Development and Validation of Hygiena's Real-Time PCR Assay for the Detection and Identification of *Aspergillus* species in Cannabis and Hemp

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BAX® System Q7   foodproof®

INTRODUCTION

Aspergillus is a common type of mold that can produce mycotoxins and cause serious health problems like aspergillosis, especially in immunocompromised individuals or people suffering from lung disease. In the USA, several cases of illness and death following the consumption of contaminated medicinal and recreational cannabis have led to the regulation of *Aspergillus* in these products.

Testing cannabis and cannabis products for *Aspergillus* allows for the timely detection and management of any potentially harmful substances, such as mold toxins.

Our kit is the perfect choice for specific and sensitive multiplex detection of *Aspergillus* in cannabis and cannabis products. Designed to completely meet all specifications of the AOAC SMPR®, the foodproof® *Aspergillus* Detection LyoKit is a lyophilized multiplex real-time PCR kit for the simultaneous detection and identification of four pathogenic *Aspergillus* species: *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus fumigatus* in a single test with only a few pipetting steps.

The method is currently being validated according to AOAC SMPR requirements for the detection of *Aspergillus* in cannabis.

PURPOSE

1. To provide a real-time PCR kit that detects the species *A. flavus, A. terreus, A. niger* and *A. fumigatus* in a single reaction and according to AOAC SMPR® requirements for the detection of *Aspergillus* in cannabis.

2. To achieve fast time-to-results: Two days to results including enrichment and DNA isolation with less than 30 minutes hands-on time.

MANUFACTURER

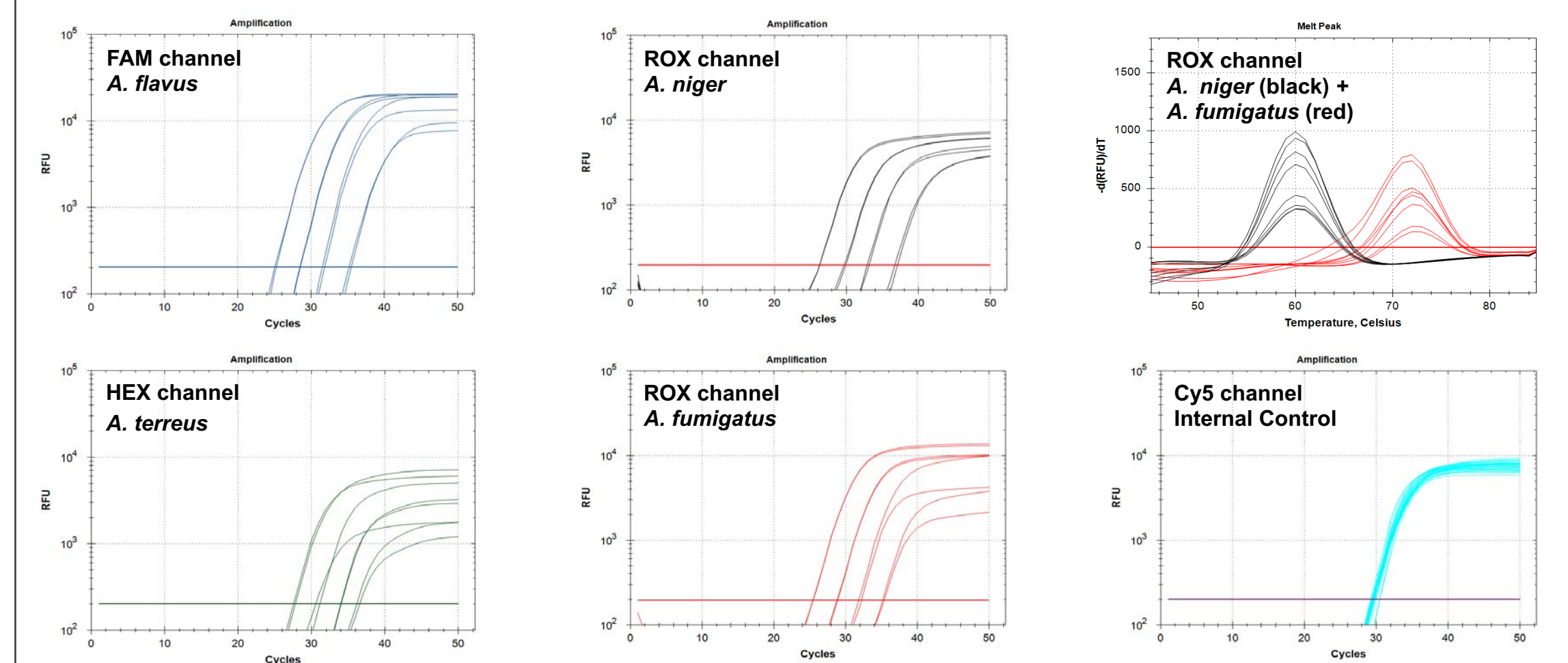Hygiena Diagnostics GmbH, Hermannswerder 17, 14473 Potsdam, Germany

foodproof® *Aspergillus* Detection LyoKit

PCR Kit:

The foodproof *Aspergillus* Detection LyoKit provides all necessary reagents for the specific detection of *A. flavus, A. terreus, A. niger* or *A. fumigatus* DNA. In addition, the kit is designed as a PCR open-platform kit and can be used with various PCR instruments.

To ensure maximum reliability of the kit and to prevent misinterpretation of negative results due to inhibition of the amplification, an Internal Control (IC) is included. A probe was designed to bind specifically the IC, allowing detection in the Cy5 channel, whereas the *A. flavus* DNA is detected in the FAM channel, the *A. terreus* DNA in the HEX channel and *A. niger* and *A. fumigatus* DNA in the ROX channel. The amplification product in the ROX channel can be attributed to either *A. niger* or *A. fumigatus* by melt curve analysis. The PCR product of *A. niger* generates a melt peak at 62 ± 3 °C; the PCR product of *A. fumigatus* generates a melt peak at 73 ± 3 °C. In case of a negative result due to inhibition of amplification by the sample DNA of interest, the amplification of the IC is suppressed as well, whereas a negative result for the sample DNA of interest and amplification of the IC clearly indicates the absence of *A. flavus, A. terreus, A. niger* or *A. fumigatus* in the sample.

The foodproof *Aspergillus* Detection LyoKit minimizes contamination risk and prevents carry-over contamination using Uracil-N-Glycosylase.

Workflow:

After sample enrichment using 1:10 w/v in Buffered Peptone Water (BPW) with chloramphenicol (100 mg/L) for 46 ± 2 hours at 37 ± 2 °C, DNA extraction was performed with either the BAX® System Lysis reagent for Yeast and Mold or the foodproof StarPrep® Two Kit. Following extraction, the lysates were analyzed with the foodproof *Aspergillus* Detection LyoKit.

Properties

Picture made with AI

Specificity:
Specificity studies of the foodproof *Aspergillus* Detection LyoKit confirmed 100% specificity for 90 target strains comprising of the four species *A. flavus (28), A. terreus (11), A. niger (26)* and *A. fumigatus (25)* and as well as more than 80 non-target strains (47 of closely related *Aspergillus* strains). All *A. flavus* strains were detected in the FAM channel, all *A. terreus* in the HEX/VIC channel and all *A. niger* and *A. fumigatus* in the ROX channel. None of the non-target strains were detected in any channel.

Sensitivity:
In the sensitivity study, the use of the PCR kit in combination with enrichment and DNA isolation resulted in relative detection limit of **1 – 10 CFU** per sample for all matrix categories tested. All PCR results were comparable to the reference culture methods for the detection of yeast and molds.

Matrix compatibility:
The foodproof *Aspergillus* Detection LyoKit was successfully tested with cannabis and hemp matrices like plant & flowers, seeds, concentrates & oils, infused edibles (cookies, chocolate, candy, coffee) and infused non-edibles (creams, shampoo, lotion).

Sensitivity Including DNA Isolation

foodproof StarPrep Two Kit DNA Extraction of Plant & Flower Mix Spiked with Target Strains After Sample Enrichment

Spiking level [cfu/ml]	*Aspergillus flavus*			*Aspergillus terreus*			*Aspergillus niger*			*Aspergillus fumigatus*			IC
	Recovery rate of 6 replicates [FAM]	Mean Cq [FAM]	CV % [FAM]	Recovery rate of 6 replicates [HEX]	Mean Cq [HEX]	CV % [HEX]	Recovery rate of 6 replicates [ROX]	Mean Cq [ROX]	CV % [ROX]	Recovery rate of 6 replicates [ROX]	Mean Cq [ROX]	CV % [ROX]	Detection [Cy5]
1 x 10⁴	100 %	30.68	0.6	100 %	31.10	0.1	100 %	29.67	0.1	100 %	29.27	0.3	ok
1 x 10³	100 %	32.89	3.0	100 %	34.26	2.2	100 %	33.03	1.7	100 %	32.15	1.4	ok
1 x 10²	100 %	36.33	3.7	100 %	37.53	3.5	83 %	36.58	1.4	100 %	35.49	4.0	ok
1 x 10¹	33 %	38.79	3.0	50 %	39.91	2.6	50 %	37.30	0.4	33 %	38.35	2.3	ok
unspiked	0 %	-	-	0 %	-	-	0 %	-	-	0 %	-	-	ok

PCR Sensitivity with DNA of the Target Species

Concentration [GE/reaction]	*Aspergillus flavus* (CBS 100927)			*Aspergillus terreus* (CBS 601.65)			*Aspergillus niger* (CBS 554.65)					*Aspergillus fumigatus* (CBS 133.61)					IC
	Recovery rate of 6 replicates [FAM]	Mean Cq [FAM]	CV % [FAM]	Recovery rate of 6 replicates [HEX]	Mean Cq [HEX]	CV % [HEX]	Recovery rate of 6 replicates [ROX]	Mean Cq [ROX]	CV % [ROX]	Tm1 [ROX] [62±3°C]	Tm2 [ROX] [73±3°C]	Recovery rate of 6 replicates [ROX]	Mean Cq [ROX]	CV % [ROX]	Tm1 [ROX] [62±3°C]	Tm2 [ROX] [73±3°C]	Detection [Cy5]
1 x 10⁴	100 %	25.48	2.1	100 %	28.44	3.6	100 %	27.10	4.8	+	-	100 %	25.98	2.0	-	+	ok
1 x 10³	100 %	28.80	1.8	100 %	31.33	1.7	100 %	29.91	1.8	+	-	100 %	29.44	2.0	-	+	ok
1 x 10²	100 %	32.10	1.7	100 %	34.60	2.0	100 %	33.06	0.9	+	-	100 %	32.73	2.0	-	+	ok
50	100 %	32.99	1.3	100 %	35.13	1.1	100 %	33.73	1.0	+	-	100 %	33.53	2.1	-	+	ok
25	100 %	34.09	2.6	100 %	36.07	1.5	100 %	34.44	0.6	+	-	100 %	34.93	2.8	-	+	ok
12	100 %	35.18	4.0	100 %	37.68	4.8	100 %	35.56	2.5	+	-	100 %	35.72	2.8	-	+	ok
6	83 %	36.15	2.7	50 %	38.70	1.4	67 %	36.07	2.9	+	-	83 %	36.12	2.9	-	+	ok
3	33 %	37.09	3.3	50 %	39.86	4.9	17 %	37.33	-	+	-	50 %	38.00	1.4	-	+	ok

PCR Detection

FAM channel *A. flavus*
ROX channel *A. niger*
ROX channel *A. niger* (black) + *A. fumigatus* (red)
HEX channel *A. terreus*
ROX channel *A. fumigatus*
Cy5 channel Internal Control

Curves show duplicates of dilution row 1x10⁴, 1x10³, 1x10², 1x10¹ GE/reaction of target strains, shown in all detection channels (FAM, HEX, ROX), including melt curve analysis (ROX) and Internal Control (Cy5).