

PathoSEEK[®] Aspergillus 5-Color and 2-Color Multiplex Detection Assays with MaGiC Lysis for Detection in Cannabis Flower and MIP Matrices

Method Developer Validation

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Abstract

Background:

Current regulations require cannabis flower and cannabis products to be free of select species of *Aspergillus*. The PathoSEEK® 5-Color and 2-Color *Aspergillus* Multiplex Assays with MaGiC Lysis Protocol are designed to detect *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus terreus* in a single qPCR (Quantitative Polymerase Chain Reaction) in cannabis flower and cannabis infused non flower matrices. Two 5-Color multiplex assays are available for use, dependent on the thermal cycler used for analysis. The PathoSEEK® 5-Color *Aspergillus* Multiplex Assay for use on the AriaMx Real-Time PCR thermal cycler (Agilent) uses the ATTO 425 Fluorophore for detection of the *Aspergillus terreus*, while the PathoSEEK® 5-Color *Aspergillus* Multiplex Assay for use on the CFX-96 (Bio-Rad) uses the Cy5.5 Fluorophore for the detection of *Aspergillus terreus*. Fluorophores for the three additional *Aspergillus* species are consistent between the two thermal cyclers. Both PCR assays will be validated in the study.

Objective:

To evaluate the PathoSEEK® 5-Color and 2-Color *Aspergillus* Multiplex Assays with MaGiC Lysis flower and MIP protocols for presence absence detection of four *Aspergillus* species in cannabis flower and marijuana-infused products (MIP).

Results:

Inclusivity and exclusivity results showed the PathoSEEK® 5-Color and 2-Color *Aspergillus* method is highly specific in discriminating target organisms found in cannabis flower and infused products from non-target organisms.

Materials

PathoSEEK® Aspergillus 2-Color Detection Assay v2 with MaGiC Lysis Kit, **P/N 420523** (contains sufficient reagents for 200 reactions)

Kit Components:

Component Name	Qty Provided	Storage Conditions
MaGiC Lysis Reagent	1 Bottle (12 mL)	RT
MaGiC Stabilization Buffer	1 Bottle (24 mL)	RT
PathoSEEK® Amplification Mix	3 Vials (67 rxns/each)	RT / -20 °C*
PathoSEEK® Aspergillus 2-Color Detection Assay v2	1 Tube (200 µL)	-20 °C

PathoSEEK® 5-Color Aspergillus Multiplex - AriaMx with MaGiC Lysis Kit, **P/N 420518** (contains sufficient reagents for 200 reactions)

Kit Components:

Component Name	Qty Provided	Storage Conditions
MaGiC Lysis Reagent	2 Bottles (12 mL each)	RT
MaGiC Stabilization Buffer	1 Bottle (24 mL)	RT
PathoSEEK® Amplification Mix	3 Vials (67 rxns each)	RT / -20 °C*
PathoSEEK® 5-Color Aspergillus Multiplex (AriaMX) Detection Assay v2	1 Tube (200 µL)	-20 °C

PathoSEEK® 5-Color Aspergillus Multiplex - CFX with MaGiC Lysis, P/N 420519 (contains sufficient reagents for 200 reactions)

Kit Components:

Component Name	Qty Provided	Storage Conditions
MaGiC Lysis Reagent	2 Bottles (12 mL each)	RT
MaGiC Stabilization Buffer	1 Bottle (24 mL)	RT
PathoSEEK® Amplification Mix	3 Vials (67 rxns each)	RT / -20 °C*
PathoSEEK® 5-Color Aspergillus Multiplex (CFX) Detection Assay v2	1 Tube (200 µL)	-20 °C

Additional Required Reagents Not in Kit:

Item P/N	Item Name	Qty Provided	Storage Conditions
420032	Potato Dextrose Broth	500 mL Bottle	2-25 °C
420033	Chloramphenicol 34 mg/mL in Solution	100 mL Bottle	-20 °C
420337	Internal Cannabis Control (ICC)*	1 Tube (50 µL)	-20 °C
420330	PathoSEEK® Aspergillus Multiplex Positive Control v3	1 Tube (50 µL)	-20 °C
420184	PCR Grade Water	500 mL Bottle	2-25 °C

*ICC is spiked into non flower matrices only

Method Developer Validation

Wet Laboratory Methodology

For the inclusivity evaluation, 50 strains of *Aspergillus* were evaluated. Target strains were cultured in Potato Dextrose Broth with Chloramphenicol for 24 hours at 37° C followed by MaGiC lysis. For exclusivity, 35 organisms were evaluated. Target strains were either cultured under optimal conditions for growth of the organism followed by DNA lysis.

Results

Of the 50 inclusivity strains tested, all 50 were correctly detected. Of the 30 exclusivity strains tested, all 35 were correctly excluded. Tables 1 and 2 present a summary of the results.

Table 1: Inclusivity Results, PathoSEEK® 5-Color and 2-Color Aspergillus Multiplex

Organism	Source	Origin	Result
<i>Aspergillus flavus</i>	ATCC 9643	Shoe sole, New Guinea	Pos
<i>Aspergillus flavus</i>	ATCC 26768	Dry cracower sausage, Poland	Pos
<i>Aspergillus flavus</i>	ATCC 16870	Butter, Japan	Pos
<i>Aspergillus flavus</i>	ATCC 16883	Cellophane. South Pacific	Pos
<i>Aspergillus flavus</i>	ATCC 11489	N/A	Pos
<i>Aspergillus flavus</i>	ATCC 11497	N/A	Pos
<i>Aspergillus flavus</i>	ATCC 204304	Human sputum, Virginia	Pos
<i>Aspergillus flavus</i>	ATCC MYA-1004	Clinical Isolate, New York City, NY	Pos
<i>Aspergillus flavus</i>	ATCC MYA-200026	Peanut cotyledons, USA	Pos
<i>Aspergillus flavus</i>	ATCC 12693	Soil	Pos
<i>Aspergillus flavus</i>	ATCC 13697	N/A	Pos
<i>Aspergillus flavus</i>	ATCC MYA-4921	N/A	Pos
<i>Aspergillus flavus</i>	ATCC MYA-3631	Human biopsy, USA	Pos
<i>Aspergillus fumigatus</i>	ATCC 16903	Human chest cavity lining, Illinois	Pos
<i>Aspergillus fumigatus</i>	ATCC 1022	Lung of chicken, Connecticut	Pos
<i>Aspergillus fumigatus</i>	ATCC 1028	Soil, Germany	Pos

<i>Aspergillus fumigatus</i>	ATCC 96918	N/A	Pos
<i>Aspergillus fumigatus</i>	ATCC 13073	Human pulmonary lesion, Maryland	Pos
<i>Aspergillus fumigatus</i>	ATCC 26934	Human with Aspergillosis disease, Montana	Pos
<i>Aspergillus fumigatus</i>	ATCC 14110	Human sputum	Pos
<i>Aspergillus fumigatus</i>	ATCC 204305	Human sputum, Virginia	Pos
<i>Aspergillus fumigatus</i>	ATCC MYA-3626	California, USA	Pos
<i>Aspergillus fumigatus</i>	ATCC MYA-3627	Jacksonville, TN, USA	Pos
<i>Aspergillus fumigatus</i>	ATCC MYA-4690	N/A	Pos
<i>Aspergillus fumigatus</i>	ATCC MYA-4915	Human sputum, Shanghai, China	Pos
<i>Aspergillus fumigatus</i>	ATCC 16424	Human lung with aspergillosis, Pennsylvania	Pos
<i>Aspergillus niger</i>	ATCC 15475	Soil	Pos
<i>Aspergillus niger</i>	ATCC 16888	N/A	Pos
<i>Aspergillus niger</i>	ATCC 9142	N/A	Pos
<i>Aspergillus niger</i>	ATCC 9029	N/A	Pos
<i>Aspergillus niger</i>	ATCC 10535	Painted pine board, Virginia	Pos
<i>Aspergillus niger</i>	ATCC 13496	Soil, Louisiana	Pos
<i>Aspergillus niger</i>	ATCC 1015	N/A	Pos
<i>Aspergillus niger</i>	ATCC 6275	Leather	Pos
<i>Aspergillus niger</i>	ATCC 66295	Jerusalem artichoke tuber, Korea	Pos
<i>Aspergillus niger</i>	ATCC 201201	Farmland, Korea	Pos
<i>Aspergillus niger</i>	ATCC 208815	Derived from ATCC 9029	Pos
<i>Aspergillus terreus</i>	ATCC 16793	Soil, Texas	Pos
<i>Aspergillus terreus</i>	ATCC 1012	Soil, Connecticut	Pos
<i>Aspergillus terreus</i>	ATCC 10690	Haversack, New Guinea	Pos
<i>Aspergillus terreus</i>	ATCC 16794	N/A	Pos
<i>Aspergillus terreus</i>	ATCC 24839	N/A	Pos
<i>Aspergillus terreus</i>	ATCC 52430	Decayed timber, India	Pos
<i>Aspergillus terreus</i>	ATCC 12238	N/A	Pos
<i>Aspergillus terreus</i>	ATCC MYA-4897	Soil, Baghdad, Iran	Pos
<i>Aspergillus terreus</i>	ATCC MYA-4898	N/A	Pos
<i>Aspergillus terreus</i>	ATCC 26604	Ankylosing spondylitis	Pos
<i>Aspergillus terreus</i>	ATCC 20542	N/A	Pos

<i>Aspergillus terreus</i>	ATCC 20064	N/A	Pos
<i>Aspergillus terreus</i>	ATCC 32587	Gamma mutant of NRRL 1960	Pos

Table 2: Exclusivity Results, PathoSEEK® 5-Color and 2-Color Aspergillus Multiplex

Organism	Source	Origin	Result
<i>Acinetobacter baumannii</i>	ATCC 19606	Urine	Neg
<i>Alternaria alternata</i>	ATCC 6663	N/A	Neg
<i>Aspergillus aculeatus</i>	ATCC 24147	N/A	Neg
<i>Aspergillus brasiliensis Varga et al.</i>	ATCC 964	Wireless radio equipment, New South Wales, Australia	Neg
<i>Aspergillus casingii</i>	ATCC 42693	Dried chillies, New Guinea	Neg
<i>Aspergillus carbonarius</i>	ATCC MYA-4641	Grape berry, Brindis, Apulia, Italy	Neg
<i>Aspergillus clavatus</i>	ATCC 1007	N/A	Neg
<i>Aspergillus deflexus</i>	ATCC 62502	Wheat, China	Neg
<i>Aspergillus fijiensis Varga et al.</i>	ATCC 20611	N/A	Neg
<i>Aspergillus niveo-glaucus</i>	ATCC 10075	N/A	Neg
<i>Aspergillus japonicus</i>	ATCC 16873	Soil, Panama	Neg
<i>Aspergillus nidulans</i>	ATCC 38163	N/A	Neg
<i>Aspergillus oryzae (Ahlburg) Cohn</i>	ATCC 10124	N/A	Pos
<i>Aspergillus parasiticus Speare</i>	ATCC 15517	N/A	Pos
<i>Aspergillus pseudo terreus Peterson et al.</i>	ATCC 10020	N/A	Pos
<i>Aspergillus tamarii</i>	ATCC 1005	tomato	Neg
<i>Aspergillus tubingensis (Schober) Mosseray</i>	ATCC 1004	N/A	Neg
<i>Aspergillus tubingensis (Schober) Mosseray</i>	ATCC 10550	N/A	Neg
<i>Aspergillus ustus</i>	ATCC 1041	Culture containment, USA	Neg
<i>Aspergillus versicolor</i>	ATCC 11730	Cellophane gas mask, India	Neg
<i>Botrytis cinerea Persoon</i>	ATCC 11542	Azalea flowers, Washington, D.C.	Neg

<i>Candida albicans</i>	ATCC 10231	Man with bronchomycosis	Neg
<i>Cryptococcus laurentii</i>	ATCC 18803	Palm wine, malaffou, Congo	Neg
<i>Cryptococcus neoformans</i>	ATCC 208821	Patient with Hodgkin's disease, New York	Neg
<i>Fusarium proliferatum</i>	ATCC 76097	Raw cotton, North Carolina	Neg
<i>Fusarium oxysporum</i>	ATCC 62506	Celery, <i>Apium graveolens</i> var. <i>dulce</i> , California, USA	Neg
<i>Fusarium solani</i>	ATCC 52628	Cardamom fruit pod, <i>Elettaria cardamomum</i> , Guatemala	Neg
<i>Mucor circinelloides</i>	ATCC 38592	N/A	Neg
<i>Mucor hiemalis</i>	ATCC 28935	Soil in spruce forest, Germany	Neg
<i>Penicillium chrysogenum</i>	ATCC 18476	Cheese?, USSR	Neg
<i>Penicillium rubens</i>	ATCC 11709	Selected from Wis. 48-701, ATCC 11707, after N-mustard exposure	Neg
<i>Penicillium venetum</i>	ATCC 16025	Hyacinthus sp. Bulb, England	Neg
<i>Pseudomonas aeruginosa</i>	ATCC 35554	N/A	Neg
<i>Rhizopus stolonifer</i>	ATCC 14037	N/A	Neg
<i>Yarrowia lipolytica</i>	ATCC 20390	Non Sporulating diploid	Neg

Limit of Detection

The method is able to detect down to 1 CFU per test portion

Proficiency Testing/Certified Reference Material Results

NSI CRM Part numbers FM-601- 604 were used to test proficiency. 24 and 48 hour enrichments of each CRM were performed in PDB + Chloramphenicol at 37°C for 24 and 48 hours.

Table 3: PathoSEEK® 2-Color Aspergillus Multiplex

Flower Matrix: 24 and 48 hour enrichment

Sample	Target FAM	24 Hours (Cq)	48 Hours (Cq)
Aspergillus Flavus in Hemp	Aspergillus niger, flavus, fumigatus and terreus	30.28	26.50
Aspergillus Flavus in Hemp	Aspergillus niger, flavus, fumigatus and terreus	30.18	26.40
Aspergillus Flavus in Hemp	Aspergillus niger, flavus, fumigatus and terreus	29.58	26.50
Aspergillus Fumigatus in Hemp	Aspergillus niger, flavus, fumigatus and terreus	31.94	30.24
Aspergillus Fumigatus in Hemp	Aspergillus niger, flavus, fumigatus and terreus	33.83	28.42
Aspergillus Fumigatus in Hemp	Aspergillus niger, flavus, fumigatus and terreus	32.47	29.21
Aspergillus Niger in Hemp	Aspergillus niger, flavus, fumigatus and terreus	32.69	32.11
Aspergillus Niger in Hemp	Aspergillus niger, flavus, fumigatus and terreus	32.11	31.36
Aspergillus Niger in Hemp	Aspergillus niger, flavus, fumigatus and terreus	31.87	31.69
Aspergillus Terreus in Hemp	Aspergillus niger, flavus, fumigatus and terreus	39.13	33.59
Aspergillus Terreus in Hemp	Aspergillus niger, flavus, fumigatus and terreus	38.77	32.74
Aspergillus Terreus in Hemp	Aspergillus niger, flavus, fumigatus and terreus	36.09	32.02
Positive Control	Aspergillus niger, flavus, fumigatus and terreus	24.46	24.01
NTC	Aspergillus niger, flavus, fumigatus and terreus	No Cq	No Cq

Table 4: PathoSEEK® 5-Color Aspergillus Multiplex

Flower Matrix: 24 and 48 hour enrichment

Sample	Target FAM	24 Hours (Cq)	48 Hours (Cq)	Target Rox	24 Hours (Cq)	48 Hours (Cq)	Target Cy5	24 Hours (Cq)	48 Hours (Cq)	Target Atto 425	24 Hours (Cq)	48 Hours (Cq)
Aspergillus Flavus in Hemp	Fumigatus	No Cq	No Cq	Niger	No Cq	No Cq	Flavus	27.68	25.26	Terreus	No Cq	No Cq
Aspergillus Flavus in Hemp	Fumigatus	No Cq	No Cq	Niger	No Cq	No Cq	Flavus	27.68	25.73	Terreus	No Cq	No Cq
Aspergillus Flavus in Hemp	Fumigatus	No Cq	No Cq	Niger	No Cq	No Cq	Flavus	27.72	25.23	Terreus	No Cq	No Cq
Aspergillus Fumigatus in Hemp	Fumigatus	31.62	28.95	Niger	No Cq	No Cq	Flavus	No Cq	No Cq	Terreus	No Cq	No Cq
Aspergillus Fumigatus in Hemp	Fumigatus	31.32	29.75	Niger	No Cq	No Cq	Flavus	No Cq	No Cq	Terreus	No Cq	No Cq
Aspergillus Fumigatus in Hemp	Fumigatus	30.71	29.05	Niger	No Cq	No Cq	Flavus	No Cq	No Cq	Terreus	No Cq	No Cq
Aspergillus Niger in Hemp	Fumigatus	No Cq	No Cq	Niger	32.76	32.51	Flavus	No Cq	No Cq	Terreus	No Cq	No Cq
Aspergillus Niger in Hemp	Fumigatus	No Cq	No Cq	Niger	32.7	32.51	Flavus	No Cq	No Cq	Terreus	No Cq	No Cq
Aspergillus Niger in Hemp	Fumigatus	No Cq	No Cq	Niger	31.9	32.99	Flavus	No Cq	No Cq	Terreus	No Cq	No Cq
Aspergillus Terreus in Hemp	Fumigatus	No Cq	No Cq	Niger	No Cq	30.97	Flavus	No Cq	No Cq	Terreus	35.83	No Cq
Aspergillus Terreus in Hemp	Fumigatus	No Cq	No Cq	Niger	No Cq	No Cq	Flavus	No Cq	No Cq	Terreus	36.5	30.78
Aspergillus Terreus in Hemp	Fumigatus	No Cq	No Cq	Niger	No Cq	No Cq	Flavus	No Cq	No Cq	Terreus	36.31	31.02
Positive Control	Fumigatus	28.88	26.9	Niger	27.74	26.62	Flavus	25.27	24.46	Terreus	27.16	27.33
NTC	Fumigatus	No Cq	No Cq	Niger	No Cq	No Cq	Flavus	No Cq	No Cq	Terreus	No Cq	No Cq

Conclusions:

The MaGiC Lysis kits in conjunction with the PathoSEEK® 5-Color and 2-Color Aspergillus Multiplex Assays are a rapid method for the detection *Aspergillus flavus*, *A. fumigatus*, *A. niger* and *A. terreus* in cannabis flower and cannabis infused products.

REVISION HISTORY

Version	Date	Description
v1	August 2024	Validation for MaGiC Lysis with Aspergillus 5 Color and 2 Color Multiplex Methods

DISCLAIMER

This test was developed, and its performance characteristics determined by Medicinal Genomics Company, for laboratory use. Any deviations from this protocol are not supported by MGC.

This test has not been validated on remediated (irradiated, ozone treated, acid treated, hydrogen peroxide treated, etc.) samples. Samples that have undergone remediation may cause discordant results between plating methods and PathoSEEK methods. When remediated samples produce a result above the action limit on qPCR, we recommend confirming viability with an approved plating method.

Results may vary based on laboratory conditions. Altitude and humidity are factors known to affect the growth of bacterial and fungal species.

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