: Targets of the AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit.

	AsperGenius® 2.0 Resistance TR Multiplex	**Target**	**Detection channel**
PN-201	TR46	*cyp*51A gene	Green
	TR34	*cyp*51A gene	Yellow
	*cyp*51A gene*	*cyp*51A gene	Orange
	Internal Control	M13 phage	Red

*The *cyp*51A gene target is specific for both *A. fumigatus* wildtype and mutant strains including the TR34/L98H and TR46/Y121F/T289A strains

5. Materials provided

The following materials are included in the AsperGenius® 2.0 Species Multiplex real-time PCR kit (PN-101; table 3) and the AsperGenius® 2.0 Resistance TR Multiplex PCR kit (PN-201; table 4). Both kits are suitable for 50 reactions and should be stored in the dark at -30 °C to -15 °C (shelf-life is indicated on the outer box label).

Table 3. Materials provided in the AsperGenius® 2.0 Species Multiplex real-time PCR kit (PN-101)

Components	Component number	Volume (µl)	Color of screw cap
2.0 Species PCR mix	CNP2115A	> 500	Amber
Taq polymerase	CNP0148A	> 50	Purple
Internal Control	CNP2100A	> 500	Black
2.0 Species Positive Control	CNP2097A	> 125	White
Dilution buffer	CNP0012B	> 950	Transparent
*AsperGenius® 2.0 Species and Resistance TR Multiplex real-time PCR kit - Inlay eIFU (Instructions for Use)**			

* Instructions for Use can be downloaded from PathoNostics' web portal using the login provided on the inlay

Table 4. Materials provided in the AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit (PN-201)

Components	Component number	Volume (µl)	Color of screw cap
2.0 Resistance TR Primer/Probe mix	CNP2096A	> 375	Red
Master mix	CNP2099A	> 625	Purple
Internal Control	CNP2100A	> 500	Black
Resistance TR Positive Control	CNP2098A	> 125	Blue
Negative Control	CNP0243B	> 950	Transparent
*AsperGenius® 2.0 Species and Resistance TR Multiplex real-time PCR kit - Inlay eIFU (Instructions for Use)**			

* Instructions for Use can be downloaded from PathoNostics' web portal using the login provided on the inlay

6. Materials required but not provided

The AsperGenius® 2.0 Multiplex real-time PCR kits are suitable for real-time PCR instruments which have a minimum of 4 different fluorescent detection channels. The kits can be used on the LightCycler® 480 II (LC480 II; Roche), the Rotor-Gene® Q (RGQ; QIAGEN), the CFX96™ (Bio-Rad), the QuantStudio™ 5 (QS5; Thermo Fisher Scientific), and the Magnetic Induction Cycler (Mic qPCR cycler; Bio molecular systems). For detection of *Aspergillus terreus,* a fifth detection channel (crimson) is required (only available on RGQ, CFX96, and QS5). Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations/quality guidelines. The materials listed below are required for optimal performance of the AsperGenius® 2.0 protocol, but are not provided.

Reagents

- DNA extraction components: NucliSENS® EasyMag® extraction reagents
- DNase free/sterile PCR grade water (negative control)
- Dithiothreitol (DTT); (Thermo Fisher Scientific product code #R0861)
- Proteinase K (Roche, product code 03 264 793 001)

Consumables

- Appropriate ceramic beads (recommendation: MagNA Lyser Green Beads from Roche, product code 03 358 941 001)
- Disposable tips containing hydrophobic filters
- Sterile DNase-free 1.5 ml vials (Eppendorf tubes)
- DNase-free 0.1 or 0.2 ml PCR tubes (QIAGEN®, product code 981103 or ThermoFisher Scientific, product code AB-0620) for use with Rotor-Gene Q
- Multiwell 96 plates (Roche, product code 04 729 692 001) for use with LightCycler® 480 II instruments
- DNAse-free Mic tubes and caps (BMS, ref 60653) for use on the Mic qPCR cycler
- Hard-Shell® PCR plates 96-well, thin-well, white shell/clear well (Bio-Rad, Hsp9601) for use on the CFX96™
- MicroAmp Fast Optical 96-well reaction plate 0.1 ml (Applied Biosystems, ref 4346906) for use on the QS5

Equipment

- Bead-beater (recommendation: MagNA Lyser, Roche)
- Adjustable pipettes: 0.1-2 µl, 2-20 µl, 20-200 µl, 200-1000 µl
- Tube rack for 1.5 ml vials (Eppendorf tubes)
- Vortex mixer
- Benchtop centrifuge with a rotor for 2.0 ml tubes
- Centrifuge capable of centrifuging PCR strips or plates
- Cooling block or ice (for Taq polymerase)
- Laminar Air Flow (LAF) cabinet
- Calibrated LightCycler® 480 II (Roche), Rotor-Gene® Q (QIAGEN), CFX96™ (Bio-Rad), QuantStudio™ 5 (Thermo Fisher Scientific) or Mic qPCR (Bio Molecular Systems) instruments
    - The LightCycler® 480 II (Roche) and QuantStudio™ 5 (Thermo Fisher Scientific) instruments require the use of an additional color compensation (LC480 II) or custom dye calibration (QS5) before the first use of the AsperGenius® 2.0 Multiplex real-time PCR kits. The Universal Color Compensation kit (PN-501) is available for end-users of the LightCycler® 480 II instrument to generate the required color compensation file. The PathoNostics Color Calibration kit (PN-507) is available for end-users of the QuantStudio™ 5 to generate the required dye calibration file.
- NucliSENS® EasyMag® extraction system

7. General precautions

Performing lab activities should always be done in compliance with general safety regulations. For more information on chemicals, consult the appropriate material safety data sheets.

The following precautions should be taken to both avoid cross-contamination and allow optimal performance and reproducibility of the assays:

- The assays should only be performed by qualified laboratory personnel.
- When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.
- Physically separate the storage and workplaces as outlined in table 5.
- Use **disposable tips** containing hydrophobic **filters** to prevent cross-contamination.
- Use DNase-free PCR vials/plates which are also free of *Aspergillus* DNA.
- Keep **Taq polymerase and Master mix** always on **ice** or on a **cooling block** when taken out of the freezer. Handle Taq polymerase and Master mix with care and mix very gently.
- When thawed, **spin down the reagents** briefly in a centrifuge and mix by gently pipetting up and down.
- The cycling program should be programmed in the real-time PCR instrument before performing the assay.
- When using a PCR plate, always spin down the PCR reactions shortly before transferring to the real-time cycler (not necessary for RGQ and Mic).
- Do not open the PCR vials/plates after PCR amplification.

8. Reagent storage and handling

The components of the AsperGenius® 2.0 Multiplex real-time PCR kits should be stored in the dark at -15 °C to -30 °C and are stable until the expiry date which is stated on the outer box label. Repeated thawing and freezing should be avoided (max 15x).

To avoid contamination, we recommend performing the experimental activities into three separate areas and store the controls in a separate room/location (table 5).

Table 5. Handling procedures in different areas

Location	Handling
Room 1	Storage Taq polymerase, Master mix, Primer/Probe mix, Negative Control Preparation real-time PCR mix
Room 2*	Storage Internal Control and Positive Control(s) DNA extraction from samples Adding DNA-extracts to the real-time PCR mix Adding Positive Control to the real-time PCR mix
Room 3	Real-time PCR reaction

**It is recommended to perform all activities in laminar air flow in order to minimize the chance of cross-contamination of Aspergillus from air.*

9. Sample storage and handling

Aspergillus detection depends on the collection of high-quality specimens, their rapid transport to the laboratory, and appropriate storage before laboratory testing. Fungal cultures, bronchoalveolar lavage (BAL), serum and plasma samples, and biopsy material are suitable for the detection of *Aspergillus* DNA.

All specimens should be transported to the laboratory as soon as possible and should preferably be processed directly or stored at -20 °C upon further processing. Multiple freeze-thaw cycles should be avoided.

10. Nucleic acid extraction and preparation

It is recommended to perform the nucleic acid pre-treatment and preparation including lysis in a laminar flow cabinet. The extraction procedure can be continued on a workbench once the sample is lysed. It is advisable to decontaminate the laminar flow cabinet after use with an appropriate decontamination solution and running the UV light for 30 minutes.

10.1 BAL samples

Appropriate material for *Aspergillus* detection is bronchoalveolar lavage (BAL). It is recommended to use **1 ml BAL** for the DNA extraction (elution volume 50 µl), but a smaller volume, like 0.5 ml, would also suffice. A specific DNA extraction protocol was developed for BAL samples. Use sterile or decontaminated materials and solutions for all steps in the process.

The procedure includes the application of materials and reagents from bioMérieux for DNA extraction using the NucliSENS® EasyMag®.

Note: *Aspergillus* DNA can be detected by only processing the supernatant of the BAL sample (see figure 2). Therefore, it is advised to firstly process the supernatant, or whole BAL, and repeat the DNA extraction with the pellet only in case of:

- Discordance with other laboratory findings
- Positive (amplification) signals for A. species and *A. fumigatus*, but in absence of signals for the resistance multiplex

DNA-extraction BAL supernatant using the NucliSENS® EasyMAG® (figure 2)

I. Vortex the BAL sample and add 100 µl of 1 M DTT to e.g. 1 ml BAL (final concentration: ~0.1 M DTT) when the BAL sample is viscous, adjust according to the amount of material
   - ❖ Incubate the BAL samples for 15 min at 37 °C
   - ❖ Note: if BAL samples are still viscous, use mechanical force (bead beating)

II. Centrifuge the BAL samples for 10 min at 13400 rpm (maximum speed)
   - ❖ Prepare the EasyMag® according to the manufacturer's instructions
   - ❖ Select 1 ml as input volume and 50 µl as elution volume
   - ❖ Select the generic protocol
   - ❖ Select the onboard lysis protocol

III. Transfer the supernatant (+/- 950 µl) to the EasyMag® tray
   - ❖ Store the cell pellet in a small remaining volume (50 µl) at -80 °C

IV. Thaw the IC, vortex gently and add 5 µl to the BAL supernatant in the EasyMag® tray

V. Start the onboard lysis (the EasyMag® will dispense the lysis buffer and incubates for 10 minutes
   - ❖ Prepare the magnetic silica bead solution

VI. After lysis, add the prepared magnetic silica beads to the lysis buffer with supernatant and resuspend
   - ❖ Start the DNA-extraction on the EasyMag®
   - ❖ After 34-40 min, transfer the eluted DNA to DNase-free tubes

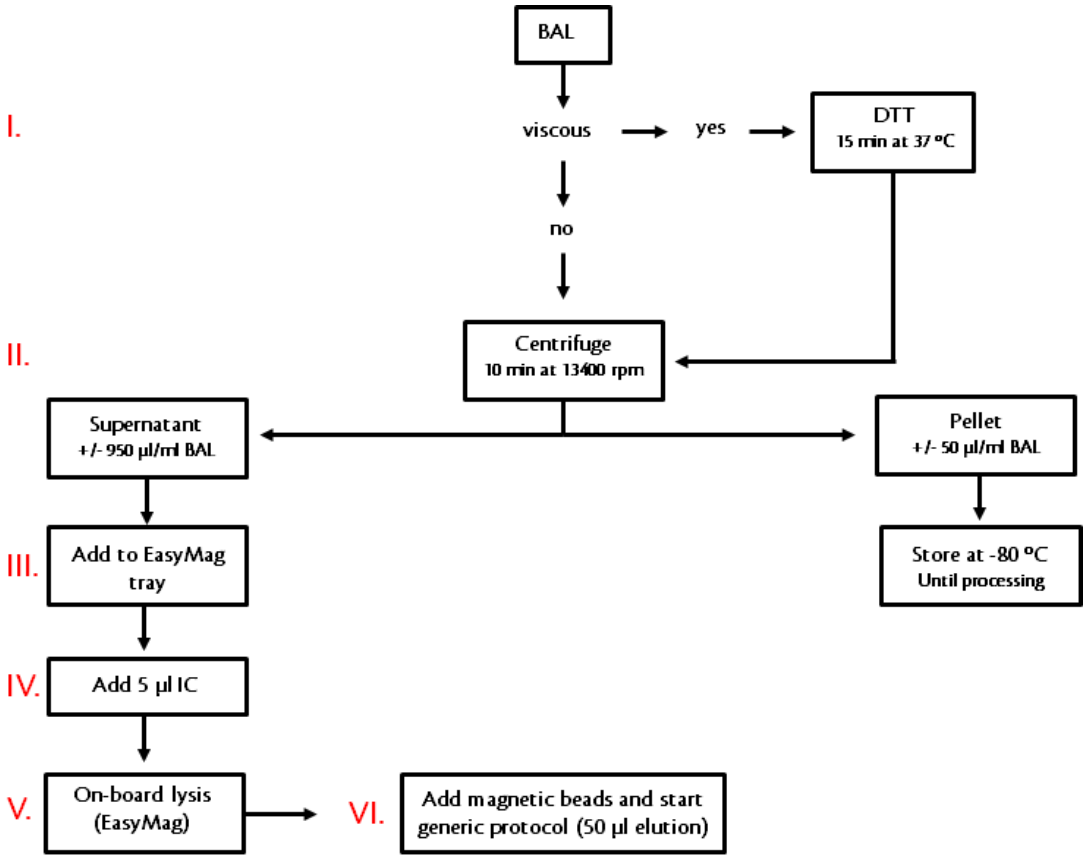


Figure 2. Flow chart of DNA-extraction from BAL supernatant. This schedule shows the DNA extraction procedure for the supernatant of 1 ml BAL.

DNA-extraction BAL pellet using the NucliSENS® EasyMAG® (figure 3)

If the supernatant from the BAL sample is not conclusive due to reasons described earlier in this section, DNA from the pellet can be extracted. The pellet contains mainly fungal cell material. To ensure efficient yield of the *Aspergillus* DNA, disruption of the fungal cell material must be enabled. For this purpose, bead-beating is used to disrupt the fungal cell structures and obtain DNA. Make sure general precautions are taken to avoid contamination.

I. Add 500 µl lysis buffer to a green bead tube
   - ❖ Transfer pellet (with pipet) to the green bead tube and incubate for 10 min at room temperature

II. Add 5 µl IC to the pellet-lysis buffer mixture present in the green bead tube and mix well

III. Bead-beat the tubes in the MagNA Lyser (Roche) for 45 sec at 6500 rpm
   - ❖ Spin down to remove foam, which is generated during bead beating in the MagNA Lyser

IV. Open tubes and add 50 µl proteinase K (30 units/mg from 20 mg/ml)
   - ❖ Vortex for five seconds
   - ❖ Incubate 10 min at 60 °C
   - ❖ Incubate 10 min at 95 °C
   - ❖ Centrifuge samples 1 min at 13400 rpm and transfer complete lysate to a new 1.5 ml tube

V. Centrifuge the tube with the lysate again 1 min at 13400 rpm to obtain a clear pellet-supernatant phase

VI. Transfer the supernatant phase containing the DNA to the EasyMag® tray
   - ❖ Note that 0.5 ml is selected as sample input volume
   - ❖ Start the onboard lysis (the EasyMag® will dispense the lysis buffer and incubates for 10 minutes
   - ❖ Prepare the magnetic silica bead solution

VII. After lysis, add the prepared magnetic silica beads to the lysis buffer with supernatant and resuspend
   - ❖ Start the DNA-extraction on the EasyMag®
   - ❖ After 34-40 min, transfer the eluted DNA to DNase-free tubes

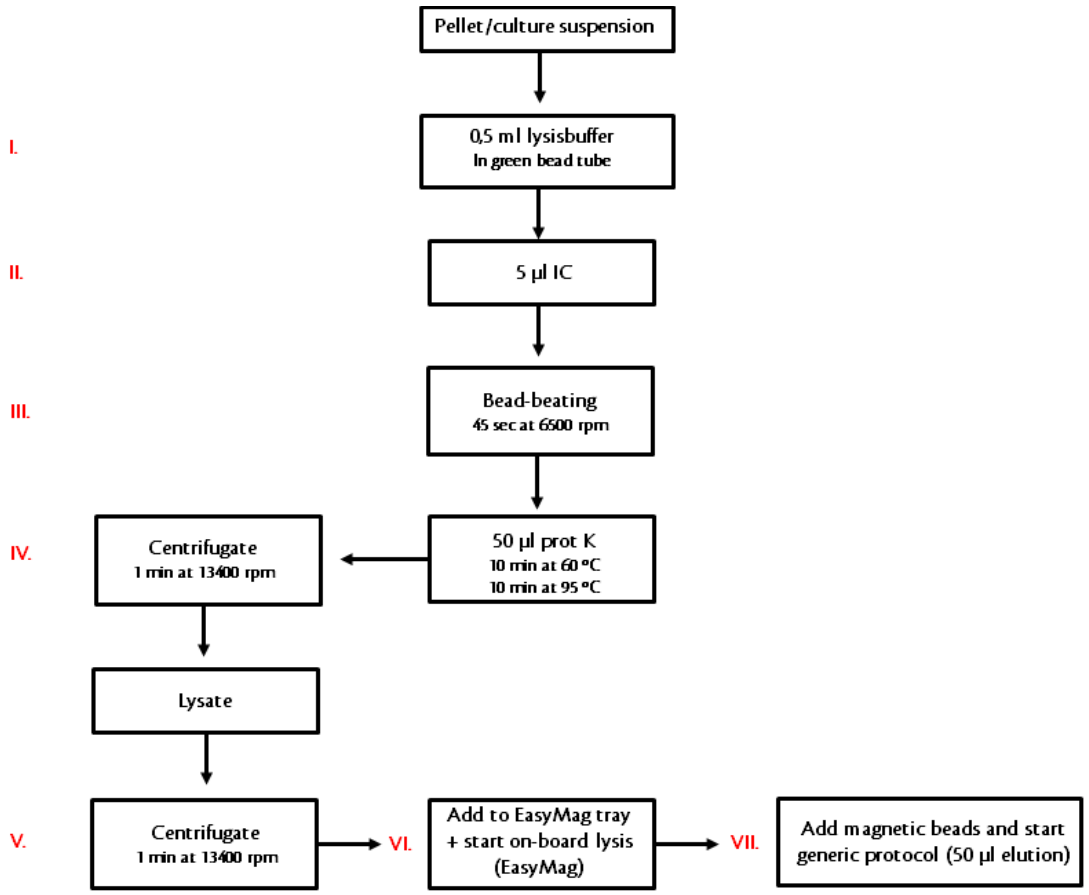


Figure 3. Flow chart DNA-extraction from BAL pellet or culture suspension. This schedule shows the DNA extraction procedure for the pellet of 1 ml BAL or culture suspension obtained from culture.

Note: Prevent freeze-thaw cycles of the extracted DNA as much as possible and store DNA extracts at 4 °C when processed within the same day. For longer periods, store the extracted DNA at -20 °C.

10.2 Other sample types

***Aspergillus* cultures**

Fungal cultures (tube or plate) can be used as well for DNA extraction and subsequent analysis with the AsperGenius® 2.0 Multiplex real-time PCR kits. Collect conidia/mycelium from a positive culture by adding 5 ml 1x tris-EDTA (pH 8) containing 0.5% Tween® 80 (polysorbate) to the surface of the culture containing fungus and incubate 15 min. Remove the culture suspension carefully from the tube or plate. It should be avoided to transfer media (like agar) into the DNA extraction process, as this might inhibit the extraction.

- See DNA extraction BAL pellet (figure 3)
    - Transfer the culture suspension (10 µl) to the green bead tube containing lysis buffer
    - No DTT is required for nucleic acid extraction from *Aspergillus* cultures

Serum and plasma samples

The AsperGenius® 2.0 Multiplex real-time PCR kits can also be used to detect Aspergillus DNA serum and plasma samples. DNA from serum and plasma samples can be extracted by using the NucliSENS® EasyMag® system as described by White *et al*. (J of Clinical Microbiology 2015;53: 2115-2121 and J of Clinical Microbiology 2017;55: 2356-2366). Please note that due to the limited *Aspergillus* burden in blood / blood derivates, it might be difficult to detect *A. fumigatus* resistance due to the detection of the *cyp*51A single-copy gene.

Tissue samples

DNA from tissue samples can be processed according to the pellet procedure (figure 3). The tissue must be added directly to the green bead tube containing lysis buffer. Use a sample with a maximum size of a pea for the DNA extraction. Biopsy material contains human DNA which could inhibit the PCR in case higher volumes are used.

10.3 Other DNA-extraction platforms

It is important to note that the extraction efficiency varies between DNA-extraction platforms and the DNA yield can be different. For some DNA extraction platforms it is recommended to prepare a pre-mix of the IC with lysis buffer before the sample is added, to guarantee optimal IC recovery. In DNA extraction systems from Roche (e.g. MagNA Pure 96 instruments), a lower extraction efficiency was observed for samples with a low *Aspergillus* load and in negative samples with IC. This was especially observed in samples with a ´clean matrix´ (e.g. IC in water used as extraction control).

DNA-extraction using the MagNA Pure 96

Various nucleic acid isolation kits from Roche are available for use in combination with MagNA Pure 96 instruments. We recommend to use large volume kits to extract DNA from 1 ml BAL samples or serum. Before the DNA-extraction is initiated on a MagNA Pure 96 instrument, we advise to prepare a mix of 5 µl IC + 15 µl lysis buffer (from the respective kit) per sample and dispense in every well in the MagNA Pure tray corresponding with the number of samples. Afterwards, add the sample to the MagNA Pure tray and make sure 50 µl elution is selected. A small volume kit (200 µl) can be used, but is not recommended due to reduced sensitivity.

QIAamp MinElute Virus Spin kit

In addition to automated DNA-extraction procedures, also filter-column based DNA-extraction methods can be used for BAL samples. The QIAamp MinElute Virus Spin Kit (Cat. Nr. 57704) shows a comparable DNA-extraction efficiency compared to the DNA-extraction procedure on the NucliSENS® EasyMAG®. Note that these filter columns are only suitable for maximal 200 µl BAL.

10.4 Controls

Internal Control (IC)

The Internal Control in the AsperGenius® 2.0 Multiplex real-time PCR kits is supplied as a M13 bacteriophage solution and is added to discriminate between true-negative samples and false-negative samples, which can be a result of nucleic acid degradation, PCR inhibition, or test failure. Make sure to mix the sample and lysis buffer before adding the IC. The lysis buffer contains a stabilization reagent which inhibits DNases, preventing the degradation of the IC. The required volume of IC to be spiked into the lysed BAL/culture is **5 µl** and elution volume should be **50 µl**. When the elution volume is changed, the amount of spiked IC has to be changed . The amount of spiked IC is irrespective of the initial volume of the sample.
In case of a sample with a high *Aspergillus* nucleic acid load and/or multiple infections, the IC may be outcompeted during the PCR reaction. This is explained by the fact that high amounts of *Aspergillus* nucleic acids consume most of the reagents in the assay. Consequently, when the IC signal is absent in the presence of one or more amplification curves indicating the presence of *Aspergillus*, the PCR is still valid.

Negative Control (NC)

A negative control (NC) or negative extraction control is recommended to determine *Aspergillus* contamination in DNA-extraction reagents. An NC only contains the IC and preferably the same sample matrix as *Aspergillus* samples. If an NC is not included in the run, then it is recommended to include at least a negative template control (NTC) reaction. An NTC exists of DNase-free water and is a control for contamination in the AsperGenius® 2.0 Multiplex real-time PCR run and also serves as a reference for the background signal.

Positive Control (PC)

The positive controls (PC) in the AsperGenius® 2.0 Multiplex real-time PCR kits consist of synthetic DNA fragments and covers the target regions which are detected in the assays. The PCs should not be extracted and are handled like normal nucleic acid extracts in the AsperGenius® 2.0 Multiplex real-time PCR assays. The PCs control for a correct real-time PCR procedure.

11. Real-time PCR Instrument Settings

The AsperGenius® 2.0 Multiplex real-time PCR kits are tested on real-time instruments LC480 II, RGQ, CFX96, QS5, and Mic qPCR cycler utilizing four or five different detection channels (see table 6). For all mentioned real-time instruments, a PCR protocol template file is available. This can be requested at PathoNostics or your local distributor.

11.1 Filter settings

The filter settings of the RGQ, LC480 II, and QS5 for fluorescence detection in the four channels are listed in table 6. These source/detector formats – also known as emission/excitation wave lengths – should be programmed in the RGQ, LC480 II, and QS5 instrument and adjusted according to the settings in table 6. The indications of the four different detection channels of the CFX96™ and Mic qPCR cycler are also shown in table 6. Detection of *A. terreus* is not possible on the LC480 II and Mic qPCR instrument, but it is possible on the Rotor Gene Q, CFX96™ and QuantStudio™ 5 instruments using 662 (Source) / 711 (Detector) detection channels. However, the QuantStudio™ 5 instrument requires an additional Custom Dye file for the instrument, which can be obtained by performing a Dye calibration using a specific reagent. This reagent is included in the PathoNostics Color Calibration kit (PN-507) and can be requested from your local distributor or PathoNostics. Programming of the instruments should be carried out according to the manufacturer's instructions. A detailed description on how to perform this Dye calibration can be found in the IFU of the PN-507 kit. Signal detection in the four/five channels must be activated.

Table 6. Filter settings for optimal detection of AsperGenius® 2.0 probes. N.A. = not available

Species multiplex	Resistance multiplex	Rotor-Gene (nm)		LC480 II (nm)		QuantStudio 5 (nm)		CFX96	Mic qPCR
		Source	Detector	Source	Detector	Source	Detector		
A. fumigatus	TR46	470	510	465	510	470	520	FAM	Green
A. flavus	TR34	530	555	533	580	520 550	558 586	HEX	Yellow
A. species	cyp51A	585	610	533	610	580	623	ROX	Orange
IC	IC	625	660	618	660	640	682	CY5	Red
A. terreus		680	710	N.A.	N.A.	662	711	Quasar 705	N.A

11.2 Additional information per PCR instrument

Rotor-Gene® Q gain settings

The correct gain settings must be determined before starting with the AsperGenius® 2.0 Multiplex real-time PCR protocol on the Rotor-Gene® Q. Correct gain settings for the Rotor-Gene® Q instrument are selected to prevent saturation in the detection channels. The gain optimization function must be used:

- Select gain optimization in the profile wizard of the Rotor-Gene software.
- Select 'perform optimization before 1st acquisition' (see figure 4).

- Click on 'optimize acquiring', this should enable gain optimization for Green, Yellow, Orange, Red and Crimson after generating the correct AsperGenius® 2.0 Multiplex real-time PCR protocol.
- Select one tube position containing the species multiplex for all five channels and select 5 to 10 FI (minimum- and maximum fluorescence intensity) and -10 to 10 for acceptable gain range (minimum and maximum).
- When the Resistance multiplex is also included in the PCR run, always select the tube which contains the Species mix for gain optimization

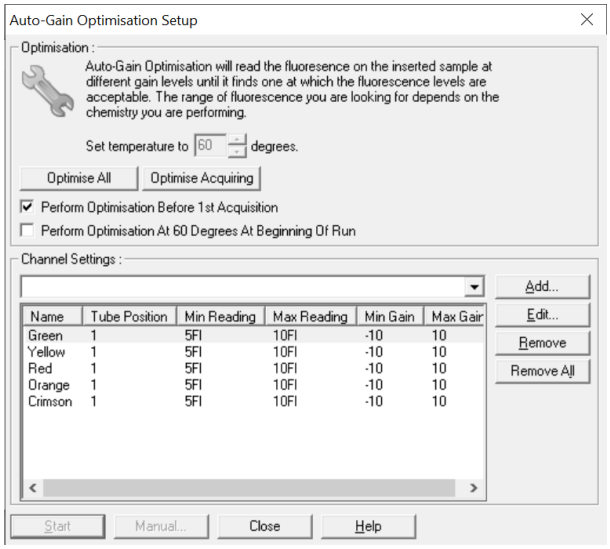


Figure 4. Gain optimisation in the Rotor-Gene software.
Example of the gain optimisation screen during amplification.

LightCycler® 480 II: Color Compensation

Due to overlap of the emission spectra of the different dyes, cross-talk between detection channels can occur. In the LC480 II instrument, the cross-talk can be compensated using a color compensation file. This required color compensation file can be generated by applying color compensation mixtures from the Universal Color Compensation kit, which is separately provided by your local distributor or PathoNostics on request (Universal Color Compensation kit; PN-501). A detailed description how to generate a Color Compensation file is provided in the Instructions For Use of the Universal Color Compensation kit. See section 11.1 for more details.

CFX96™: Select the correct plate type

When creating a new plate, make sure that **Scan Mode** is set to **All Channels**, and subsequently enable the detection channels prepared by the manufacturer: FAM, HEX, ROX, Cy5, and Quasar. Ensure that the correct plate type has been selected before starting the AsperGenius® 2.0 PCR run.

QuantStudio™ 5: Select the correct properties

Use the following settings in the properties screen of the QuantStudio™ Design & Analysis Software:

- Experiment type: Relative Standard Curve
- Chemistry: Taqman® Reagents
- Run Mode: Standard

Dye calibration:

See section 11.1 for more details.

Please refer to the IFU of the PathoNostics Color Calibration kit (PN-507) for detailed instructions on how to perform the crimson dye calibration.

11.3 Real-time PCR program

The cycling program should be programmed in the real-time PCR instrument before performing the AsperGenius® 2.0 Multiplex real-time PCR reactions. The cycling program for the LC480 II, CFX96, and QS5 is shown in table 7. The cycling program for the RGQ and Mic qPCR is shown in table 8.

Table 7. Real-time PCR program on the LC480 II, CFX96, and QS5

Step	Time	Temperature	Function
Taq activation	30 seconds	95°C	Taq activation/ Initial denaturation
Cycling (45x)	10 seconds 30 seconds	95°C 60°C*	Denaturation Annealing and extension

*activate fluorescent detection (see table 6 for detection channels)

Table 8. Real-time PCR program on the RGQ and Mic qPCR

Step	Time	Temperature	Function
Taq activation	30 seconds	95°C	Taq activation/ Initial denaturation
Cycling (45x)	10 seconds 30 seconds	95°C **62**°C*	Denaturation Annealing and extension

*activate fluorescent detection (see table 6 for detection channels)

An overview of protocol duration is shown in table 9.

Table 9. Approximate duration of the real-time PCR program.

Real-time PCR instrument	Duration PCR program (in min)
LC480 II	65
RGQ	95
CFX96	70
Mic qPCR	65
QS5	65

12 Procedure

Recommendations

- Thaw template DNA (if frozen) and all reagents, and keep on ice.
- Prepare a slightly larger amount than required for the various mixes to compensate for pipetting losses.

12.1 Room 1: preparation of the real-time PCR mixes

The real-time PCR reaction is performed in a final volume of 25 µl.

- Prepare the real-time PCR mixes according to table 10 for testing AsperGenius® 2.0 Species and according to table 11 for testing AsperGenius® 2.0 Resistance.
  Note: always prepare a slight excess of PCR mix due to loss of volume whilst pipetting; e.g., prepare for 11 reactions when 10 are needed. This is to ensure that at least 10 reactions can be performed with 20 µl per reaction.
- Mix the real-time PCR mix gently but thoroughly and dispense 20 µl mix for the per sample/control into a PCR vial or well from a plate. Keep the PCR vials or plates on ice or on a cooling block.

Table 10. Real-time PCR mix for AsperGenius® 2.0 Species Multiplex real-time PCR kit (PN-101)

Component	Volume/reaction (µl)	Volume for 10 samples (µl)
2.0 Species PCR mix	10	100
Taq polymerase	1	10
Dilution buffer	9	90
Total Volume	20	200

Table 11. Real-time PCR mix for AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit (PN201)

Component	Volume/reaction (µl)	Volume for 10 samples (µl)
Resistance TR 2.0 Primer/Probe mix	7.5	75
Master mix	12.5	125
Total Volume	20	200

12.2 Room 2: Addition of DNA to the PCR mixtures

- Add 5 µl of extracted DNA from the sample (containing IC) to the dispensed Species mix and 5 µl to the dispensed Resistance TR mix.
- Add 5 µl Negative Control to the Species mix and add 5 µl Negative Control to the Resistance TR mix.
- Add 5 µl 2.0 Species Positive Control to the Species mix and add 5 µl 2.0 Resistance TR Positive Control to the Resistance TR mix.
- Close the PCR vials or seal the real-time PCR plate and spin down briefly (only necessary for a real-time PCR plate). Try to avoid air bubbles in the plate.

12.3 Room 3: Start the real-time PCR instrument

- Transfer the PCR vials or real-time PCR plate to the real-time PCR instrument.
- Program (or install) the real-time cycler program and select start (the real-time PCR cycling program is shown in table 7 and 8)

13 Interpretation of results; PCR instrument related issues

A more detailed protocol to perform data analysis is available for all tested real-time instruments. This can be requested at PathoNostics or your local distributor. More detailed information about the settings of the different real-time cyclers is explained below.

Thresholds: Rotor-Gene® Q

Before determining the C_t-value with the Rotor-Gene® Q software, check whether the baseline is positioned correctly and adjust if necessary by using the slope correct function. The C_t-value is determined by using the threshold function. The recommended thresholds for amplification curves are listed in table 12. The threshold locations are based on the maximal fluorescence intensity, but can differ between samples. An example of an amplification curve obtained with the Rotor-Gene® Q is illustrated in figure 5.

Example of an amplification curve

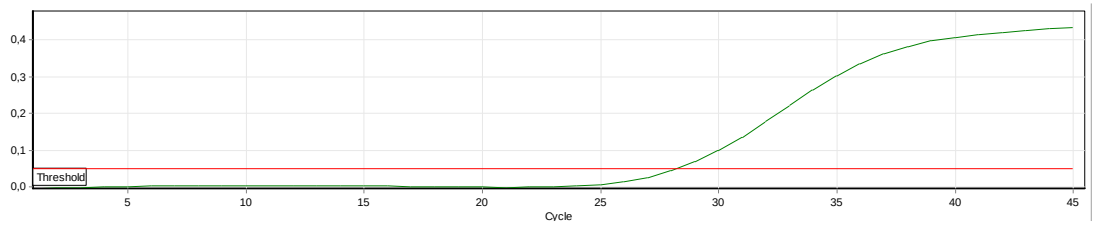


Figure 5. Amplification curve. Example of an amplification curve on the RGQ, obtained with the *A. fumigatus* probe in the green detection channel.

Table 12. Thresholds Rotor-Gene amplification curves

Max FI (norm. fluorescence)	Threshold location
< 0.2	0.025
0.2-0.6	0.05
0.6-1.0	0.1
>1.0	0.15

Cross-talk correction for detection channel "Crimson"

Cross-talk from internal control signals (red channel) can partially be seen in the crimson channel (*A. terreus)*.

To correct for this cross-talk, a custom raw channel needs to made, in which Red is being normalised to Crimson, followed by a normalisation of Crimson to the newly made Red-to-Crimson raw channel.

Click de options button in the bottom of the raw channel window of "Cycling A.Red" and select "Normalise to Cycling A.Crimson" (figure 6).

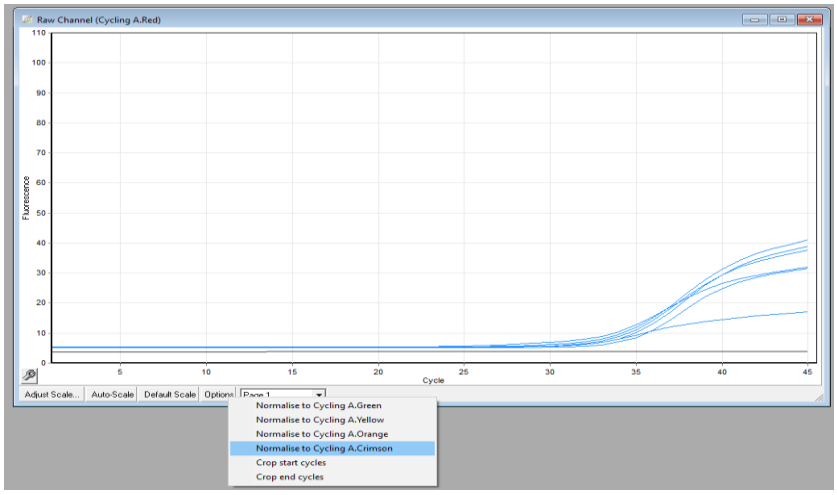


Figure 6. Creating raw channel "Normalise to Cycling A.Crimson"

A new raw channel has been added to list of channels (see figure 7). Do not open this channel; this channel is only required for the final correction.

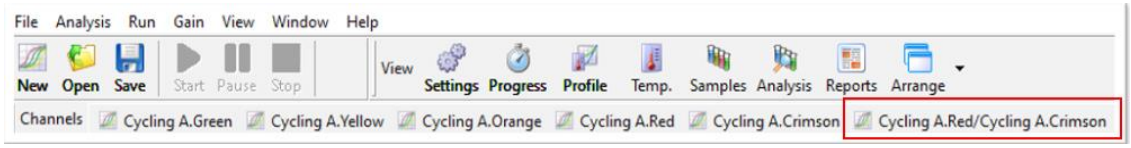


Figure 7. List of raw channels in Rotor-gene Q software

Open the "Cycling A.Crimson" raw channel and select "Normalise to Cycling A.Red/Cycling A.Crimson" for the final correction (figure 8).

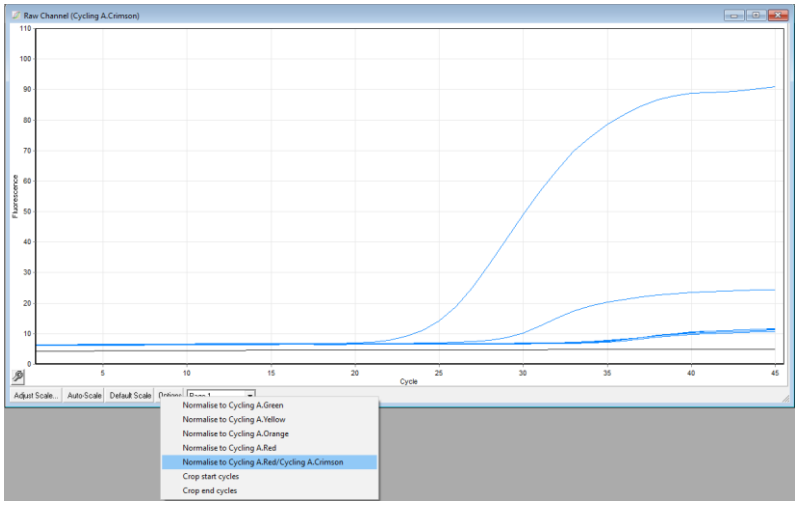


Figure 8. Creating raw channel "Normalise to Cycling A.Red/Cycling A.Crimson"

A new raw channel will be added to the list channels: "Cycling A.Crimson/Cycling A.Red/Cycling A.Crimson" (see figure 9). This channel can be used for data analysis of *A. terreus* (Crimson).

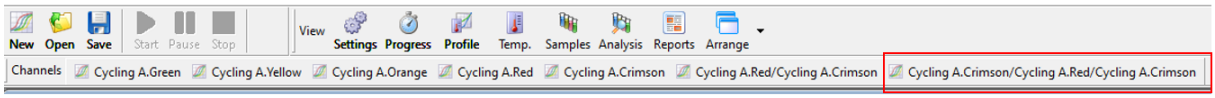


Figure 9. List of raw channels in Rotor-gene Q software

Thresholds: LightCycler® 480 II

To determine the C_t-values with the LC480 II software, we recommend to use the 2nd derivative function. This function automatically determines the C_t-value. However, note that with this 2nd derivative function, samples could falsely called positive by the LC480 software. Therefore, always manually inspect whether a positive reaction is accompanied with a an amplification curve, and deselect if needed. In addition, all amplification curves with C_t-values > 40 are called as 40 with the 2nd derivative function.

Thresholds: CFX96™

To determine the C_t-values with the CFX software, we recommend to use the Cq determination mode and select **regression**. This function automatically determines the C_t-value. However, the **single threshold** mode can also be used to confirm whether the regression mode called the correct C_t-values.

Thresholds: QS5

Ct-values are determined by an automated threshold. This can be adjusted to manual in the settings menu but this is not recommended, as this is not standardized.

Thresholds: Mic qPCR

To automatically determine the Ct-values on the Mic qPCR cycler, the auto set threshold function can be selected. This is, however, not always possible depending on other selected parameters. For the most reliable analysis settings on the Mic qPCR cycler it is recommended to contact PathoNostics or your local distributor to obtain an optimal analysis template.

14 Interpretation of results

14.1 Species multiplex

Data interpretation of the AsperGenius® 2.0 Species Multiplex real-time PCR kit is shown in table 13. All *Aspergillus* targets are detected by probes specific for the 28S multicopy gene. If no fluorescence signal is observed in any detection channel, when using the AsperGenius® 2.0 Species Multiplex real-time PCR kit, the DNA might be degraded, the reaction might be inhibited, or a manual error has occurred. See section 17 "troubleshooting" on how to proceed.

Table 13. Interpretation of signals in AsperGenius® 2.0 Species Multiplex real-time PCR kit.
A. fum = Aspergillus fumigatus, *A. fla = Aspergillus flavus*, *A.* spp = *Aspergillus* species, *A. ter = Aspergillus terreus*

AsperGenius® 2.0 Species amplification signals						
Target	***A. fum***	***A. fla***	***A.* spp**	**IC**	***A. ter***	**Result**
	+	-	+	+		*A. fumigatus*
	-	+	+	+		*A. flavus*
	-	-	+	+		*Aspergillus* species
			+	+	+	*A. terreus*
	-	-	-	+		No *Aspergillus* species, IC Positive
	+/-	+/-	+/-	-	+/-	IC outcompeted by high DNA load, result is still valid
	-	-	-	-		Invalid

Note: The *A.* species probe detects all *Aspergillus* species and is slightly more sensitive than the target specific probes for *A. fumigatus*, *A. flavus*, and *A. terreus*. When samples result in high C_t-values for the *A.* species probe (Ct ≥ 35), signals for *A. fumigatus, A. flavus*, or *A. terreus* might not be observed. Nevertheless, the sample extract could still contain one of these *Aspergillus* species.

14.2 Resistance TR multiplex

In the event that the AsperGenius® 2.0 Species Multiplex real-time PCR is positive for *A. fumigatus*, positive signals can be expected with the AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit as well. The C_t-values obtained with AsperGenius® 2.0 Species Multiplex real-time PCR kit are always lower than Ct-values obtained with the AsperGenius® 2.0 Resistance TR multiplex due to the multi- and single-copy gene effect. **Therefore, the AsperGenius® 2.0 Resistance TR PCR could be negative when C_t-values ≥ 35 have been called with the 2.0 Species assay.**

Data interpretation of the AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit is shown in table 14. The probes detect tandem repeats (TR) in the promotor region of the *cyp*51A (single-copy) gene, which are associated with azole resistance. The TR34 probe is specific for the TR34/L98H resistance mechanism and the TR46 probe for the TR46/Y121F/T289A resistance mechanism. The *cyp*51A gene probe reacts with all *A. fumigatus* wildtype strains but also with *A. fumigatus* strains that contain one of the aforementioned resistance mechanisms.
If no fluorescence signal is observed in any detection channel when using the AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit, then the DNA might be degraded, the reaction might be inhibited, or a manual error has occurred. See section 17 “troubleshooting” how to proceed.

Table 14. Interpretation of signals in AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit.

AsperGenius® 2.0 Resistance TR amplification signals					
Target	**TR46**	**TR34**	**CYP51A**	**IC**	**Result**
	+	-	+	+	*A. fumigatus* TR46/Y121F/T289A
	-	+	+	+	TR34/L98H
	-	-	+	+	*A. fumigatus* wildtype
	-	-	-	+	No *A. fumigatus* detected, IC Positive
	+/-	+/-	+/-	-	IC outcompeted by high DNA load, result is still valid
	-	-	-	-	Invalid

14.3 Controls

In order to verify the efficiency of the DNA extraction (IC) and PCR procedure (IC and PC), the C_t-values for all controls included in the AsperGenius® 2.0 Multiplex real-time PCR kit must be within the acceptable range (table 15). The C_t-values for the IC strongly depend on the sample matrix and the DNA-extraction procedure.

Table 15. C_t value ranges for controls. C_t-values are determined by using the recommended threshold settings described in this manual.

Control	Target	Channel	C_t values
Species 2.0 PC	*A. fumigatus*	Green/465-510/FAM	27.0-33.0
	A. flavus	Yellow/533-580/HEX/VIC	26.0-32.0
	A. species	Orange/533-610/ROX	26.0-32.0
	Internal control	Red/618-660/Cy5	26.0-31.0
	A. terreus	Quasar/Crimson/ATTO700	27.0-33.0
Resistance TR PC	TR46	Green/465-510/FAM	26.0-33.0
	TR34	Yellow/533-580/Hex/VIC	26.0-32.0
	cyp51A	Orange/533-610/ROX	27.0-32.0
	Internal control	Red/618-660/Cy5	26.0-31.0
IC*	M13	Red/618-660/Cy5	30.0-37.0

* A strong positive *Aspergillus* sample might outcompete the IC and result in higher C_t values than listed.

Please note that the C_t-values were obtained with in-house calibrated PCR instruments. Other PCR instruments can differ slightly from the C_t-values provided. Nevertheless, both the positive and internal controls must always be detected when included in an AsperGenius® 2.0 experiment.

The Ct values for the IC are higher for the AsperGenius® 2.0 Species Multiplex real-time PCR than for the AsperGenius® 2.0 Resistance TR Multiplex real-time PCR, which is caused by the Taq polymerase/Master mix included in each of these kits. Therefore, the overall Ct value range for the IC is set from 30 to 37.

15 Limitations

The AsperGenius® 2.0 Multiplex real-time PCR kits aid in the diagnosis of Invasive Pulmonary Aspergillosis. Negative results do not necessarily indicate absence of (fungal) infection. Negative results should not be used as the sole basis for diagnosis, therapy, or other treatment decisions. Positive results do not exclude co-infection with other pathogens. The pathogen(s) detected may not be the definite cause of disease. Other laboratory testing and assessment of clinical presentation must be included in the final diagnosis.

16 Performance Characteristics

16.1 Analytical performance

16.1.1 Limit of detection (LoD)

The limit of detection or analytical sensitivity was determined for both the AsperGenius® 2.0 Species and AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kits using a dilution series of genomic DNA. The final limit of detection was confirmed by testing 20 replicates with a positivity rate of ≥ 95%. All replicates were spiked with the Internal Control to mimic an actual clinical sample. Quantification of the targets was performed with an in-house droplet digital PCR. An overview of the LoD values for all targets are shown in table 16.

Table 16: The minimum number of copies per reaction that can successfully be determined by the AsperGenius® 2.0 Multiplex real-time PCR kits on five different real-time instruments.

Assay	Target	CFX	RGQ	LC480	MIC	QS5
		copies/ reaction	copies/ reaction	copies/ reaction	copies/ reaction	copies/ reaction
Species (PN-101)	*A. fumigatus*	6	6	6	6	6
	A. flavus	5	5	5	5	5
	A. terreus	16	16	16	16	3
	A. niger	5	5	5	5	5
Resistance TR (PN-201)	*A. fumigatus* WT	3	3	3	3	3
	A. fumigatus TR34	9	9	9	9	9
	A. fumigatus TR46	3	3	3	3	3

16.1.2 Specificity and inclusivity

The analytical specificity and inclusivity of the AsperGenius® 2.0 Species and Resistance TR Multiplex real-time PCR kits was determined by testing DNA of various clinically relevant pathogens including various fungal strains, yeasts, and bacteria. All DNA samples tested are listed in table 17.

Table 17. Species tested with the AsperGenius® 2.0 Species and Resistance TR Multiplex real-time PCR kits.

Pathogen	AsperGenius 2.0 test result
Aspergillus fumigatus	*Aspergillus fumigatus*
Aspergillus fumigatus TR34	*Aspergillus fumigatus* TR34
Aspergillus fumigatus TR46	*Aspergillus fumigatus* TR46
Aspergillus fumigatus TR53	*Aspergillus fumigatus* wild-type
Aspergillus fumigatus TR92	*Aspergillus fumigatus* TR46
Aspergillus flavus	*Aspergillus flavus*
Aspergillus terreus	*Aspergillus terreus*
Aspergillus niger	*Aspergillus* species
Aspergillus lentulus	*Aspergillus* species
Aspergillus versicolor	*Aspergillus* species
Penicillium chrysogenum	*Aspergillus* species*
Penicillium marneffei	Negative
Pneumocystis jirovecii	Negative
Fusarium solani	Negative
Mucor hiemalis	Negative
Cunninghamella bertholletiae	Negative
Lichtheimia corymbifera	Negative
Rhizomucor miehei	Negative
Rhizomucor pusillus	Negative
Rhizopus microsporus	Negative
Rhizopus oryzae	Negative
Scopulariopsis brevicaulis	Negative
Malassezia caprae	Negative
Saccharomyces cerevisiae	Negative
Cryptococcus neoformans	Negative
Candida albicans	Negative
Candida parapsilosis	Negative
Candida glabrata	Negative
Candida guilliermondii	Negative
Candida tropicalis	Negative
Candida krusei	Negative
Bordetella pertussis	Negative
Escherichia coli	Negative
Haemophilus influenzae	Negative
Moraxella catarrhalis	Negative
Pseudomonas aeruginosa	Negative
Staphylococcus aureus	Negative
Streptococcus pneumoniae	Negative
Klebsiella pneumoniae	Negative
Legionella pneumoniae	Negative
Mycoplasma pneumoniae	Negative
Mycobacterium tuberculosis	Negative

* *Penicillium chrysogenum* is also detected by the 28S *A.* species probe due to the genetic similarities of both species in the 28S gene.

16.1.3 Robustness

The verification of the robustness allows the determination of the total failure rate of the AsperGenius® 2.0 Species and Resistance TR Multiplex real-time PCR kits by monitoring the presence of the IC in a sample extract. The robustness was assessed by testing multiple BAL samples according to the procedure as described in chapter 10.1. The IC was correctly recovered from all tested BAL samples (100%) with both the AsperGenius® 2.0 Species and Resistance TR Multiplex real-time PCR kits.

16.1.4 Accuracy and precision

To verify the accuracy and precision of the AsperGenius® 2.0 Species and Resistance TR Multiplex real-time PCR kits, three different production lots were tested on the PC and IC signals on two different real-time PCR instruments (CFX96 and RGQ). The arithmetic mean, standard deviation (stdev) and coefficient of variation were calculated. The results are listed in Table 18 – Table 21.

Table 18. Reproducibility of the AsperGenius® 2.0 Species Multiplex real-time PCR kit on the CFX96.

Target	CFX								
	Lot 1		Lot 2		Lot 3		Total		
	Mean	stdev	Mean	stdev	Mean	stdev	Mean	stdev	CV (%)
PC *A. fumigatus*	30.45	0.21	30.42	0.09	29.89	0.10	30.25	0.30	0.98
PC *A. flavus*	29.38	0.38	29.07	0.07	28.79	0.08	29.08	0.32	1.11
PC A. spp	28.73	0.13	28.69	0.06	28.43	0.08	28.62	0.16	0.56
PC IC	28.30	0.27	28.47	0.03	28.18	0.06	28.32	0.19	0.68
PC *A. terreus*	29.63	0.13	29.64	0.05	29.27	0.07	29.51	0.19	0.66
Internal control	33.71	0.15	34.25	0.31	34.30	0.27	34.09	0.36	1.06

Table 19. Reproducibility of the AsperGenius® 2.0 Species Multiplex real-time PCR kit on the RGQ.

Target	RGQ								
	Lot 1		Lot 2		Lot 3		Total		
	Mean	stdev	Mean	stdev	Mean	stdev	Mean	stdev	CV (%)
PC *A. fumigatus*	30.10	0.10	30.35	0.20	29.98	0.09	30.14	0.20	0.68
PC *A. flavus*	30.20	0.21	30.15	0.20	29.98	0.15	30.11	0.19	0.64
PC A. spp	27.23	0.05	27.50	0.18	27.19	0.10	27.31	0.18	0.67
PC IC	27.42	0.13	27.60	0.17	27.29	0.06	27.44	0.18	0.64
PC *A. terreus*	29.12	0.07	28.79	0.22	28.44	0.07	28.78	0.32	1.10
Internal control	32.21	0.36	32.69	0.38	32.34	0.37	32.42	0.41	1.26

Table 20. Reproducibility of the AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit on the CFX96.

Target	CFX96								
	Lot 1		Lot 2		Lot 3		Total		
	Mean	stdev	Mean	stdev	Mean	stdev	Mean	stdev	CV (%)
PC *A. fumigatus* TR46	29.61	0.11	29.39	0.08	29.55	0.08	29.52	0.13	0.43
PC *A. fumigatus* TR34	28.99	0.10	29.03	0.06	29.11	0.04	29.04	0.08	0.28
PC *A. fumigatus* Cyp51A	28.18	0.10	27.94	0.09	28.14	0.08	28.09	0.14	0.49
PC IC	27.46	0.05	27.42	0.05	27.49	0.02	27.46	0.05	0.18
Internal control	33.17	0.15	31.49	0.52	33.12	0.19	32.59	0.87	2.67

Table 21. Reproducibility of the AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit on the RGQ.

Target	RGQ								
	Lot 1		Lot 2		Lot 3		Total		
	Mean	stdev	Mean	stdev	Mean	stdev	Mean	stdev	CV (%)
PC *A. fumigatus* TR46	29.22	0.46	28.78	0.22	29.07	0.18	29.02	0.34	1.17
PC *A. fumigatus* TR34	28.46	0.12	28.12	0.29	28.38	0.14	28.32	0.23	0.83
PC *A. fumigatus* Cyp51A	27.95	0.14	26.99	0.28	28.22	0.19	27.72	0.58	2.11
PC IC	26.70	0.10	26.08	0.30	26.74	0.13	26.51	0.36	1.36
Internal control	32.19	0.40	31.49	0.52	32.52	0.45	32.07	0.61	1.90

16.2 Clinical Performance

QCMD proficiency panels ASPDNA18S, ASPDNA 19S, ASPDNA20S, ASPDNA21S, ASPDNA22S, and ASPDNA23S were all tested with the AsperGenius® 2.0 Species Multiplex real-time PCR kit. A volume of 0.5 ml of each QCMD sample was extracted using NucliSENS® EasyMAG® with an elution volume of 50 µl. Results are presented in table 22, together with the sample content according to QCMD.

Table 22: Results QCMD panels using AsperGenius® 2.0 Species Multiplex real-time PCR kit

Sample	AsperGenius 2.0 Species (PN-101)					Sample content
	A. fum	*A. flav*	*A.* spp	IC	*A. terr*	
ASPDNA 18S-01	36.6		33.5	33.5		*A. fumigatus*
ASPDNA 18S-02	31.8		29.3	31		*A. fumigatus*
ASPDNA 18S-03			25.6	31.8		*A. niger*
ASPDNA 18S-04				34.9		Negative
ASPDNA 18S-05			29.2	33.2		*A. niger*
ASPDNA 18S-06				30		Negative
ASPDNA 18S-07	31		28.8	32.5		*A. fumigatus*
ASPDNA 18S-08	34.8		32.2	33.8		*A. fumigatus*
ASPDNA 19S-01	31.2		28.2	30.1		*A. fumigatus*
ASPDNA 19S-02				neg		Negative
ASPDNA 19S-03	35.3		32.8	34.7		*A. fumigatus*
ASPDNA 19S-04	35.1		31.7	30.8		*A. fumigatus*
ASPDNA 19S-05				37.3		Negative
ASPDNA 19S-06	38.9		35.8	36.4		*A. fumigatus*
ASPDNA 19S-07			31.4	36.2		*A. niger*
ASPDNA 19S-08			26.7	31.6		*A. niger*
ASPDNA20S-01	31.9		29	29.2		*A. fumigatus*
ASPDNA20S-02				29.2		Negative
ASPDNA20S-03	32.7		30.3	33.1		*A. fumigatus*
ASPDNA20S-04	37.3		32.2	29		*A. fumigatus*
ASPDNA20S-05	37.3		33.5	32		*A. fumigatus*
ASPDNA20S-06				32.6		Negative
ASPDNA20S-07			26.1	29.9		*A. niger*
ASPDNA20S-08			29.7	31.6		*A. niger*
ASPDNA21S-01			29.9	33.1		*A. niger*
ASPDNA21S-02	38.9		37.1	30.8		*A. fumigatus*
ASPDNA21S-03				35.8		Negative
ASPDNA21S-04	36.1		34.9	30.0		*A. fumigatus*
ASPDNA21S-05			32.9	34.1		*A. niger*
ASPDNA21S-06				30.1		Negative
ASPDNA21S-07	35.5		35.2	35.2		*A. fumigatus*
ASPDNA21S-08	31.3		30.7	30.0		
ASPDNA22S-01	30.1		30.6	30.7		*A. fumigatus*
ASPDNA22S-02	30.5		30.5	31.4		*A. fumigatus*
ASPDNA22S-03			21.3	30.7		*A. niger*
ASPDNA22S-04				30.9		
ASPDNA22S-05				31.0		
ASPDNA22S-06	32.3		32.2	30.7		*A. fumigatus*
ASPDNA22S-07	32.5		31.2	30.9		*A. fumigatus*
ASPDNA22S-08			25.1	31.9		*A. niger*
ASPDNA23S-01	32.5		32.3	35.0		*A. fumigatus*

ASPDNA23S-02				35.9		
ASPDNA23S-03	37.0		35.7	36.1		*A. fumigatus*
ASPDNA23S-04	34.0		33.6	N.A.		*A. fumigatus*
ASPDNA23S-05			29.5	40.4*		*A. niger*
ASPDNA23S-06				37.2		
ASPDNA23S-07			33,2	38.2		*A. niger*
ASPDNA23S-08	33.2		32.6	30.3		*A. fumigatus*

** Ct value affected due to strong Aspergillus signal in adjacent channel*

In addition to the QCMD proficiency testing, a validation study was performed at the PathoNostics facilities with 55 BAL samples. These samples were tested together with the first generation CE-IVD AsperGenius® Species Multiplex real-time PCR kit (PN-001) as reference. Results obtained with PN-001 and PN-101 of all 55 BAL samples were equal: 28 BAL samples revealed negative test results in both assays, whereas 27 were identified as *A. fumigatus* with both assays.

17 Troubleshooting

This section may be helpful in solving any problems that may arise (table 23).

Table 23. Troubleshooting.

Problem	Possible cause	Recommendations
Positive controls remain negative	One of the components was not added	Ensure that all components have been added
	The control was not stored properly	Check storage conditions and the expiry date of the kit (indicated on the box label)
	Positive control was added to the wrong reaction tube	Ensure that positive control was added to the correct reaction tube.
	Incorrect PCR profile / programming	Check programming of real-time cycler
		Check your work steps / pipetting scheme and check calibration of real-time PCR machine and pipettes.
IC remains negative in *Aspergillus*-negative samples	Nucleic acid degradation / inhibition	Repeat the extraction according to the protocol and ensure that all instruments are properly decontaminated
	Incorrect PCR mixture	Check if sufficient Primer/Probe mix, Taq polymerase, Master mix, and sample are added
	PCR conditions do not comply with the protocol	Check PCR conditions and repeat the PCR with correct settings if necessary

	Incorrect elution volume / insufficient IC added DNA extraction procedure is used with unknown efficiency	In case the elution is performed in 50 µl, check if 5 µl IC is added to the starting material. Contact PathoNostics for experience with the particular DNA extraction platform. It can be recommended to repeat the extraction with 10 µl IC, depending on the extraction procedure.
Negative Control generates a fluorescence signal	Carry over / contamination	Repeat the entire experiment with fresh reagents, handle samples, kit components and consumables as prescribed and spin down reagents before pipetting If the problem still occurs, repeat also the DNA-extraction Make sure you performed all steps in a laminar air flow safety cabinet II in order to prevent contamination. Add positive controls strictly at the last step. Make sure that work spaces and instruments are decontaminated regularly.
Very weak fluorescence signals also for controls	Incorrect instrument settings Incorrect real-time PCR mix	Check annealing/denaturation temperature and channel / gain settings Check if the PCR mixtures are prepared according to the protocol Check expiry date and storage conditions
Unexpected amplification curves for the PC	Incorrect instrument settings	Use Color Compensation (LC480)
The Resistance TR multiplex is negative while the Species multiplex is positive	Limited amount of *Aspergillus* DNA present in the supernatant	Test the pellet and/or use a higher volume of BAL for DNA-extraction to increase the sensitivity

18 Notice to the purchaser

The AsperGenius® 2.0 Multiplex real-time PCR kit products are manufactured by PathoNostics B.V., Maastricht, The Netherlands within quality systems accredited to ISO 13485:2016. This product is sold for use by the end-user only and may only be re-sold, distributed or re-packaged with the written approval of PathoNostics and only by licenced distributors.

Reagents of different AsperGenius® 2.0 Multiplex real-time PCR kit lots should not be combined. If a AsperGenius® 2.0 Multiplex real-time PCR kit is received in a damaged packaging or in absence of dry ice, please contact PathoNostics or your local PathoNostics distributor.

Materials used in the kit, including reagents, samples and used vials should be disposed of according to local regulations.

Other language versions of these Instructions for Use can be requested from PathoNostics or your local PathoNostics distributor. Any serious incident that has occurred in relation to a AsperGenius® 2.0 Multiplex real-time PCR kit shall be reported to PathoNostics and the competent authority of the country in which the user and/or the patient is established.

18.1 Introduced modifications since last revision

Since IFU version IFU AsperGenius 2.0 PN-101 and PN-201 v2.1 EN, the following modification were introduced:

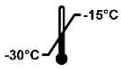
- Use of the PathoNostics Color Calibration kit is added in section 4 and 11.1
- Sample input of volumes <1.0 ml has been added to section 10.1.
- Minor update in section 11 regarding the crimson channel detection on the QS5.
- The Ct range of the PC in section 14.3 has been set wider.
- Analytical specificity A. terreus on QS5 has been added to section 16.1.1.
- Section 18.1 is added.
- Important note regarding absence of a signal in section 14.2 has been removed as this statement appeared to be incorrect, as well as in table 14.
- Information about the CFX settings (scan mode) has been added in section 12.
- ASPDNA22S and ASPDNA23S EQA data has been added to table 22.
- Specific information regarding color compensation and dye calibration has been added to Section 6.
- Additional information regarding Ct calling on the CFX96 has been added (use of single threshold).
- Statement about providing installation guides by PathoNostics has been removed in section 11 as these are not available.
- Cross-talk correction for detection channel “Crimson” has been added to section 14 for analysis on the Rotor-gene Q.

AsperGenius® 2.0 Species Multiplex real-time PCR kit

Product no.: PN-101

50 reactions

Basic UDI 87193267509PN-101E9



Store at –30 °C to –15 °C

Keep away from sunlight

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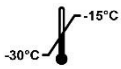
Email: info@pathonostics.com, Web: www.pathonostics.com

AsperGenius® 2.0 Resistance TR Multiplex real-time PCR kit

Product no.: PN-201

50 reactions

Basic UDI 87193267509PN-201EE



Store at -30 °C to -15 °C

Keep away from sunlight

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