iQ-Check *Aspergillus* Kit

User Guide

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

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BIO-RAD

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Section 1
Introduction

Due to increasing state-level legislation concerning medicinal and recreational marijuana across the U.S. and Canada, 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. 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 System, with analysis using CFX Manager IDE Software.

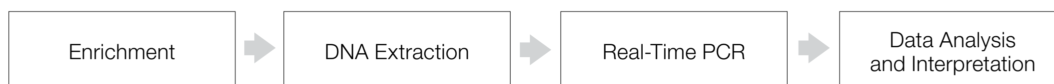
PCR is a powerful technique used to generate many copies of target DNA. The PCR reaction consists of cycles of heating and cooling to denature the DNA followed by the binding of primers to a specific target region. DNA polymerase, along with these primers and deoxynucleotide triphosphates (dNTPs), then extends the DNA, creating copies of the target DNA. These copies are called amplicons.

In real-time PCR, probes are used to detect specific targets on the DNA during the amplification step. These probes are linked to a dye that emits fluorescence only when it is hybridized to the target sequence. In the absence of target DNA, no fluorescence will be detected. As the number of amplicons increases with each round of amplification, fluorescence intensity also rises. The optical module of the real-time PCR instrument measures this fluorescence at the annealing step during each PCR cycle while CFX Manager IDE 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 sequence and is detected by a specific probe coupled with another fluorophore.

The iQ-Check *Aspergillus* Kit is a multiplex PCR system with an internal control (HEX fluorophore). The flexibility of CFX Manager IDE Software allows the detection of *A. flavus*, *A. fumigatus* and *A. niger* together (FAM fluorophore) and *A. terreus* alone (Texas Red fluorophore) as required by some regulations.

The test includes four main steps:



Section 3
Kit Components

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

Reagent ID	Reagent	Quantity Provided
A	Lysis reagent I	1 bottle, 20 ml
B	Fluorescent probes	1 tube, 0.55 ml
C	Amplification mix	1 tube, 4.4 ml
D	PCR negative control	1 tube, 0.5 ml
E	PCR positive control	1 tube, 0.25 ml
F	Lysis beads	1 tube, 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
- 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/or 95–100°C
  - Cell disruptor, such as a Disruptor Genie (Scientific Industries, Inc.)
- For extraction in deep well plates:
  - Heating thermoshaker* capable of maintaining 37 ± 2°C and/or 95–100°C, with a mixing speed of at least 1,300 rpm
  - Benchtop centrifuge with deep well rotor (capable of 2,250 x g)
- Vortex apparatus
- Magnetic stir plate

- For the Standard II Extraction protocol:
  - DW 40 Deepwell Microplate Washer (catalog #90137)
  - Deep well plate rotor
- 20, 200, and 1,000 µl micropipets
- Bio-Rad real-time PCR system*; for example, the CFX96 Touch Deep Well System (#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, BPW:
  - 500 g (#3564684)
  - 225 ml x 6 bottles (#3554179)
  - 2.3 L x 5 bags (#3555789)
  - 5 L x 2 bags (#3555790)
- Chloramphenicol, 0.3 g/L (Sigma-Aldrich C0378, C1919, or equivalent)
- Ethanol, 96%
- Tergitol 7 or equivalent surfactant
- Filter sample bags
- iQ-Check Purification Reagent (#12012383)
- iQ-Check Free DNA Removal Solution (#3594970)
- For extraction in tubes:
  - 1.5 ml conical screwcap tubes, sterile (for example, #2240110XTU)
- For extraction in a deep well plate:
  - iQ-Check Deep Well Microplates (#3594900)
  - Plastic sealing film (for example, #3590139)
  - Pre-Pierced Plate Sealing Film (#3600040, North America only) or equivalent
- 200 µl wide-opening pipet tips
- 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 serological 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
- iQ-Check *Aspergillus* Kit
  - All substances or mixtures in the test kit are classified products according to the Globally Harmonized System (GHS). 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
- 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. Due to excessive heat, some components may pose a risk of personal injury 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 the 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 (autoclave for 20 min at 120°C). Consult the Safety Data Sheet for additional information and local regulations for disposal requirements

- 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, and other such items) from one workstation to another
   - Always use a positive and 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
   - Check instruments to ensure they are functioning correctly
   - 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 do not leave fingerprints or write on tube caps to avoid interference with data acquisition

- Bio-Rad strongly recommends that you 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)

Section 7
Protocol

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

Enrichment conditions are detailed in Table 1. DNA extraction can be performed according to two different protocols. The Easy II protocol requires a 100 µl sample volume. The Standard II protocol requires a 1 ml sample volume. Both include a grinding step.

Sample Enrichment

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

Table 1. Sample enrichment conditions.

Scope (matrices)	Enrichment	Surfactant
Cannabis and cannabis-infused products	*n** g of sample in 9 x *n* ml BPW + chloramphenicol**	N/A
Concentrates and cannabis-infused oils and fats	*n* g of sample in 9 x *n* ml BPW + chloramphenicol**	1% v/v

* *n* = number of grams of matrix to be tested

** 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).

1. Homogenize with a lab paddle blender for 30 sec.
2. Incubate without shaking for 48 ± 3 hr at 37°C.
3. Homogenize by hand before transferring sample.
4. Follow DNA extraction protocol below.

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

iQ-Check Free DNA Removal Treatment

The iQ-Check Free DNA Removal Solution (catalog #3594970) provides an ideal way to get rid of free DNA. Follow Bio-Rad's recommendations in the user guide.

DNA Extraction

General recommendations:

- 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
- Avoid shaking the enrichment bag and collecting large fragments of debris from matrices. If a fat layer forms, collect the sample just below this layer
- Open tubes and wells carefully to avoid cross-contamination
- Cool the deep well plate before pipetting directly through pre-pierced sealing film
- Reconstitute lysis reagent:
  - Carefully pour all contents from reagent F (lysis beads) into reagent A (lysis reagent)

- Use consumables with a tip wide enough to allow pipetting of the homogenized lysis reagent
- Reagent F (lysis beads) is included in the iQ-Check *Aspergillus* Kit
- The reconstituted lysis reagent (reagents A + F) has a shelf life of 6 months when stored at 4°C

Standard II Protocol

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

   Note: Shake suspension to homogenize the culture, 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.
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 agitator-incubator 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.

If you choose to temporarily stop the procedure, this is the recommended stopping point. The supernatant can be stored for up to 1 year at –20°C. Before reusing it, always allow it to thaw and homogenize and then centrifuge at 10,000–12,000 x g for 5 min.

Easy II Protocol

1. Aliquot 100 µl of homogenized lysis reagent (reagents A + F) to wells of a deep well plate.
2. Add 100 µl of enriched sample.

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

3. Mix the solution by pipetting up and down until homogenized.
4. Seal the deep well plate with pre-pierced sealing film.
5. Incubate deep well plate in the heat block at 95–100°C for 15–20 min under agitation at 1,300 rpm.

If you choose to temporarily stop the procedure, this is the recommended stopping point. The supernatant can be stored for up to 1 year at –20°C. Before reusing it, always allow it to thaw and homogenize and then centrifuge at 10,000–12,000 x g for 5 min.

Real-Time PCR

1. For instrument and software setup, follow instructions in the CFX Manager IDE Software User Manual.
   a. For the detection of *A. flavus*, *A. fumigatus*, *A. niger*, and *A. terreus*, select *Aspergillus* from the list of assay targets.
   b. For the detection of *A. flavus*, *A. fumigatus*, and *A. niger* only, select *Aspergillus* AK from the list of assay targets.
2. Prepare PCR mix.
   a. Prepare PCR mix containing the amplification solution (reagent C) and the fluorescent probes (reagent B) according to 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 Section 11 Appendix — PCR Mix Calculation Guide to find the correct volumes to use for each reagent.

      Note: The PCR mix (reagents B + C) must be used immediately. It is stable for 1 hr maximum at 2–8°C.
   b. Pipet 45 µl of this PCR mix into each well according to your plate setup.
   c. 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.
   d. Add 5 µl of purified or diluted sample, reagent D (negative control), or reagent E (positive control). Do not vortex the sample before pipetting. Pipet carefully to avoid bubbles at the bottom of the wells. Hermetically seal the wells of the plate or tube strips. As an optional step, centrifuge the sealed PCR plate or the PCR tube strips (quick spin) to eliminate any bubbles.
   e. Place the plate or tube strips in the thermal cycler. Be sure to place the plate correctly: A1 well at the upper left corner. Close the reaction module.
3. Start the PCR run.

To start the PCR run, follow instructions in the CFX Manager IDE Software User Manual.

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.

Table 2. Interpretation of experimental controls.

	Aspergillus **Detection (FAM and Texas Red channels)**	**Internal Control Detection (HEX channel)**
Negative control	Cq = N/A*	23 ≤ Cq ≤ 35
Positive control	26 ≤ Cq ≤ 36	Not significant

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

If the negative or positive control results differ from those in the table above (invalid control), repeat the PCR test.

Samples

A sample must have a Cq value ≥10 for the FAM fluorophore (targets: *A. flavus*, *A. fumigatus,* and/or *A. niger*) and/or the Texas Red fluorophore (target: *A. terreus*) to be considered positive. If the Cq value for both channels is less than 10, check the raw data and verify that the curve is a regular amplification curve (a flat base line followed by a rapid 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:

- An *Aspergillus* sample is considered negative if the FAM and Texas Red Cq values are N/A and the internal control Cq ≥ 23
- An internal control that also has no Cq value (Cq = N/A) indicates a probable inhibition of the PCR reaction. 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 is a regular amplification curve. If the curve does not have the expected shape, repeat the PCR test

Interpretation of the sample results is summarized in Table 3.

Table 3. Interpretation of sample results.

Aspergillus **Detection (FAM and Texas Red channels)**	**Internal Control Detection (HEX channel)**	**Interpretation**
Cq ≥ 10	Not significant	Positive
Cq = N/A	Cq ≥ 23	Negative
Cq = N/A	Cq = N/A	Inhibition*

* When both the *Aspergillus* and internal control detection channels give a Cq value N/A, the sample must be tested again.

Section 8
Confirmation of Positive Results

All positive iQ-Check results should be confirmed following the laboratory's confirmation protocols and local 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.

Section 10
References

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

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