CERTIFICATION

AOAC® *Performance Tested*SM

Certificate No.

022103

The AOAC Research Institute hereby certifies the method known as:

GENE-UP® AspergillusPRO

manufactured by

Invisible Sentinel®
3711 Market Street, Suite 910
Philadelphia, PA 19104 USA

This method has been evaluated in the AOAC® *Performance Tested Methods*SM Program and found to perform as stated by the manufacturer contingent to the comments contained in the manuscript. This certificate means that an AOAC® Certification Mark License Agreement has been executed which authorizes the manufacturer to display the AOAC *Performance Tested*SM certification mark along with the statement - "THIS METHOD'S PERFORMANCE WAS REVIEWED BY AOAC RESEARCH INSTITUTE AND WAS FOUND TO PERFORM TO THE MANUFACTURER'S SPECIFICATIONS" - on the above-mentioned method for a period of one calendar year from the date of this certificate (November 22, 2021 – December 31, 2022). Renewal may be granted at the end of one year under the rules stated in the licensing agreement.

Scott Coates

Scott Coates, Senior Director
Signature for AOAC Research Institute

November 22, 2021
Date

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Invisible Sentinel®
3711 Market Street, Suite 910
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METHOD NAME
GENE-UP® *AspergillusPRO*

CATALOG NUMBER
IS1086

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APPLICABILITY OF METHOD
Analytes – *Aspergillus flavus, A. fumigatus, A. niger* and *A. terreus*.

Matrixes – Cannabis flower (delta 9-tetrahydrocannabinol >0.3%, 10 g & 1 g).

Performance claims - The GENE-UP AspergillusPRO method met the requirements of the *Standard Method Performance Requirement* (SMPR) *for Detection of Aspergillus in Cannabis and Cannabis Products* 2019.001 (2).

REFERENCE GUIDELINES
AOAC International SMPR 2019.001, Standard Method Performance Requirements for Detection of *Aspergillus* in Cannabis and Cannabis Products. (2)

ORIGINAL CERTIFICATION DATE
February 21, 2021

CERTIFICATION RENEWAL RECORD
Renewed annually through December 2022.

METHOD MODIFICATION RECORD
1. **November 2021 Level 1**
2. **May 2022 Level 1**

SUMMARY OF MODIFICATION
1. **Editorial/clerical changes.**
2. **Editorial/clerical changes.**

Under this AOAC® *Performance Tested*[SM] License Number, 022103 this method is distributed by:
NONE

Under this AOAC® *Performance Tested*[SM] License Number, 022103 this method is distributed as:
NONE

PRINCIPLE OF THE METHOD (1)
GENE-UP® AspergillusPRO is a real-time PCR assay for the detection of the four most prevalent pathogenic *Aspergillus* species in cannabis flower. The GENE-UP AspergillusPRO assay utilizes a multiplex detection method that targets *Aspergillus terreus* on the FAM channel, *Aspergillus niger* on the HEX channel, *Aspergillus flavus* on the ROX channel, and *Aspergillus fumigatus* on the Cy5 channel. Additionally, the assay has an internal amplification control (IAC) on the Cy5.5 channel. GENE-UP AspergillusPRO couples the advantages of the real-time PCR format with a streamlined enrichment and sampling protocol to enable detection of target organisms in matrix after 24 or 48 hours of enrichment. The addition of Buffer G into the methodology reduces potential inhibition with the detection of the target species.
GENE-UP AspergillusPRO is intended for use by personnel familiar with basic sample collection and preparation techniques associated with pathogenic organism detection during production and packaging. GENE-UP AspergillusPRO kits are specifically designed to be easy-to-use and reduce the need for advanced training in molecular biology.

DISCUSSION OF THE VALIDATION STUDY (1)
The GENE-UP AspergillusPRO successfully detected *Aspergillus* species from cannabis flower at both 24 and 48 h of enrichment. No statistical difference was observed between the candidate and confirmed results at either time point. For the validation study, 1 false positive result was observed with the 10 g test portions at both 24 and 48 h of enrichment. The primary enrichment for this sample was re-subcultured after storage at 2-8°C and no growth of any organism was observed on the agar plates. This may indicate that the PCR result was the result of cross contamination of the lysate during PCR preparation procedure. For the 1 g data set, 1 false negative result was observed at the 24 h time frame. The enrichment was reanalyzed after storage at 2-8°C and a positive result was obtained indicating the sample most likely contained low levels of the target organism. Since there was no cause to omit the original data point, it was included in the statistical analysis. The assay has minimal hands-on time required for both the 24- and 48-hour time points. Pre-aliquoted Digest and PCR reagent tubes further decreased the amount of time to process samples. Integration of Buffer G into the procedure reduces potential for inhibition in the PCR analysis through a combination of dilution and enhancement of enzymatic lysis.
In the inclusivity and exclusivity evaluations, all inclusivity organisms were correctly identified. Certain exclusivity organisms were detected by the candidate method. However, these strains have previously been identified as extremely close neighbors and were originally deposited as the target strains with which they cross reacted. The lot-to-lot consistency and stability study show no significant differences observed between the lots evaluated. Using POD analysis, the robustness study shows no statistically significant differences between the 8 treatment combinations and the nominal condition for the assay. The enrichment for this sample was subcultured multiple times and no recovery of the target organism was obtained.

Table 3. Inclusivity Results for the GENE-UP AspergillusPRO (1)

Organism	Reference #	Origin	*A. flavus*	*A. terreus*	*A. niger*	*A. fumigatus*
Aspergillus flavus	BMX[a] 700082	Unknown	+	-	-	-
Aspergillus flavus	BMX 700083	Unknown	+	-	-	-
Aspergillus flavus	BMX 700231	Unknown	+	-	-	-
Aspergillus flavus	BMX 700250	Unknown	+	-	-	-
Aspergillus flavus	KSU[b] A1446	Peanut cotyledons, USA	+	-	-	-
Aspergillus flavus	KSU A1120	Peanut cotyledons, USA	+	-	-	-
Aspergillus flavus	ATCC[c] 9643	Shoe Sole , New Guinea	+	-	-	-
Aspergillus flavus	NRRL[d] 453	Brazil nuts	+	-	-	-
Aspergillus flavus	NRRL 1957	Cellophane, South Pacific	+	-	-	-
Aspergillus flavus	NRRL 5565	Turkey feed mix	+	-	-	-
Aspergillus flavus	ATCC 204304	Human sputum, Virginia	+	-	-	-
Aspergillus flavus	ATCC 15547	Raw Spanish peanuts, California,	+	-	-	-
Aspergillus flavus	ATCC 22546	Moldy corn, Iowa	+	-	-	-
Aspergillus terreus	NRRL 255	Soil Connecticut	-	+	-	-
Aspergillus terreus	NRRL 265	Soil, Texas	-	+	-	-
Aspergillus terreus	NRRL 571	Cloth, New Guinea	-	+	-	-
Aspergillus terreus	NRRL 680	Soil	-	+	-	-
Aspergillus terreus	KSU A1156	Unknown	-	+	-	-
Aspergillus terreus	ATCC 1012	Soil, Connecticut	-	+	-	-
Aspergillus terreus	ATCC 20542	Unknown	-	+	-	-
Aspergillus terreus	DTO[e] 032-G7	Environmental Sample	-	+	-	-
Aspergillus terreus	DTO 008-G3	Blended almond pits, Netherlands	-	+	-	-
Aspergillus terreus	DTO 235-A4	Indoor Air sample, Netherlands	-	+	-	-
Aspergillus terreus	DTO 017-A1	Viral Media Contamination, Netherlands	-	+	-	-
Aspergillus terreus	DTO 032-G8	Environmental, New Mexico	-	+	-	-
Aspergillus niger	BMX 700313	Tannin-gallic acid fermentation, Connecticut, USA	-	-	+	-
Aspergillus niger	ATCC 16888	Painted pine board, Virginia	-	-	+	-
Aspergillus niger	ATCC 10535	USA	-	-	+	-
Aspergillus niger	KSU A1143	Mc Dermott, Mellon Inst., Pennsylvania	-	-	+	-
Aspergillus niger	KSU A1516	USA	-	-	+	-
Aspergillus niger	KSU A732	unknown	-	-	+	-
Aspergillus niger	KSU A1513	USA	-	-	+	-
Aspergillus niger	KSU A1121	Tannin-gallic acid fermentation, Connecticut, USA	-	-	+	-
Aspergillus niger	NRRL 326	Mc Dermott, Mellon Inst., Pennsylvania	-	-	+	-
Aspergillus niger	ATCC 1015	K. Yakoyama, Japan	-	-	+	-
Aspergillus niger	NRRL 341	unknown	-	-	+	-
Aspergillus niger	KSU A1099	Leather	-	-	+	-
Aspergillus niger	ATCC 6275	Tannin-gallic acid fermentation, Connecticut, USA	-	-	+	-
Aspergillus fumigatus	BMX 700000	Unknown	-	-	-	+
Aspergillus fumigatus	BMX 700084	Unknown	-	-	-	+
Aspergillus fumigatus	BMX 700085	Unknown	-	-	-	+
Aspergillus fumigatus	BMX 700220	Unknown	-	-	-	+
Aspergillus fumigatus	KSU A1152	Unknown	-	-	-	+
Aspergillus fumigatus	KSU A1259	Clinical Isolate, UK	-	-	-	+
Aspergillus fumigatus	KSU A1435	Clinical Isolate	-	-	-	+
Aspergillus fumigatus	KSU A1461	Unknown	-	-	-	+
Aspergillus fumigatus	KSU A1241	Environmental Ireland	-	-	-	+
Aspergillus fumigatus	NRRL 5109	Pus, Chicago, Illinois	-	-	-	+
Aspergillus fumigatus	ATCC 204305	Human sputum, Virginia	-	-	-	+
Aspergillus fumigatus	NRRL 163	PUS, Chicago, Illinois	-	-	-	+
Aspergillus fumigatus	NRRL 5517	Pus Chicago, Illinois	-	-	-	+

[a]BMX – bioMérieux Culture Collection, Hazelwood, MO

[b]KSU- Kansas State University Culture Collection, Manhattan, KS

[c]ATCC – American Type Culture Collection, Manassas, VA

[d] NRRL – ARS Culture Collection, Peoria, IL

[e]DTO - Applied and Industrial Mycology department Westerdijk Institute Culture Collection, Utrecht, Netherlands; (+) – Positive detection of the target; (-) – No detection of the target

Table 4. Exclusivity Results for the GENE-UP AspergillusPRO (1)

Organism	Reference #	Origin	*A. flavus*	*A. terreus*	*A. niger**	*A. fumigatus*
Altenaria alternata	BMX 700 081	Unknown	-	-	-	-
Aspergillus alabamensis	DTO 010-C2	Soli, Citrus Grove FL USA	-	-	-	-
Aspergillus aureoterreus	NRRL 1923	Soil, Argentina	-	-	-	-
Aspergillus brasiliensis	BMX 700 087	Unknown	-	-	-[g]	-
Aspergillus caesiellus	ATCC 42693	Dried chillies, New Guinea	-	-	-	-
Aspergillus carbonarius	DTO 294-D2	Coffee Beans, Thailand	-	-	-	-
Aspergillus carneus	DTO 051-D8	Air Sample, DC, USA	-	-	-	-
Aspergillus clavatus	DTO 389-E6	Indoor Environment, Germany	-	-	-	-
Aspergillus deflectus	DTO 026-I1	Soil, Brazil	-	-	-	-
Aspergillus fijiensis	DTO[e] 131-A7	Indoor Environment, Thailand	-	-	-	-
Aspergillus fischeri	NRRL 181	Soil, Argentina	-	-	-	+
Aspergillus glaucus	DTO 355-H1	Air Sample, Puerto Rico	-	-	-	-
Aspergillus hortai	NRRL 274	Soil, Argentina	-	-	-	-
Aspergillus luchuensis	CY20S[d]	Unknown	-	-	-	-
Aspergillus nidulans	BMX 700 086	Unknown	-	-	-	-
Aspergillus oryzae	ATCC[a] 10124	Unknown	+	-	-	-
Aspergillus parasiticus	NRRL[b] 465	Unknown	+	-	-	-
Aspergillus pseudoterreus	NRRL 4017	Soil, Argentina	-	-	-	-
Aspergillus tubingensis	BMX[c] 700 127	Unknown	-	-	-	-
Aspergillus tubingensis	ATCC 10550	Soil, Argentina	-	-	-	-
Aspergillus ustus	DTO 414-I5	Swab, Netherlands	-	-	-	-
Aspergillus versicolor	NRRL 238	Soil, Argentina	-	-	-	-
Aspergillus welwitschiae	CY20S	Unknown	-	-	+	-
Candida albicans	BMX 300 494	Unknown	-	-	-	-
Cladosporium sphaerospermum	ISCC 7.2	Wine, CA	-	-	-	-
Cryptococcus laurentii	BMX 300 821	Unknown	-	-	-	-
Cryptococcus neoformans	BMX 301 418	Unknown	-	-	-	-
Fusarium oxysporum spp complex	BMX 700 095	Unknown	-	-	-	-
Fusarium proliferatum	BMX 700 096	Unknown	-	-	-	-
Fusarium solani spp complex	BMX 700 097	Unknown	-	-	-	-
Mucor circinelloides	BMX 700 235	Unknown	-	-	-	-
Mucor hiemalis	DTO 187-H6	Environmental Sample, Netherlands	-	-	-	-
Penicillium brevicompactum	ISCC 3.1	Wine, CA	-	-	-	-
Penicillium chrysogenum	DTO 382-H1	Margarine, Belgium	-	-	-	-
Penicillium grisefulum	ISCC[f] 7.1	Wine, CA	-	-	-	-
Penicillium rubens	DTO 418-D1	Contamination Agar, Netherlands	-	-	-	-
Penicillium venetum	DTO 200-I6	Sputum, Netherlands	-	-	-	-
Rhizopus stolonifer	DTO 356-B5	Wrap, Netherlands	-	-	-	-
Yarrowia lipolytica	DTO 124-G4	Swab, Egg Processing, Netherlands	-	-	-	-
Altenaria alternata	BMX 700 081	Unknown	-	-	-	-

[a]ATCC – American Type Culture Collection, Manassas, VA

[b]NRRL – ARS Culture Collection, Peoria, IL' [c]BMX – bioMérieux Culture Collection, Hazelwood, MO

[d]CY- BCN Research Laboratories Culture Collection, Rockford, TN

[e]DTO - Applied and Industrial Mycology department Westerdijk Institute Culture Collection, Utrecht, Netherlands

[f]ISCC- Invisible Sentinel Culture Collection, Philadelphia, PA; (+) – Positive detection of the target; (-) – No detection of the target

[g]Detected in non-selective media. Strain not detected following test method enrichment protocol in Aspergillus Enrichment Broth

Table 5: GENE-UP AspergillusPRO Presumptive vs Confirmed Results (Paired) – POD Results Cannabis 10 g (1)

Matrix and Inoculum	Time Point / Lysis	MPN[a] / Test Portion	N[b]	x[c]	Presumptive		x	Confirmed		dPOD$_{cp}$[f]	95% CI[g]
					POD$_{cp}$[d]	95% CI		POD$_{cc}$[e]	95% CI		
Cannabis flower 10g (*Aspergillus niger* ATCC 16888*)*	24 hours	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
		1.25 (0.73 - 2.05)	20	13	0.65	0.43, 0.82	12	0.60	0.39, 0.78	0.05	(-0.11, 0.21)
		6.25 (3.65 - 10.3)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)
	48 hour	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
		1.25 (0.73 - 2.05)	20	13	0.65	0.43, 0.82	12	0.60	0.39, 0.78	0.05	(-0.11, 0.21)
		6.25 (3.65 - 10.3)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)

[a]MPN = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator, with 95% confidence interval.
[b]N = Number of test potions.
[c]x = Number of positive test portions.
[d]POD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.
[e]POD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.
[f]$dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

Table 6: GENE-UP AspergillusPRO Presumptive vs Confirmed Results (Paired) – POD Results Cannabis 1 g (1)

Matrix and Inoculum	Time Point / Lysis	MPN[a] / Test Portion	N[b]	x[c]	Presumptive		x	Confirmed		dPOD$_{cp}$[f]	95% CI[g]
					POD$_{cp}$[d]	95% CI		POD$_{cc}$[e]	95% CI		
Cannabis flower 1g (*Aspergillus niger* ATCC 16888*)*	24 hour	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
		1.90 (1.19 - 3.16)	20	14	0.70	0.48, 0.86	15	0.75	0.53, 0.89	-0.05	(-0.21, 0.11)
		11.4 (2.97 - 43.3)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)
	48 hour	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(-0.47, 0.47)
		1.90 (1.19 - 3.16)	20	15	0.75	0.53, 0.89	15	0.75	0.53, 0.89	0.00	(-0.13, 0.13
		11.4 (2.97 - 43.3)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(-0.47, 0.47)

[a]MPN = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator, with 95% confidence interval.
[b]N = Number of test potions.
[c]x = Number of positive test portions.
[d]POD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.
[e]POD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.
[f]$dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

REFERENCES CITED

1. Johnson, R., Joelsson, A., and Brown, A., Validation of the GENE-UP® AspergillusPRO for the Detection of *Aspergillus* in Cannabis Flower, AOAC® *Performance Tested*℠ certification number 022103.
2. AOAC International SMPR 2019.001, Standard Method Performance Requirements for Detection of *Aspergillus* in Cannabis and Cannabis Products. http://www.eoma.aoac.org/SMPR/upload/116/SMPR%202019_001.pdf (accessed January 2021)