
Bodie Allen, Rebecca Davenport, Flore Margotteau, Oliver Pinkos, Virginia Lauren, Kristel Barbet, Nathalie Bernard, Hélène De Salla, Josh Withworth, Mike Clark, Laurent Jallu, Sophie Fenu and Christophe Quilling

1 Bio-Rad, Marnes-la-Coquette, France 2 Bio-Rad, Hercules, CA USA

Methods

Method Design

As shown in Figure 1, the Aspergillus method includes enrichment of the cannabis matrix in buffered Pippette Water (BPW), DNA extraction and purification, real-time PCR amplification and detection, and automated data interpretation. The challenge was to develop an efficient enrichment method and a novel multiple PCR assay capable of simultaneously detecting four unique genetic targets in a single tube reaction. A. fumigatus, A. flavus and A. niger were screened with primers and probes detected in the FAM fluorophore channel. A. terreus was detected in the Texas Red fluorophore channel. An internal control was also designed consisting of a heterologous target sequenced double stranded probe coupled with HEX fluorophore that is co-amplified with primers identical to Aspergillus targets. A positive control with both FAM and Texas Red targets was also included to each PCR run to pass the specifications and validate the run.

Assessment on Cannabis Matrices

The method was also assessed with cannabis matrices at the Colorado Department of Public Health and Environment laboratories (CPDHE). Five grams of cannabis flower, 5–6 g of infused non-edibles, 5–6 g of infused edibles or 1 g of solvent-based concentrated was prepared with a starting concentration equivalent to 3.50 x 10^5 CFU/ml. The LOD of A. flavus was 65 CFU/ml. The LOD of A. fumigatus, A. niger and A. terreus was 176, 284 and 2633 CFU/ml respectively. The double-inoculum had an LOD of 6 x 10^5 CFU/ml for A. niger and 3.6 x 10^5 CFU/ml for A. terreus demonstrating the assay's capability to successfully distinguish between the two species of Aspergillus.

Assessment on Cannabis Matrices

The CPDHE Laboratory study analyzed four categories of unspiked cannabis matrices with iQ-Check Aspergillus. It was found that 48 ± 3 hr was the optimal enrichment time for accurate Aspergillus detection (Table 2). This parameter enabled the detection in FAM or Texas Redchannel with the other time parameters that showed detection only in FAM channel. In some of the matrices, Aspergillus was detected after 48 hr of enrichment and standard extraction method followed by a purification step with a iQ-Purification Reagent (iQ-PR).

Promising results were obtained from the method on cannabis matrices and the ability to detect Aspergillus in naturally contaminated flowers.

Proficiency Testing

One sample out of the 4 provided was detected positive using the IQ-Check Aspergillus method. Native and diluted 1:10 DNA enabled the detection in FAM channel indicating the presence of A. flavus, A. fumigatus and/or A. niger after enrichment of 48 hr at 37°C. Native DNA was detected at 23.97 Cq values and diluted DNA at 26.62 (Fig. 2 and Table 3). Replicates for each extract showed similar results with a standard deviation of 0.1 and 0.2 Cq for native and diluted DNA respectively. This results show a good repeatability of the method. Furthermore, cultures on agar plates confirmed these results. All these results were confirmed by the Emerald report.

Conclusions

A novel solution with a simplified real time PCR assay to qualitatively detect Aspergillus species from cannabis matrices in 48 hr using BPW was developed by Bio-Rad. Preliminary results demonstrate that iQ-Check Aspergillus is an effective method for the detection of A. flavus, A. fumigatus, A. niger and A. terreus in a multiple format. The inclusion of an internal control confirmed the absence of false negative results. Supplementary data obtained with the Emerald proficiency testing and the study conducted by the CPDHE Laboratory showed the efficiency of the Aspergillus method in cannabis matrices and significant time savings as compared to the FDA BAM method (Fig. 4). Furthermore, the method enables the user to distinguish A. flavus from A. fumigatus and A. niger.

References


Table 1. Inclusivity and exclusivity study.

A: Inclusivity study
B: Exclusivity study
+ positive sample with Cq values lower than 35
- positive sample with Cq values higher than 33
- negative sample

Table 2. Comparison of incubation time for spiked cannabis matrices.

Table 3. iQ-Check Aspergillus with unspiked cannabis matrices.

Table 4. Emerald proficiency testing.

+ positive sample - negative sample

Results

Inclusivity/Exclusivity

The specificity of the real-time PCR assay was validated with a comprehensive panel of inclusivity and exclusivity strains. Forty-six exclusivity species of yeast and molds represented a large panel of non-targeted Aspergillus or non-Aspergillus strains. Twenty-one Aspergillus species composed the inclusivity panel. Each exclusivity strain was tested at a concentration of 10^3 cfu/ml vs. 10^6 cfu/ml for the inclusivity strains to mimic what may be found in an enrichment. Five microliters of the purified DNA was directly added to the PCR well and analyzed with iQ-Check Aspergillus real-time PCR assay.

Limit of Detection

The analytical sensitivity of a PCR method can be expressed as the Limit of Detection (LOD), defined as the lowest concentration at which the test can consistently detect the analyte. The LOD of a PCR method can be defined as the lowest concentration at which the test can consistently detect the analyte. In this study, 10 replicates of a dilution series were analyzed to determine the LOD of the PCR method. The LOD of the PCR method was determined to be 6.40 x 10^3 CFU/ml for A. flavus. A. parasiticus and 3.6 x 10^5 CFU/ml for A. terreus.

Inclusivity/Exclusivity

Twenty one inclusivity species were tested and validated. Of the forty six exclusivity species assessed, 5 species were positive in FAM channel (Table 1): Aspergillus oryzae, Aspergillus parasiticus, Aspergillus tamarii were detected with high Cq values contrary to Aspergillus paniceolides and Aspergillus wandersi. These species have been verified in dedicated studies (1). A. oryzae, A. parasiticus and A. tamarii are phylogenetically close to A. flavus, A. parasiticus is close to A. niger and A. wandersi belongs to the A. niger section. It means that the positive result may indicate a true Aspergillus inclusivity species especially for species of flavus or niger sections. Furthermore, the aspergillosis disease prevalence of those strains is unknown. Regarding the close phylogenetic link between some Aspergillus species (e.g. the flavus exclusivity species described), the inclusivity/exclusivity study was validated and guarantees functionality of the specificity of the Aspergillus kit.

Limit of Detection (LOD)

The limit of detection (LOD) was determined for each species and a cocktail of A. niger and A. terreus. The LOD of A. flavus was 65 CFU/ml. The LOD of A. niger and A. terreus was 176, 284 and 2633 CFU/ml respectively.

Fig. 1. Flowchart of iQ-Check Aspergillus.

Fig. 2. Cannabis matrices detection in FAM channel. Blue, positive control; Orange, FAM - native DNA. Green, FAM - native DNA (diluted at 1:100) with a mean Cq value of 25.62; Red, iQ-Check Aspergillus in FAM channel. A. detection in Texas Red channel. C. detection in HEX channel.