# CERTIFICATION

## AOAC® *Performance Tested*SM

Certificate No.

**032104**

**The AOAC Research Institute hereby certifies the method known as:**

## iQ-Check *Aspergillus* and iQ-Design *Aspergillus* Real-Time PCR Kits

**Corporate Location**
**Bio-Rad Laboratories**
**2000 Alfred Nobel Drive**
**Hercules, CA 94547 USA**

**Manufacturing Location**
**Bio-Rad Laboratories**
**925 Alfred Nobel Drive**
**Hercules, CA 94547 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 (April 29, 2022 – 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

May 6, 2022
Date

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**Hercules, CA 94547**
**USA**

**METHOD NAME**
iQ-Check *Aspergillus* and iQ-Design *Aspergillus* Real-Time PCR Kits

**CATALOG NUMBERS**
12010806, 17006992, 12015336, 12015260, 12015337

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Modification April 2022 reviewed internally by AOAC Research Institute.

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

**Matrixes – Cannabis flower (10 g, delta 9-tetrahydrocannabinol[(THC] >0.3%;), Cannabis concentrate, Solvent based (5 g), and Cannabis concentrate, Nonsolvent based (5 g)**

**Performance claims - The iQ-Check *Aspergillus* and iQ-Design *Aspergillus* real-time PCR kit meets the method performance requirements outlined in AOAC SMPR® 2019.001, *Standard Method Performance Requirements* for Detection of *Aspergillus* in Cannabis and Cannabis Products for cannabis flower and cannabis concentrates (2) and Appendix J of the Official Methods of Analysis Manual (3).**

**STANDARD METHOD PERFORMANCE REQUIREMENTS**
**AOAC International SMPR 2019.001, Standard Method Performance Requirements for Detection of *Aspergillus* in Cannabis and Cannabis Products. (2)**

**ORIGINAL CERTIFICATION DATE**
**March 19, 2021**

**CERTIFICATION RENEWAL RECORD**
**Renewed annually through December 2022.**

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

**SUMMARY OF MODIFICATION**
1. **Editorial/clerical changes for clarity.**
2. **Addition of iQ-Design *Aspergillus* Speciation Solution.**

**Under this AOAC® *Performance Tested*SM License Number, 032104 this method is distributed by:**
**NONE**

**Under this AOAC® *Performance Tested*SM License Number, 032104 this method is distributed as:**
**NONE**

**PRINCIPLE OF THE METHOD (1)**
The Bio-Rad iQ-Check test kits are based on gene amplification and detection by the use of real-time PCR technology. Ready-to-use PCR reagents contain oligonucleotides (primers and probes) specific for target analytes, as well as DNA polymerase and nucleotides. The iQ-Check *Aspergillus* kit is designed to detect *A. flavus, A. fumigatus, A. niger,* and *A. terreus* in a variety of matrices. Detection and data analysis are optimized for use with Bio-Rad real-time PCR instruments, such as the CFX96 Touch Deep Well system.
PCR is a powerful technique used to generate many copies of target DNA. During the PCR reaction, several cycles of heating and cooling facilitate DNA denaturation, primer binding to the target region, and DNA polymerase extension of the DNA, creating copies (amplicons) of the target region. A synthetic DNA internal control is included in the reaction mix. This control is amplified with a specific probe at the same time as the target analytes. It allows for the validation of any negative result.

**DISCUSSION OF THE VALIDATION STUDY (1)**

*Cannabis Flower*.— The iQ-Check *Aspergillus* real-time PCR kit successfully detected *Aspergillus* species from 10 g sample portions of cannabis flower when incubated in 90 mL BPW with chloramphenicol (0.3 g/L) at 48 h. Using POD analysis, no statistically significant differences were observed between the number of positive samples detected by the candidate methods and the confirmed results for all test portions analyzed with or without FDRS. The iQ-Check *Aspergillus* real-time PCR kit successfully detected targeted *Aspergillus* from Lot 1 of the cannabis flower when incubated in 90 mL BPW with chloramphenicol (0.3 g/L) at 48 h following FDRS treatment. The same 14 samples from Lot 1 were positive post enrichment when analyzed with and without FDRS and by cultural confirmations. The changes in Cq values between conditions indicate that the FDRS is removing free DNA from the samples without impacting the confirmations. For Lot 2 samples, four of the PCR positive samples analyzed without FDRS became negative after FDRS treatment. One of these samples was confirmed negative by the culture method indicating the FDRS worked as indicated by removing a false positive result. The other three samples were confirmed positive by the culture method indicating potential false negative results. These discrepant results can be related to two scenarios described below.

1) Testing of the different extraction conditions require using two different aliquots of 1 mL of enrichment. Normal distribution of low-level organisms in the enrichment could result in the target organism not homogenously distributed between the two different aliquots. For Lot 2 samples, the fractional positive level is already at the lower end of the acceptable range.
2) If the heat block used for DNA extraction does not reach the 95–100°C as indicated in the user guide before starting the lysing step, the enzymatic action of the FDRS will not be deactivated and will degrade DNA from lysed cells. Laboratories are advised to ensure heat block temperatures reach 95–100°C before starting the DNA extraction.

*Cannabis Concentrates*.— The iQ-Check *Aspergillus* real-time PCR kit successfully detected *Aspergillus* species from 5 g sample portions of cannabis concentrates solvent-based and cannabis concentrates nonsolvent-based when incubated in 45 mL BPW with chloramphenicol (0.3 g/L) at 48 h. Using POD analysis, no statistically significant differences were observed between the number of positive samples detected by the candidate methods and the confirmed results for all test portions.

In the inclusivity and exclusivity evaluations, all inclusivity organisms were correctly identified. All the exclusivity organisms were correctly excluded with the exception of *A. oryzae* (ATCC 10124) and *A. parasiticus* (ATCC 15517). Both strains have been identified as very close neighbors and are deposited as *Aspergillus flavus*. The lot-to-lot consistency and stability study show no significant differences observed across the shelf life of the kits for three different lots of kits at each time point tested.

The detection of *Aspergillus* in 48 h is challenge even for highly sensitive methods like PCR. To overcome this challenge, the iQ-Check Standard extraction protocol is used as it includes a step to concentrate the target organism. The iQ-Check *Aspergillus* real-time PCR method is easy to perform but the Standard extraction protocol does have additional hands-on time when compared to the Easy extraction protocols for the other iQ-Check kits. The method provides results in a few hours post incubation of the enrichment for up to 94 sample replicates compared to traditional agar methods that take a minimum of five days for identification. The CFX Manager IDE software is user friendly with the ability to track lot information and sample identification quickly and with ease. Since results are displayed in real-time, the user is able to quickly and accurately determine if results will be valid before the end of the run. The software also provides the user the option to analyze each individual Cq curve to help aid in problem solving any issues within an individual reaction. PCR inhibition is commonly seen when testing cannabis flower. The internal control that is included in each PCR reaction validates negative results by interpreting the sample as inhibited when PCR inhibition occurs. This advantage of the software allows the user to know when to retest the sample the with the iQ-Check Purification Reagent.

**Table 3. Inclusivity Results for the iQ-Check *Aspergillus* Assay (1)**

| Organism | Source | Origin | PCR Result |
|---|---|---|---|
| *A. flavus* | CECT[1] 20802 | Walnuts, USA | + |
| *A. flavus* | CECT 20400 | Sugar cane, Cuba | + |
| *A. flavus* | CECT 2949 | Shoe sole, Papua New Guinea | + |
| *A. flavus* | ATCC[2] 16883 | Cellophane, South Pacific | + |
| *A. flavus* | CECT 2684 | Unknown | + |
| *A. flavus* | CECT 20403 | Cuba | + |
| *A. flavus* | CECT 2685 | Unknown | + |
| *A. flavus* | CECT 2687 | Unknown | + |
| *A. flavus* | CECT 2686 | Corn, USA | + |
| *A. flavus* | CECT 20402 | Cuba | + |
| *A. flavus* | CECT 20401 | Sugar cane, Cuba | + |
| *A. flavus* | MUCL[3] 9068 | Melted cheese, Belgium | + |
| *A. flavus* | MUCL 14492 | Unknown | + |
| *A. flavus* | MUCL 47419 | Soil, Cuba | + |
| *A. fumigatus* | CECT 2071 | Unknown | + |
| *A. fumigatus* | CECT 20228 | Unknown | + |
| *A. fumigatus* | CECT 20190 | Unknown | + |
| *A. fumigatus* | ATCC 34506 | Soil | + |
| *A. fumigatus* | CECT 20827 | Olive, Spain | + |
| *A. fumigatus* | CECT 20366 | Compost, Spain | + |
| *A. fumigatus* | DSM[4] 21023 | Twig of *Juniperus communis* | + |
| *A. fumigatus* | DSM 790 | Unknown | + |
| *A. fumigatus* | ATCC 36607 | Clinical isolate | + |
| *A. fumigatus* | ATCC 14110 | Human sputum | + |
| *A. fumigatus* | MUCL 978 | Soil, Belgium | + |
| *A. fumigatus* | MUCL 8004 | Dead twig, Belgium | + |
| *A. fumigatus* | MUCL 46660 | Silage, Belgium | + |
| *A. niger* | CECT 2775 | Plant galls, China | + |
| *A. niger* | CECT 2088 | USA | + |
| *A. niger* | ATCC 16888 | Unknown | + |
| *A. niger* | CECT 2090 | Northern America | + |
| *A. niger* | CECT 2806 | Unknown | + |
| *A. niger* | CECT 2807 | Leather, Unknown | + |
| *A. niger* | CECT 2907 | Bran, Unknown | + |
| *A. niger* | CECT 20385 | Unknown | + |

| | | | |
|---|---|---|---|
| *A. niger* | DSM 63263 | Radio set, Australia | + |
| *A. niger* | DSM 737 | Unknown | + |
| *A. niger* | MUCL 28699 | Seed, Sudan | + |
| *A. niger* | MUCL 15973 | Wheat flour | + |
| *A. niger* | MUCL 44639 | Unknown | + |
| *A. terreus* | CECT 20365 | Sewage farm mud, Spain | + |
| *A. terreus* | CECT 20194 | Spain | + |
| *A. terreus* | CECT 2808 | Haversack, Papua New Guinea | + |
| *A. terreus* | ATCC 1012 | Soil, Connecticut | + |
| *A. terreus* | DSM 62071 | Optic glass, Pakistan | + |
| *A. terreus* | CECT 20404 | Sugar cane, Cuba | + |
| *A. terreus* | CECT 20405 | Sugar cane, Cuba | + |
| *A. terreus* | CECT 20406 | Cuba | + |
| *A. terreus* | CECT 20407 | Cuba | + |
| *A. terreus* | CECT 20408 | Cuba | + |
| *A. terreus* | MUCL 14006 | Soil, Zaïre | + |
| *A. terreus* | MUCL 21932 | Humic soil, Africa | + |
| *A. terreus* | MUCL 38642 | Soil | + |

[1] Spanish Type Culture Collection. Valencia, Spain
[2] American Type Culture Collection, Manassas, VA
[3] Belgian Coordinated Collections of Microorganisms, Brussels, Belgium
[4] The Leibniz Institute DSMZ, Brunswick, Germany
(+) = Positive detection of the target

**Table 4. Exclusivity Results for the iQ-Check *Aspergillus* Assay (1)**

| Organism | Source | Origin | PCR Result |
|---|---|---|---|
| *Acinetobacter baumanii* | DSM[2] 30007 | Urine | - |
| *Alternaria alternata* | DSM 1102 | *Prunus malus*, Japan | - |
| *Aspergillus aculeatus* | CECT[3] 2968 | Soil, India | - |
| *Aspergillus alabamensis* | ATCC[4] 3633 | Human | - |
| *Aspergillus brasiliensis* Varga et al. | ATCC 9642 | Wireless Radio Equipment, Australia | - |
| *Aspergillus caesiellus* | CECT 20807 | Dried chillies, Papua New Guinea | - |
| *Aspergillus carbonarius* | CECT 2086 | Northern America | - |
| *Aspergillus carneus* | DSM 1518 | Unknown | - |
| *Aspergillus clavatus* | CECT 2674 | Unknown | - |
| *Aspergillus deflectus* | CBS[5] 109.55 | Soil, Brazil | - |
| *Aspergillus fijiensis* | ATCC 20611 | Unknown | - |
| *Aspergillus glaucus* | CBS 516.65 | Unpainted board, USA | - |
| *Aspergillus japonicus* | DSM 2345 | Unknown | - |
| *Aspergillus nidulans* | CBS 114.63 | Human nail, India | - |
| *Aspergillus oryzae*[1] | ATCC 10124 | Unknown | + |
| *Aspergillus parasiticus*[1] | ATCC 15517 | Rat colon carcinomas | + |
| *Aspergillus pseudoterreus* | ATCC 10020 | Soil Texas | - |
| *Aspergillus steynii* | CECT 20510 | Pollen of bee, Spain | - |
| *Aspergillus tubingensis* | ATCC 1004 | Unknown | - |
| *Aspergillus tubingensis* | ATCC 10550 | Unknown | - |
| *Aspergillus ustus* | DSM 1349 | Soil | - |
| *Aspergillus versicolor* | CECT 2903 | Unknown | - |
| *Botrytis cinerea Persoon* | DSM 877 | Unknown | - |
| *Candida albicans* | ATCC 10231 | Man with bronchomycosis | - |
| *Cryptococcus laurentii* | ATCC 18803 | Palm wine, Congo | - |
| *Cryptococcus neoformans* | DSM 11959 | Cerebrospinal fluid, USA | - |
| *Fusarium proliferatum* | CECT 20944 | Rice caryopses, Spain | - |
| *Fusarium oxysporum* | DSM 62306 | *Allium cepa*, rotting bulb, USA | - |
| *Fusarium solani* | DSM 10696 | Human corneal ulcer, Nigeria | - |
| *Mucor circinelloides* | DSM 1191 | Fermenting rice | - |
| *Mucor hiemalis* | DSM 2655 | Unknown | - |
| *Penicillium rubens / chrysogenum* | DSM 1075 | Moldy fruit of cantaloupe, USA | - |
| *Pseudomonas aeruginosa* | ATCC 10145 | Unknown | - |
| *Rhizopus stolonifer* | DSM 2194 | Unknown | - |
| *Scopulariopsis acremonium* | DSM 1987 | Wheat field soil, Germany | - |
| *Yarrowia lipolytica* | CECT 1469 | Unknown | - |

[1] *A. oryzae* ATCC 10124 and *A. parasiticus* ATCC 15517 strains are deposited as *Aspergillus flavus*
[2]The Leibniz Institute DSMZ, Brunswick, Germany
[3] Spanish Type Culture Collection. Valencia, Spain
[4] American Type Culture Collection, Manassas, VA
[5] Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands
(-) = No detection of target
(+) = Positive detection in FAM Channel

**Table 5. iQ-Check *Aspergillus* Results – Presumptive vs. Confirmed (1)**

| Matrix | Strain | MPN[a]/ Test Portion | N[b] | Presumptive | | | Confirmed | | | dPOD$_{CP}$[f] | 95% CI[g] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | X[c] | POD$_{CP}$[d] | 95% CI | X | POD$_{CC}$[e] | 95% CI | | |
| Cannabis Flower, 10g, Lot 1 (No FDRS Treatment) | Natural contamination (*A. flavus* and *A. fumigatus*) | 0.73 (0.41, 1.25) | 20 | 14 | 0.70 | 0.48, 0.86 | 14 | 0.70 | 0.48, 0.86 | 0.00 | -0.13, 0.13 |
| Cannabis Flower, 10g, Lot 1 (FDRS Treatment) | Natural contamination (*A. flavus* and *A. fumigatus*) | 0.73 (0.41, 1.25) | 20 | 14 | 0.70 | 0.48, 0.86 | 14 | 0.70 | 0.48, 0.86 | 0.00 | -0.13, 0.13 |
| Cannabis Flower, 10g, Lot 2 (No FDRS Treatment) | Natural contamination (*A. flavus* and *A. fumigatus*) | 0.51 (0.25 - 0.96) | 20 | 8 | 0.40 | 0.22, 0.61 | 7 | 0.35 | 0.18, 0.57 | 0.05 | -0.11, 0.21 |
| Cannabis Flower, 10g, Lot 2 (FDRS Treatment) | Natural contamination (*A. flavus* and *A. fumigatus*) | 0.51 (0.25 - 0.96) | 20 | 4 | 0.20 | 0.08, 0.42 | 7 | 0.35 | 0.18, 0.57 | -0.15 | -0.35, 0.05 |
| Cannabis Concentrate - solvent based, 5 g | *Aspergillus flavus* ATCC 16883 | 0.0 | 5 | 0 | 0.00 | 0.00, 0.43 | 0 | 0.00 | 0.00, 0.43 | 0.00 | -0.47, 0.47 |
| | | 1.28 (0.74 - 2.15) | 20 | 15 | 0.75 | 0.53, 0.89 | 13 | 0.65 | 0.43, 0.82 | 0.10 | -0.08, 0.28 |
| | | 3.65 (1.55 - 8.55) | 5 | 5 | 1.00 | 0.57, 1.00 | 5 | 1.00 | 0.57, 1.00 | 0.00 | -0.47, 0.47 |
| Cannabis Concentrate - nonsolvent based, 5 g | *Aspergillus fumigatus* ATCC 9197 | 0.0 | 5 | 0 | 0.00 | 0.00, 0.43 | 0 | 0.00 | 0.00, 0.43 | 0.00 | -0.47, 0.47 |
| | | 0.57 (0.25 - 1.01) | 20 | 8 | 0.40 | 0.22, 0.61 | 7 | 0.35 | 0.18, 0.57 | 0.05 | -0.11, 0.21 |
| | | 2.22 (0.94 - 5.25) | 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 calculated using the LCF MPN calculator provided by AOAC RI, with 95% confidence interval

[b]N = Number of test portions

[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 and confirmed 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

**DISCUSSION OF MODIFICATION APPROVED APRIL 2022 (4)**

*Cannabis Flower*.— The iQ-Design *Aspergillus flavus, Aspergillus fumigatus,* and *Aspergillus niger* assays successfully identified the target *Aspergillus* species from 10 g sample portions of cannabis flower when incubated in 90 mL BPW with chloramphenicol (0.3 g/L) at 48 h using the same DNA extract following iQ-Check *Aspergillus* real-time PCR kit screening. Using POD analysis, no statistically significant differences were observed between the number of positive samples detected by the candidate methods and the confirmed results for all test portions analyzed with or without FDRS. The iQ-Design *Aspergillus* speciation real-time PCR assays successfully detected targeted *Aspergillus* from Lot 1 of the cannabis flower when incubated in 90 mL BPW with chloramphenicol (0.3 g/L) at 48 h following FDRS treatment. The same 14 samples from Lot 1 were positive post enrichment when analyzed with and without FDRS and by cultural confirmations. For Lot 2 samples, four of the PCR positive samples analyzed without FDRS became negative after FDRS treatment. One of these samples was confirmed negative by the culture method indicating the FDRS worked as indicated by removing a false positive result. The other three samples were confirmed positive by the culture method indicating potential false negative results. These discrepant results can be related to two scenarios described below.

1) Testing of the different extraction conditions require using two different aliquots of 1 mL of enrichment. Normal distribution of low-level organisms in the enrichment could result in the target organism not homogenously distributed between the two different aliquots. For Lot 2 samples, the fractional positive level is already at the lower end of the acceptable range.
2) If the heat block used for DNA extraction does not reach the 95–100°C as indicated in the user guide before starting the lysing step, the enzymatic action of the FDRS will not be deactivated and will degrade DNA from lysed cells. Laboratories are advised to ensure heat block temperatures reach 95–100°C before starting the DNA extraction.

*Cannabis Concentrates*.— The iQ-Design *Aspergillus flavus, Aspergillus fumigatus,* and *Aspergillus niger* assays successfully identified the target *Aspergillus* species from 5 g sample portions of cannabis concentrates solvent-based and cannabis concentrates nonsolvent-based when incubated in 45 mL BPW with chloramphenicol (0.3 g/L) at 48 h using the same DNA extract following iQ-Check *Aspergillus* real-time PCR kit screening. Using POD analysis, no statistically significant differences were observed between the number of positive samples detected by the candidate methods and the confirmed results for all test portions.

In the inclusivity and exclusivity evaluations, all inclusivity organisms were correctly identified. All the exclusivity organisms were correctly excluded with the exception of *A. oryzae* (ATCC 10124) and *A. parasiticus* (ATCC 15517) which were detected by the iQ-Design *Aspergillus flavus* assay. Both strains have been identified as very close neighbors and were originally deposited as *Aspergillus flavus* in the ATCC database indicating a close phylogenic relationship to the target organisms.

The detection of *Aspergillus* in 48 h is challenge even for highly sensitive methods like PCR. To overcome this challenge, the iQ-Check Standard extraction protocol is used as it includes a step to concentrate the target organism. The iQ-Check *Aspergillus* real-time PCR method for screening followed by the iQ-Design *Aspergillus* speciation assays are easy to perform providing results in a few hours post incubation of the enrichment for up to 94 sample replicates compared to traditional agar methods that take a minimum of five days for identification. The CFX Manager IDE software is user friendly with the ability to track lot information and sample identification quickly and with ease. Since results are displayed in real-time, the user is able to quickly and accurately determine if results will be valid before the end of the run. The software also provides the user the option to analyze each individual Cq curve to help aid in problem solving any issues within an individual reaction. PCR inhibition is commonly seen when testing cannabis flower. The internal control that is included in each PCR reaction validates negative results by interpreting the sample as inhibited when PCR inhibition occurs. This advantage of the software allows the user to know when to retest the sample the with the iQ-Check Purification Reagent or a 1:10 dilution of the DNA extract.

**Table 3. Inclusivity Results for the iQ-Design *Aspergillus* Speciation Assays (4)**

| Organism | Source | Origin | iQ-Design *A. flavus* Assay Result | iQ-Design *A. fumigatus* Assay Result | iQ-Design *A. niger* Assay Result |
|---|---|---|---|---|---|
| *A. flavus* | CECT[1] 20802 | Walnuts, USA | + | - | - |
| *A. flavus* | CECT 20400 | Sugar cane, Cuba | + | - | - |
| *A. flavus* | CECT 2949 | Shoe sole, Papua New Guinea | + | - | - |
| *A. flavus* | ATCC[2] 16883 | Cellophane, South Pacific | + | - | - |
| *A. flavus* | CECT 2684 | Unknown | + | - | - |
| *A. flavus* | CECT 20403 | Cuba | + | - | - |
| *A. flavus* | CECT 2685 | Unknown | + | - | - |
| *A. flavus* | CECT 2687 | Unknown | + | - | - |
| *A. flavus* | CECT 2686 | Corn, USA | + | - | - |
| *A. flavus* | CECT 20402 | Cuba | + | - | - |
| *A. flavus* | CECT 20401 | Sugar cane, Cuba | + | - | - |
| *A. flavus* | MUCL[3] 9068 | Melted cheese, Belgium | + | - | - |
| *A. flavus* | MUCL 14492 | Unknown | + | - | - |
| *A. flavus* | MUCL 47419 | Soil, Cuba | + | - | - |
| *A. fumigatus* | CECT 2071 | Unknown | - | + | - |
| *A. fumigatus* | CECT 20228 | Unknown | - | + | - |
| *A. fumigatus* | CECT 20190 | Unknown | - | + | - |
| *A. fumigatus* | ATCC 34506 | Soil | - | + | - |
| *A. fumigatus* | CECT 20827 | Olive, Spain | - | + | - |
| *A. fumigatus* | CECT 20366 | Compost, Spain | - | + | - |
| *A. fumigatus* | DSM[4] 21023 | Twig of *Juniperus communis* | - | + | - |
| *A. fumigatus* | DSM 790 | Unknown | - | + | - |
| *A. fumigatus* | ATCC 36607 | Clinical isolate | - | + | - |
| *A. fumigatus* | ATCC 14110 | Human sputum | - | + | - |
| *A. fumigatus* | MUCL 978 | Soil, Belgium | - | + | - |
| *A. fumigatus* | MUCL 8004 | Dead twig, Belgium | - | + | - |
| *A. fumigatus* | MUCL 46660 | Silage, Belgium | - | + | - |
| *A. niger* | CECT 2775 | Plant galls, China | - | - | + |
| *A. niger* | CECT 2088 | USA | - | - | + |
| *A. niger* | ATCC 16888 | Unknown | - | - | + |
| *A. niger* | CECT 2090 | Northern America | - | - | + |
| *A. niger* | CECT 2806 | Unknown | - | - | + |
| *A. niger* | CECT 2807 | Leather, Unknown | - | - | + |
| *A. niger* | CECT 2907 | Bran, Unknown | - | - | + |
| *A. niger* | CECT 20385 | Unknown | - | - | + |
| *A. niger* | DSM 63263 | Radio set, Australia | - | - | + |
| *A. niger* | DSM 737 | Unknown | - | - | + |
| *A. niger* | MUCL 28699 | Seed, Sudan | - | - | + |
| *A. niger* | MUCL 15973 | Wheat flour | - | - | + |
| *A. niger* | MUCL 44639 | Unknown | - | - | + |

[1] Spanish Type Culture Collection. Valencia, Spain
[2] American Type Culture Collection, Manassas, VA
[3] Belgian Coordinated Collections of Microorganisms, Brussels, Belgium
[4] The Leibniz Institute DSMZ, Brunswick, Germany
(+) = Positive detection of the target

**Table 4. Exclusivity Results for the iQ-Design *Aspergillus* Speciation Assays (4)**

| Organism | Source | Origin | iQ-Design *A. flavus* Assay Result | iQ-Design *A. fumigatus* Assay Result | iQ-Design *A. niger* Assay Result |
|---|---|---|---|---|---|
| *Acinetobacter baumanii* | DSM[2] 30007 | Urine | - | - | - |
| *Alternaria alternata* | DSM 1102 | *Prunus malus*, Japan | - | - | - |
| *Aspergillus aculeatus* | CECT[3] 2968 | Soil, India | - | - | - |
| *Aspergillus alabamensis* | ATCC[4] 3633 | Human | - | - | - |
| *Aspergillus brasiliensis* Varga et al. | ATCC 9642 | Wireless Radio Equipment, Australia | - | - | - |
| *Aspergillus caesiellus* | CECT 20807 | Dried chillies, Papua New Guinea | - | - | - |
| *Aspergillus carbonarius* | CECT 2086 | Northern America | - | - | - |
| *Aspergillus carneus* | DSM 1518 | Unknown | - | - | - |
| *Aspergillus clavatus* | CECT 2674 | Unknown | - | - | - |
| *Aspergillus deflectus* | CBS[5] 109.55 | Soil, Brazil | - | - | - |
| *Aspergillus fijiensis* | ATCC 20611 | Unknown | - | - | - |
| *Aspergillus glaucus* | CBS 516.65 | Unpainted board, USA | - | - | - |
| *Aspergillus japonicus* | DSM 2345 | Unknown | - | - | - |
| *Aspergillus nidulans* | CBS 114.63 | Human nail, India | - | - | - |
| *Aspergillus oryzae*[1] | ATCC 10124 | Unknown | + | - | - |
| *Aspergillus parasiticus*[1] | ATCC 15517 | Rat colon carcinomas | + | - | - |
| *Aspergillus pseudoterreus* | ATCC 10020 | Soil Texas | - | - | - |
| *Aspergillus steynii* | CECT 20510 | Pollen of bee, Spain | - | - | - |
| *Aspergillus terreus* | CECT 20365 | Sewage farm mud, Spain | - | - | - |
| *Aspergillus terreus* | CECT 20194 | Spain | - | - | - |
| *Aspergillus terreus* | CECT 2808 | Haversack, Papua New Guinea | - | - | - |
| *Aspergillus terreus* | ATCC 1012 | Soil, Connecticut | - | - | - |
| *Aspergillus terreus* | DSM 62071 | Optic glass, Pakistan | - | - | - |
| *Aspergillus terreus* | CECT 20404 | Sugar cane, Cuba | - | - | - |
| *Aspergillus terreus* | CECT 20405 | Sugar cane, Cuba | - | - | - |
| *Aspergillus terreus* | CECT 20406 | Cuba | - | - | - |
| *Aspergillus terreus* | CECT 20407 | Cuba | - | - | - |
| *Aspergillus terreus* | CECT 20408 | Cuba | - | - | - |
| *Aspergillus terreus* | MUCL[6] 14006 | Soil, Zaïre | - | - | - |
| *Aspergillus terreus* | MUCL 21932 | Humic soil, Africa | - | - | - |
| *Aspergillus terreus* | MUCL 38642 | Soil | - | - | - |
| *Aspergillus tubingensis* | ATCC 1004 | Unknown | - | - | - |
| *Aspergillus tubingensis* | ATCC 10550 | Unknown | - | - | - |
| *Aspergillus ustus* | DSM 1349 | Soil | - | - | - |
| *Aspergillus versicolor* | CECT 2903 | Unknown | - | - | - |
| *Botrytis cinerea Persoon* | DSM 877 | Unknown | - | - | - |
| *Candida albicans* | ATCC 10231 | Man with bronchomycosis | - | - | - |
| *Cryptococcus laurentii* | ATCC 18803 | Palm wine, Congo | - | - | - |
| *Cryptococcus neoformans* | DSM 11959 | Cerebrospinal fluid, USA | - | - | - |
| *Fusarium proliferatum* | CECT 20944 | Rice caryopses, Spain | - | - | - |
| *Fusarium oxysporum* | DSM 62306 | *Allium cepa*, rotting bulb, USA | - | - | - |
| *Fusarium solani* | DSM 10696 | Human corneal ulcer, Nigeria | - | - | - |
| *Mucor circinelloides* | DSM 1191 | Fermenting rice | - | - | - |
| *Mucor hiemalis* | DSM 2655 | Unknown | - | - | - |
| *Penicillium rubens / chrysogenum* | DSM 1075 | Moldy fruit of cantaloupe, USA | - | - | - |
| *Pseudomonas aeruginosa* | ATCC 10145 | Unknown | - | - | - |
| *Rhizopus stolonifer* | DSM 2194 | Unknown | - | - | - |
| *Scopulariopsis acremonium* | DSM 1987 | Wheat field soil, Germany | - | - | - |
| *Yarrowia lipolytica* | CECT 1469 | Unknown | - | - | - |

[1] *A. oryzae* ATCC 10124 and *A. parasiticus* ATCC 15517 strains are deposited as *Aspergillus flavus*

[2]The Leibniz Institute DSMZ, Brunswick, Germany

[3] Spanish Type Culture Collection. Valencia, Spain

[4] American Type Culture Collection, Manassas, VA

[5] Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands

[6] Belgian Coordinated Collections of Microorganisms, Brussels, Belgium

(-) = No detection of target

(+) = Positive detection in FAM Channel

**Table 5. iQ-Design Results – Presumptive vs. Confirmed (4)**

| Matrix | Strain | Kit | MPN[a]/ Test Portion | N[b] | Presumptive | | | Confirmed | | | dPOD$_{CP}$[f] | 95% CI[g] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | X[c] | POD$_{CP}$[d] | 95% CI | X | POD$_{CC}$[e] | 95% CI | | |
| Cannabis Flower, 10g, Lot 1 (No FDRS Treatment) | Natural contamination *A. flavus* | iQ-Design *A. flavus* | 0.73 (0.41, 1.25) | 20 | 5 | 0.25 | 0.11, 0.47 | 5 | 0.25 | 0.11, 0.47 | 0.00 | -0.13, 0.13 |
| Cannabis Flower, 10g, Lot 1 (FDRS Treatment) | Natural contamination *A. flavus* | iQ-Design *A. flavus* | 0.73 (0.41, 1.25) | 20 | 5 | 0.25 | 0.11, 0.47 | 5 | 0.25 | 0.11, 0.47 | 0.00 | -0.13, 0.13 |
| Cannabis Flower, 10g, Lot 1 (No FDRS Treatment) | Natural contamination *A. fumigatus* | iQ-Design *A. fumigatus* | 0.73 (0.41, 1.25) | 20 | 9 | 0.45 | 0.26, 0.66 | 9 | 0.45 | 0.26, 0.66 | 0.00 | -0.13, 0.13 |
| Cannabis Flower, 10g, Lot 1 (FDRS Treatment) | Natural contamination *A. fumigatus* | iQ-Design *A. fumigatus* | 0.73 (0.41, 1.25) | 20 | 9 | 0.45 | 0.26, 0.66 | 9 | 0.45 | 0.26, 0.66 | 0.00 | -0.13, 0.13 |
| Cannabis Flower, 10g, Lot 2 (No FDRS Treatment) | Natural contamination *A. flavus* | iQ-Design *A. flavus* | 0.51 (0.25 - 0.96) | 20 | 3 | 0.15 | 0.05, 0.36 | 3 | 0.15 | 0.05, 0.36 | 0.00 | -0.13, 0.13 |
| Cannabis Flower, 10g, Lot 2 (FDRS Treatment) | Natural contamination *A. flavus* | iQ-Design *A. flavus* | 0.51 (0.25 - 0.96) | 20 | 2 | 0.10 | 0.03, 0.30 | 3 | 0.15 | 0.05, 0.36 | -0.05 | -0.21, 0.11 |
| Cannabis Flower, 10g, Lot 2 (No FDRS Treatment) | Natural contamination *A. fumigatus* | iQ-Design *A. fumigatus* | 0.51 (0.25 - 0.96) | 20 | 5 | 0.25 | 0.11, 0.47 | 4 | 0.20 | 0.08, 0.42 | 0.05 | -0.11, 0.21 |
| Cannabis Flower, 10g, Lot 2 (FDRS Treatment) | Natural contamination *A. fumigatus* | iQ-Design *A. fumigatus* | 0.51 (0.25 - 0.96) | 20 | 2 | 0.10 | 0.03, 0.30 | 4 | 0.20 | 0.08, 0.42 | -0.10 | -0.28, 0.08 |

[a]MPN = Most Probable Number is calculated using the LCF MPN calculator provided by AOAC RI, with 95% confidence interval
[b]N = Number of test portions
[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 and confirmed 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. iQ-Design Results – Presumptive vs. Confirmed (4)**

| Matrix | Strain | Kit | MPN[a]/ Test Portion | N[b] | Presumptive | | | Confirmed | | | dPOD$_{CP}$[f] | 95% CI[g] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | X[c] | POD$_{CP}$[d] | 95% CI | X | POD$_{CC}$[e] | 95% CI | | |
| Cannabis Concentrate - solvent based, 5 g | *Aspergillus flavus* ATCC 16883 | iQ-Design *A. flavus* | 0.0 | 5 | 0 | 0.00 | 0.00, 0.43 | 0 | 0.00 | 0.00, 0.43 | 0.00 | -0.47, 0.47 |
| | | | 1.28 (0.74 - 2.15) | 20 | 15 | 0.75 | 0.53, 0.89 | 13 | 0.65 | 0.43, 0.82 | 0.10 | -0.08, 0.28 |
| | | | 3.65 (1.55 - 8.55) | 5 | 5 | 1.00 | 0.57, 1.00 | 5 | 1.00 | 0.57, 1.00 | 0.00 | -0.47, 0.47 |
| Cannabis Concentrate - nonsolvent based, 5 g | *Aspergillus fumigatus* ATCC 9197 | iQ-Design *A. fumigatus* | 0.0 | 5 | 0 | 0.00 | 0.00, 0.43 | 0 | 0.00 | 0.00, 0.43 | 0.00 | -0.47, 0.47 |
| | | | 0.57 (0.25 - 1.01) | 20 | 8 | 0.40 | 0.22, 0.61 | 7 | 0.35 | 0.18, 0.57 | 0.05 | -0.11, 0.21 |
| | | | 2.22 (0.94 - 5.25) | 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 calculated using the LCF MPN calculator provided by AOAC RI, with 95% confidence interval
[b]N = Number of test portions
[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 and confirmed 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

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1. Clark, M., Validation of the Bio-Rad iQ-Check *Aspergillus* Real-Time PCR Kit for the Detection of Select *Aspergillus* Species from Select Cannabis Matrices, AOAC® *Performance Tested*SM certification number 032104.
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
3. *Official Methods of Analysis* (2019), 21st Edition, Appendix J, AOAC INTERNATIONAL, Rockville, MD.
4. Clark, M., Method Modification of the Bio-Rad iQ-Check *Aspergillus* Real-Time PCR Kit for the Confirmation of *Aspergillus flavus, Aspergillus fumigatus,* and *Aspergillus niger* from Select Cannabis Matrices Using iQ-Design *Aspergillus* Species Assays , AOAC® *Performance Tested*SM certification number 032104. Approved April 2022.