iQ-Check *Aspergillus* Kit

User Guide

Test for the real-time PCR detection of *Aspergillus flavus*, *A. fumigatus*, *A. niger*, and *A. terreus* in cannabis and cannabis-infused products

Catalog #12010806

BIO-RAD

Table of Contents

Section 1
Introduction

Due to increased regulations and legislation on the local and federal level concerning medicinal and recreational marijuana, consumers now have access to cannabis edibles and inhalables. These products can be contaminated with yeast and molds, including *Aspergillus* spp. Inhalation of *Aspergillus* spores may worsen asthma or lead to aspergillosis, a group of diseases especially dangerous to those with a weakened immune system, damaged lungs, or allergies. These diseases include invasive, noninvasive, and semi-invasive aspergillosis.

Not all of the hundreds of different *Aspergillus* species are dangerous to humans. Four species have been identified as hazards in cannabis and cannabis-infused products — *A. flavus*, *A. fumigatus*, *A. niger*, and *A. terreus*.

Section 2
The iQ-Check *Aspergillus* Technology

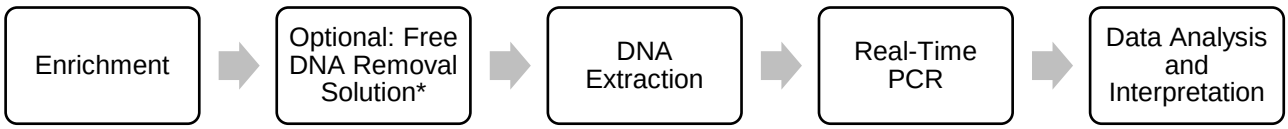
The iQ-Check *Aspergillus* Kit is a multiplex test based on gene amplification and detection by real-time PCR. The kit's ready-to-use PCR reagents contain oligonucleotides (primers and probes) specific for *A. flavus*, *A. fumigatus*, *A. niger*, and *A. terreus*, as well as DNA polymerase and nucleotides. Detection and data analysis are optimized for use with a Bio-Rad real-time PCR instrument, such as the CFX96 Touch Deep Well Real-Time PCR Detection System, with analysis using CFX Manager IDE Software.

PCR is a powerful technique used to generate many copies of target DNA. During the PCR reaction, several cycles of heating and cooling allow DNA denaturation followed by primers annealing to the target region, at which point DNA polymerase uses these primers and deoxynucleotide triphosphates (dNTPs) to extend the DNA, creating copies of the target DNA. These copies are called amplicons.

In real-time PCR, specific probes are used to detect DNA during the amplification step by hybridizing to the amplicons. These probes are linked to a fluorophore that fluoresces only when hybridized to the target sequence. FAM is the fluorophore linked to the probe hybridizing to the *A. flavus*, *A. fumigatus,* and *A. niger*–specific DNA sequences while Texas Red is linked to the probe hybridizing to the *A. terreus*–specific DNA sequence. In the absence of target DNA, no fluorescence will be detected. As the amount of amplicons increases with each round of amplification, fluorescence intensity also increases. The optical module measures this fluorescence at the annealing step during each PCR cycle while the associated software plots the fluorescence intensity versus number of cycles.

A synthetic DNA internal control is included in the reaction mix to validate any negative results. This control is amplified at the same time as the *Aspergillus* target DNA sequences and is detected by a third fluorophore.

This test allows the qualitative detection of *Aspergillus* in select matrices samples previously enriched by culture. It includes the following five main steps:



* Please refer to the user guide of the iQ-Check Free DNA Removal Solution (#10000058391) for the conditions of use.

Section 3
Kit Components

The iQ-Check *Aspergillus* Kit contains sufficient reagents for 96 tests (94 samples).

Reagent ID	Reagent	Quantity Provided, ml
A	Lysis reagent	1 bottle, 20
B	Fluorescent probes	1 tube, 0.55
C	Amplification mix	1 tube, 4.4
D	PCR negative control	1 tube, 0.5
E	PCR positive control	1 tube, 0.25
F	Lysis beads	1 bottle (17.6 g)

Section 4
Shelf Life and Storage

Once received, the kit must be stored at 2–8°C. Reagents stored at this temperature can be used until the expiration date indicated on the tubes.

Section 5
Materials Required but Not Supplied

Equipment

- Lab paddle blender for homogenizing test samples
- Incubator for sample microbiological enrichment
- Specific for extraction in sterile 1.5 ml conical screwcap tubes
    - Benchtop centrifuge capable of 10,000–12,000 x g
    - Dry heat block at 37 ± 2°C and 95–100°C
    - Cell disruptor, for example Disruptor Genie (Scientific Industries, Inc.)
- Specific for extraction in deep well plate
    - Heating thermoshaker* capable of maintaining 37 ± 2°C and 95–100°C, with a mixing speed of at least 1,300 rpm
    - DW 40 Deepwell Microplate Washer (catalog #3590137)
    - Benchtop centrifuge with deep well rotor (capable of 2,250 x g)
- Vortexer

- Magnetic stir plate
- 20, 200, and 1,000 µl micropipets
- Tips for repeat pipettors; sterile, individually packaged
- Bio-Rad real-time PCR system*; for example, the CFX96 Touch Deep Well System (catalog #3600037)

Note: We recommend using an uninterrupted power supply (UPS) with the thermal cycler.

* Contact Bio-Rad Technical Support for information on recommended instruments.

Supplies

- Enrichment medium: buffered peptone water (for example, BPW Plus catalog #3564684, dehydrated, 500 g; 3554179, 225 ml x 6 bottles; 3555790, 5 L x 2 bags; 3555795, 3 L x 4 bags. BPW Standard catalog #12013259, dehydrated, 500 g; 12013258 dehydrated, 5 kg; 12013260 5L x 2 bags)
- Chloramphenicol, 0.3 g/L (MilliporeSigma C0378, C1919, or equivalent)
- iQ-Check Free DNA Removal Solution (catalog #3594970)
- iQ-Check Purification Reagent (catalog #12012383)
- Ethanol, 96%
- Filter sample bags
- Specific for extraction in tubes
    - 1.5 ml conical screwcap tubes, sterile (for example, catalog #2240110XTU)
- Specific for extraction in a deep well plate
    - 96-well deep well plate (iQ-Check Deep Well Microplates, catalog #3594900)
    - Plastic sealing film (TeSeE NSP Plastic Sealing Film, catalog #3590139)
- Surfactant or emulsifying agent (MilliporeSigma 15S7 or equivalent)
- PCR plate sealing film (X-Pierce Films, catalog #3593977, or Pre-Pierced Plate Sealing Film, #3600040, North America only)
- PCR plates, tubes, sealing tape, and caps
- Sterile filter tips adaptable to 20, 200, and 1,000 µl micropipets
- Tips for Combitip pipets or equivalent repeat pipettors; sterile, individually packaged
- 1 and 10 ml pipets
- 2 and 5 ml sterile test tubes
- Powder-free gloves

- Distilled sterile water
- Bleach, 5%
- Cleaning agent such as DNA AWAY or RNase AWAY

Section 6
Safety Precautions and Recommendations for Best Results

- This test must be performed by trained personnel
- Samples and enrichment cultures must be handled as potentially infectious material and discarded according to local rules and regulations
- All potentially infectious material should be autoclaved before disposal
- The quality of results depends on strict compliance with Good Laboratory Practices (for example, the EN ISO 7218 standard), especially concerning PCR:
    - Never circulate laboratory equipment (pipets, tubes, etc.) from one workstation to another
    - Always use a positive control and a negative control for each series of amplification reactions
    - Do not use reagents after their expiration date
    - Vortex reagents from the kit before using them to ensure homogeneity
    - Periodically verify the accuracy and precision of pipets, as well as correct functioning of the instruments
    - Change gloves often, especially if you suspect they are contaminated
    - Clean work spaces periodically with 5% bleach and other decontaminating agents such as DNA AWAY
    - Use powder-free gloves and avoid fingerprints and writing on tube caps. Both will interfere with data acquisition
- It is strongly advised to follow the general requirements described in the standard EN ISO 22174:2005 (Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food pathogens — General requirements and definitions)
- iQ-Check *Aspergillus* Kit
    - All substances or mixtures in the test kit are classified products, according to the Globally Harmonized System. Contact with acids may cause release of toxic gases. No special precautions are necessary if used correctly. If the product is inhaled, supply fresh air and consult a doctor in case of complaints. After eye contact with the product, rinse opened eye for several minutes under running water. If the products are swallowed, induce vomiting and call for medical help. Exclusivity results indicated that *A. oryzae* (ATCC 10124) and *A. parasiticus* (ATCC 15517) tested positive in the FAM Channel.

- CFX96 Touch Deep Well Real-Time PCR Detection System

  - Improper use of the CFX96 Touch Deep Well Real-Time PCR Detection System may cause personal injury or damage to the instrument. Some components may pose a risk of personal injury due to excessive heat if improperly handled. For safe use, the CFX96 Touch Deep Well Real-Time PCR Detection System must be operated only by qualified laboratory personnel who have been appropriately trained. Servicing of instrument must be performed only by Bio-Rad Field Service Engineers

- Enrichment

  - The user should read, understand, and follow all safety information in the instructions for the iQ-Check Aspergillus Kit. Retain the safety instructions for future reference. To reduce the risks associated with exposure to chemicals and biohazards, perform testing in a properly equipped laboratory under the control of trained personnel. Always follow standard laboratory safety practices, including wearing appropriate protective apparel and eye protection while handling reagents and contaminated samples. Avoid contact with the contents of the enrichment media and reagent tubes after amplification. Dispose of enriched samples according to current industry standards

  - *Aspergillus* species are Biosafety Level 2 organisms. Biological samples such as enrichments have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations on disposal of biological waste. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities utilizing the appropriate safety equipment (for example, physical containment devices). Individuals should be trained in accordance with applicable regulatory and company/institution requirements before working with potentially infectious materials

  - When testing is complete, all materials and media possibly containing pathogens should be decontaminated following current industry standards for the disposal of contaminated waste (that is, autoclave for 20 min at 121°C). Consult the Safety Data Sheet for additional information and local regulations for disposal

Section 7
Protocol

It is strongly recommended to read the entire protocol before starting the test.

The following table outlines the different protocols that can be used depending on the application and the scope of the validation:

Scope (matrices)	Enrichment	DNA Extraction		Certification
		Method	Format	
Cannabis flower and cannabis-infused products	BPW + chloramphenicol* 45–51 hr at 37 ± 1°C	Standard II	Tube/Deep Well	AOAC PTM**
Concentrates and cannabis-infused oils and fats	BPW + chloramphenicol 45–51 hr at 37 ± 1°C Add 1% v/v surfactant	Standard II	Tube/Deep Well	AOAC PTM**

* Prepare a chloramphenicol working solution by dissolving 360 mg of powder in 12 ml of 96% ethanol. Add 10 ml of chloramphenicol solution to 1 L of sterilized BPW (0.3 g/L).

**AOAC PTM covers cannabis flower (10 g) and cannabis concentrates (5 g)

A. Sample Enrichment

Media should be at room temperature (20–25ºC) before use. A filtered sample bag should be used for all matrices tested.

1. Homogenize *n* g of sample in 9 x *n* ml prewarmed BPW supplemented with 0.3 g/L chloramphenicol (for example, 10 g in 90 ml) in a stomacher bag with incorporated filter.

2. Incubate for 45–51 hr at 37 ± 1°C.

Note: If homogenization of samples using a 1:10 enrichment is difficult, a 1:30 enrichment may be used.

B. Free DNA Removal Treatment

The iQ-Check Free DNA Removal Solution (FDRS) provides an ideal way to get rid of free DNA.

1. Dispense 100 µl activated FDRS and 1 ml of incubated sample to a 1.5 ml microcentrifuge tube or deep well plate.
2. Incubate for 15–30 min at 37 ± 2°C without agitation.
3. Proceed to DNA Extraction. Be sure that the thermoshaker or heat block has reached 95–100°C to properly inactivate the iQ-Check Free DNA Removal Reagent.

C. DNA Extraction

General recommendations:

1. Turn on the heat block or thermoshaker to preheat before starting the test. Set it to 95–100°C. Keep the lysis reagent in suspension while pipetting by stirring at medium speed on a magnetic stir plate.

2. Open tubes and wells carefully to avoid any possible cross contamination.

3. Cool the deep well plate before pipetting directly through pre-pierced sealing film.

4. Gently shake the lysis reagent by hand first to resuspend the resin. Then pipet while it is stirring at medium speed with the magnetic bar contained in the bottle, in order to keep it in suspension.

Note: Use the magnetic bar to keep the lysis reagent in suspension. Pipet while it is stirring at medium speed.

5. Reconstitute the final lysis reagent as follows:
    a. Carefully pour all the contents from reagent F (lysis beads) into reagent A (lysis reagent).

    b. Use consumables with a wide enough tip to allow pipetting of the homogenized lysis reagent.

    c. The lysis reagent mixed with lysis beads (reagents A + F) has a shelf life of 6 months when stored at 4°C.

Standard II Protocol

If using FDRS, begin with step 2.

1. Collect 1 ml of decanted enriched sample in a tube or a well of a deep well plate. If using a deep well plate, seal it with a plastic film.

Note: Shake suspension to homogenize the culture, and then allow any debris to settle before collecting the sample.

2. Centrifuge tubes at 10,000–12,000 x g for 5 min or deep well plates at 2,250 x g for 20 min. Discard supernatant manually (tubes or plates) or using the DW 40 Deepwell Microplate Washer (plates only).

3. Add 200 µl of homogenized lysis reagent (reagents A + F) to the pellet and resuspend by pipetting the reagent up and down 5 times. Close tubes or seal deep well plate with pre-pierced sealing film.

Note: Gently shake the lysis reagent by hand first to resuspend the beads

4. Place tube in a cell disruptor for 3 ± 1 min, then incubate in a heat block with tube adaptor at 95–100°C for 10–15 min. For deep well plates, place in a plate thermoshaker at 1,300–1,600 rpm at 95–100°C for 10–15 min.

Note: If a heat block with a tube adaptor is unavailable, after the cell disruption step, transfer the entire volume of sample from the tube to a deep well plate and proceed with the deep well plate instructions.

5. For tubes, vortex at high speed, then centrifuge at 10,000–12,000 x g for 5 min. For deep well plates, centrifuge at 2,250 x g for 2 min or allow to settle undisturbed for 30 min.

This is the recommended stopping point for temporarily stopping the procedure.

The supernatant can be stored for up to 1 year at –20°C. Always allow it to thaw and homogenize, and then centrifuge at 10,000–12,000 x g for 5 min before reusing.

D. Real-Time PCR

Instrument and Software Setup

- For the detection of *A. flavus*, *A. fumigatus*, *A. niger*, and *A. terreus*, select “Aspergillus” from the list of assay targets
- For the detection of *A. flavus*, *A. fumigatus*, and *A. niger* only, select “Aspergillus AK” from the list of assay targets

PCR Mix Preparation

1. Prepare the PCR mix containing the amplification solution (reagent C) and the fluorescent probes (reagent B). The volume of PCR mix needed depends on the number of samples and controls to be analyzed. At least one positive and one negative control must be included in each PCR run. Use the pipetting table in Appendix – PCR Mix Calculation Guide to find the correct volumes to use for each reagent.

Note: Use the PCR mix (reagent B + C) immediately after preparation. It is stable for 1 hr maximum at 2–8°C.

2. Pipet 45 µl of the PCR mix into each well according to your plate setup.

3. To purify DNA, combine 50 µl of DNA extracted from each sample with 200 µl of iQ-Check Purification Reagent. Pipet up and down 5 times to homogenize. Alternatively, a 1:10 dilution of extracted DNA can

be made using sterile water.

Note: Cannabis flower is known to cause PCR inhibition. It is highly recommended to purify the DNA extract to avoid inhibition.

4. Add 5 µl of purified or diluted DNA extract, reagent D (negative control), or reagent E (positive control). Do not vortex the sample before pipetting. Hermetically seal the wells of the plate or strips. It is important to avoid bubbles at the bottom of the wells by pipetting carefully. As an optional step, centrifuge the sealed PCR plate or tube strips (quick spin) to eliminate any bubbles.

5. Place the PCR plate or tube strips in the thermal cycler. Be sure to place the plate with the A1 well at the upper left corner. Close the reaction module.

Run PCR

To start the PCR run, follow instructions in the real-time PCR system user guide for iQ-Check Kits.

E. Data Analysis

Data can be analyzed directly at the end of the PCR run or at a later time by opening the stored data file. Follow instructions in the corresponding CFX Manager IDE Software User Manual for opening data files and setting the data analysis parameters.

Interpreting Results

Once the data analysis parameters have been set, results are interpreted by analyzing the Cq values of each sample (the cycle at which the amplification curve crosses the threshold).

CFX Manager IDE Software allows complete automated analysis for Bio-Rad real-time PCR detection systems.

Controls

Verify the positive and negative controls before interpreting sample results.

For the experiment to be valid, the controls must have the results shown in Table 2. Otherwise the PCR reaction must be repeated.

	***A. flavus*, *A. fumigatus*, *A. niger* (FAM channel)**	***A. terreus* (Texas Red channel)**	**Internal control detection (HEX channel)**
Negative control	Cq = N/A*	Cq = N/A*	23 ≤ Cq ≤ 35
Positive control	26 ≤ Cq ≤ 36	26 ≤ Cq ≤ 36	N/A

The software indicates a Cq value of N/A (not applicable) when the fluorescence of a sample does not rise significantly above the background noise, and hence does not cross the threshold.

If results of negative and positive controls differ from those in the Controls table (invalid control), repeat the run and analysis described in D. Real-Time PCR and E. Data Analysis in Section 7 Protocol.

Samples

A positive *Aspergillus* sample must have a Cq value ≥10 in the FAM channel (targets: *A. flavus*, *A. fumigatus,* and/or *A. niger*) and/or the Texas Red fluorophore (target: *A. terreus*).

- If the Cq value is < 10, verify the raw data curve is a regular amplification curve (with a flat baseline, followed by a rapid exponential increase of fluorescence, and then a flattening out). If the curve seems correct, it may be considered a positive *Aspergillus* sample

If there is no Cq value (Cq = N/A) for FAM and Texas Red, or if the curve is not a typical amplification curve, the internal control for that sample must then be analyzed:

- If there is no Cq value for FAM and Texas Red and the internal control has a Cq ≥23, this sample is considered a negative *Aspergillus* sample
- If the internal control also has no Cq value (Cq = N/A), this probably indicates inhibition of the PCR reaction. Dilute the sample (perform a 1:10 dilution in distilled sterile water using 10 µl of DNA extract), use 5 µl of the dilution for amplification, and repeat the PCR test
- If the Cq value for the internal control is <23, it is not possible to interpret the result. Verify that the threshold was correctly placed and that the raw data curve is a regular amplification curve. If the curve does not have a characteristic shape, it will be necessary to repeat the PCR test.

Interpretation of sample results is summarized in the following table:

A. flavus*, *A. fumigatus*, *A. niger **(FAM Channel)**	***A. terreus*** **(Texas Red channel)**	**Internal control detection (HEX channel)**	**Interpretation**
Cq ≥ 10	Cq ≥ 10	N/A	Positive for all 4 targets
Cq ≥ 10	Cq = N/A	N/A	Positive for *A. flavus*, *A. fumigatus*, *A. niger*
Cq = N/A	Cq ≥ 10	N/A	Positive for *A. terreus*
Cq = N/A	Cq = N/A	Cq > 23	Negative for all 4 targets
Cq = N/A	Cq = N/A	Cq = N/A	Inhibition*

* When both *Aspergillus* and internal control detection give a Cq value = N/A (not applicable), the sample lysate must be tested again but diluted (1:10) in distilled water.

An invalid interpretation can be given when validation criteria are not met. Check the raw data and proceed as if the sample was inhibited.

Section 8
Confirmation of Positive Results

All positive iQ-Check Kit results should be confirmed following AOAC SMPR 2019.001 or the laboratory's confirmation protocols and state and/or regional requirements.

Section 9
Confirmation of Single Colonies Using iQ-Check Kit

The iQ-Check *Aspergillus* Kit may also be used to confirm single isolated colonies on agar plates.

1. Pick isolated conidia or hyphae from dichloran rose bengal chloramphenicol (DRBC) agar or potato dextrose agar (PDA) with a toothpick, sterile loop, or other adapted consumable (for example, a pipet tip).

2. Resuspend the colony in 100 µl tryptone salt or distilled sterile water in a microcentrifuge tube. Homogenize using a vortexer.

3. Use 5 µl of the suspension with 45 µl of PCR mix (see Section 7, Real-Time PCR) and follow the rest of the iQ-Check *Aspergillus* Kit protocol for data and result interpretation. DNA extraction is not necessary.

Section 10
Test Performance and Validations

PERFORMANCE TESTED AOAC RESEARCH INSTITUTE LICENSE NUMBER 032104

The iQ-Check *Aspergillus* Kit is validated by the AOAC Research Institute under the Performance Tested Method Program for detection of *A. flavus*, *A. fumigatus*, *A. niger*, and *A. terreus* in cannabis flower, cannabis concentrates-solvent based and cannabis concentrates-nonsolvent based. A positive result with iQ-Check should be considered presumptive, and it is recommended it be confirmed by standard reference methods. Certificate number: 032104

Section 11
References

AOAC International (2019). Standard Method Performance Requirements for Detection of *Aspergillus* in Cannabis and Cannabis Products, AOAC SMPR 2019.001. www.eoma.aoac.org/SMPR/upload/116/SMPR%202019_001.pdf, accessed August 8, 2019.

United States Food and Drug Administration (2001). Bacteriological Analytical Manual, Chapter 18: Yeast, Molds, and Mycotoxins. www.fda.gov/food/laboratory-methods-food/bam-yeasts-molds-and-mycotoxins, accessed August 8, 2019.

Section 12
Revision History

Release date	Document number	Change
September 2019	10000116839 Ver A	· New document
April 2021	10000116839 Ver B	· AOAC validation · Removal of Easy Protocol · Formatting changes

Appendix — PCR Mix Calculation Guide

To find the correct volumes to use when preparing the PCR mix, add the total number of samples and controls to be analyzed and find the corresponding volumes of reagent B and reagent C in the table.

Total Number of Samples and Controls	Probes Reagent B, µl	Amplification Mix Reagent C, µl	Total Number of Samples and Controls	Probes Reagent B, µl	Amplification Mix Reagent C, µl	Total Number of Samples and Controls	Probes Reagent B, µl	Amplification Mix Reagent C, µl
1	5	40	33	178	1,400	65	351	2,800
2	11	86	34	184	1,500	66	356	2,900
3	16	130	35	189	1,500	67	362	2,900
4	22	173	36	194	1,600	68	367	2,900
5	27	216	37	200	1,600	69	373	3,000
6	32	259	38	205	1,600	70	378	3,000
7	38	302	39	211	1,700	71	383	3,100
8	43	346	40	216	1,700	72	389	3,100
9	49	389	41	221	1,800	73	394	3,200
10	54	432	42	227	1,800	74	400	3,200
11	59	475	43	232	1,900	75	405	3,200
12	65	518	44	238	1,900	76	410	3,300
13	70	562	45	243	1,900	77	416	3,300
14	76	605	46	248	2,000	78	421	3,400
15	81	648	47	254	2,000	79	427	3,400
16	86	691	48	259	2,100	80	432	3,500
17	92	734	49	265	2,100	81	437	3,500
18	97	778	50	270	2,200	82	443	3,500
19	103	821	51	275	2,200	83	448	3,600
20	108	864	52	281	2,200	84	454	3,600
21	113	907	53	286	2,300	85	459	3,700
22	119	950	54	292	2,300	86	464	3,700
23	124	994	55	297	2,400	87	470	3,800
24	130	1,000	56	302	2,400	88	475	3,800
25	135	1,100	57	308	2,500	89	481	3,800
26	140	1,100	58	313	2,500	90	486	3,900
27	146	1,200	59	319	2,500	91	491	3,900
28	151	1,200	60	324	2,600	92	497	4,000
29	157	1,300	61	329	2,600	93	502	4,000
30	162	1,300	62	335	2,700	94	508	4,100
31	167	1,300	63	340	2,700	95	513	4,100
32	173	1,400	64	346	2,800	96	518	4,100

Visit bio-rad.com/iqcheck for more information.

Bio-Rad Laboratories, Inc.

Life Science Group

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