

Detection of Aflatoxigenic *Aspergillus flavus* using a Probe-Based Real-Time QPCR

Application Note

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Abstract

Aflatoxin producing fungi, such as *Aspergillus*, are common food contaminants, and various methods exist to detect their presence. Early detection of food contaminants is important and essentially required to prevent food spoilage during storage or transport, and thus reduce the economic impact caused by rejection of consignments. In this study, we have developed a probe based qPCR for detecting toxin-producing molds using the Agilent Mx3005P QPCR instrument. Primers and probes detect the specific region of the O-methyltransferase (*omt-1*) gene of the aflatoxin metabolic pathway. The linear detection range of this assay was determined to be from 0.01 pg to 10 ng of fungal gDNA. This assay could be used for specific detection of other aflatoxigenic *Aspergillus* species from various food samples.



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Introduction

Aflatoxins, the low molecular weight compounds produced by the aflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus* are one of the most harmful among the mycotoxins. These filamentous fungi grow on a wide variety of agricultural commodities such as maize, groundnuts, soybeans, chilli peppers, and so forth. They pose adverse effects on human health and livestock. The conditions used for the storage of grains influence the promotion of fungal species growth. Conventional microbiology techniques used for grain testing require extended incubation time (days), special expertise, and equipment with LC/MS detection as the gold standard technology for toxin detection. It has been observed that samples tested negative by LC/MS before shipment reveal the presence of the toxins at the receiving port. The fungal spores present in very low numbers grow and produce toxins during the transit. To overcome this issue, there is need for a sensitive assay to detect the presence of these

toxin-producing fungi by alternative methods to prevent and minimize the risk of consignment rejection. This application note describes a method to detect the toxin producing *A. flavus* by using a probe that targets the toxin pathway gene, O-Methyltransferase (*omt-1*) (Figure 1).

Materials and methods

DNA, primers, and probes

The aflatoxin-producing strain of *A. flavus* was obtained from University of Mysore, India. DNA was extracted by using a DNeasy plant mini kit with slight modifications. Briefly, fungal mycelial mat was powdered using mortar-pestle in liquid nitrogen. The powder was dissolved in digestion buffer AP1 and subjected to zymolase treatment for 1 hour. Genomic DNA was isolated as per the kit's instructions. Purified DNA was quantified using NanoDrop 1000 and Qubit (Invitrogen).

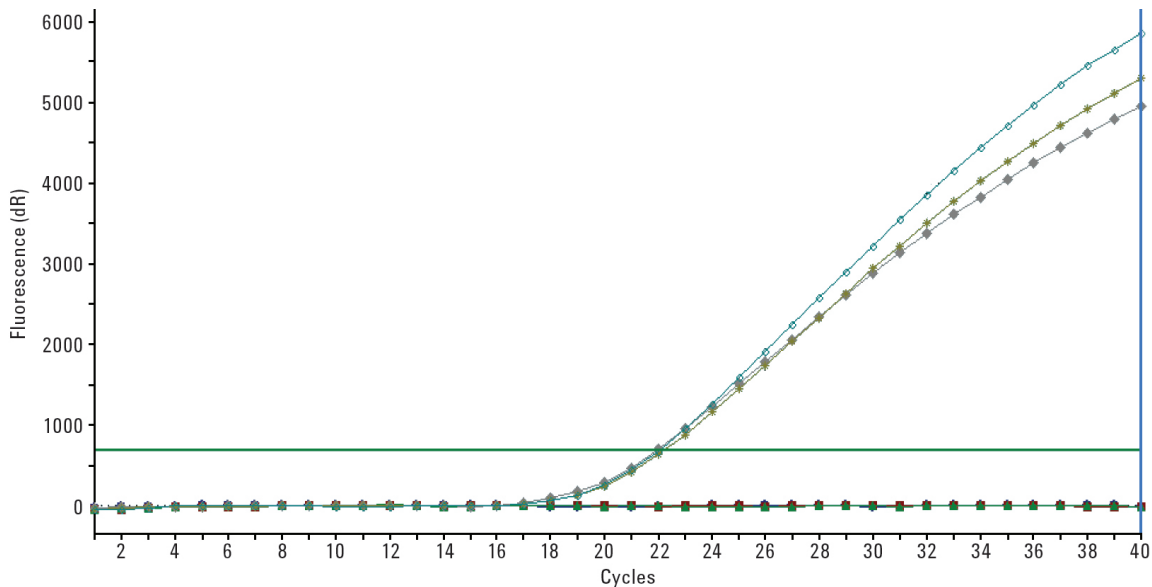


Figure 1. *Omt-1* amplification plots for a positive control and a no template control performed in triplicates.

Based on the conserved regions of the *omt-1* gene (GeneBank accession number L25835.1), forward and reverse primers were designed using Beacon Designer software v8 (Premier Biosoft). A HEX-labeled *omt-1* probe was used as described previously [1]. The sequences of the primers and probe used for qPCR amplification are provided in Table 1.

Table 1. Nucleotide Sequences of *omt-1* Primers and Probe used in the Present Study

Primers and probes	Nucleotide sequences (5'–3')
Forward- <i>omt-1</i>	GATCGTAGGTAATCCACTC
Reverse- <i>omt-1</i>	GGTAGACTGCTGAAATCA
<i>omt-1</i> probe	[HEX]-CCACTGGTAGAGGAGATGT-[BHQ1]

qPCR assay

A real-time quantitative PCR assay was performed by adding 10 µL of 2x Brilliant III ultra-fast QPCR Master Mix (Agilent Technologies, La Jolla, CA) to each well containing 10 ng of fungal template DNA, 600 nM of forward and reverse primers and 375 nM of *omt-1* probe in a 20 µL reaction volume using the Agilent Mx30005P instrument. After a denaturation step at 95 °C for 3 minutes, the cycling profile used was 95 °C for 20 seconds, 52 °C for 20 seconds, and 60 °C for 20 seconds for 40 cycles of amplification. The experiment was performed in triplicates and repeated three times. No Template Controls (NTC) were used to detect DNA contaminations.

The amplicon size of the resulting PCR products was analyzed on an Agilent 2100 Bioanalyzer system using an Agilent DNA 1000 assay according to the manufacturer's protocol.

Results and Discussion

Real-time QPCR detection of the *omt-1* gene present in aflatoxigenic fungi was performed using an Mx3005P instrument. The goal was to enable sensitive detection of a wide range of aflatoxigenic *Aspergillus* strains in various food matrices. The probe sequence for the *omt-1* amplicon was based on previous studies [1] and primer sequences were adapted to allow for good performance of the assay on the Mx system. Out of the four different sets of primers designed, *omt-1* primer pairs gave the best performance. The probe and primer concentrations were standardized and the primer concentration of 600 nM and the probe concentration of 375 nM were optimal for the assay. Specific amplification of the target sequence was observed with clear NTC as shown in Figure 1. A standard curve with serial dilutions of a genomic DNA obtained from a positive control sample showed very good correlation, with a slope of -3.441 , an efficiency of 95.3 %, and R^2 value of 1.0, which are in the suggested range [2] (Figure 2). The PCR products revealed a clear single band at the expected amplicon size (Figure 3). This probe based QPCR method allows fast analysis from sample to result with sensitive detection of fungal contaminations.

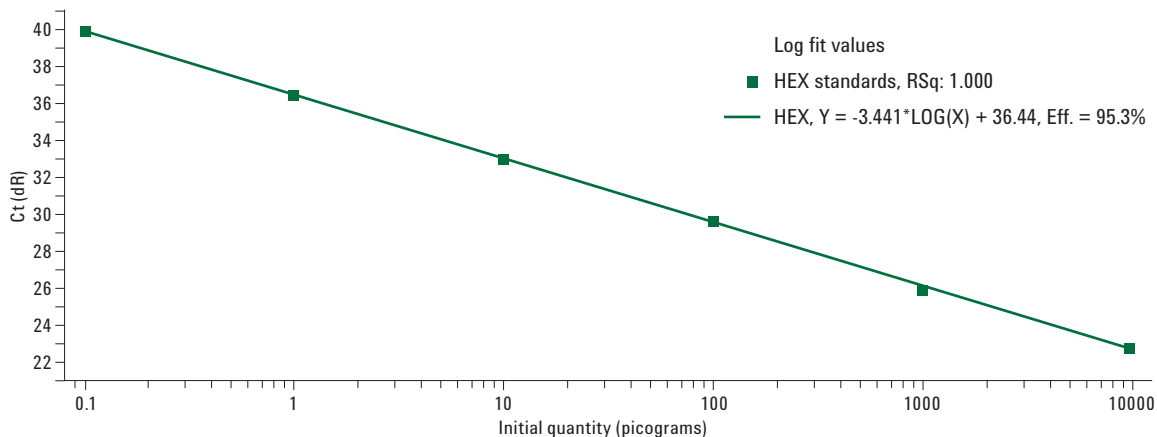


Figure 2. Standard curve of \log_{10} DNA concentration against the cycle threshold (Ct) for different DNA dilutions from *A. flavus*.

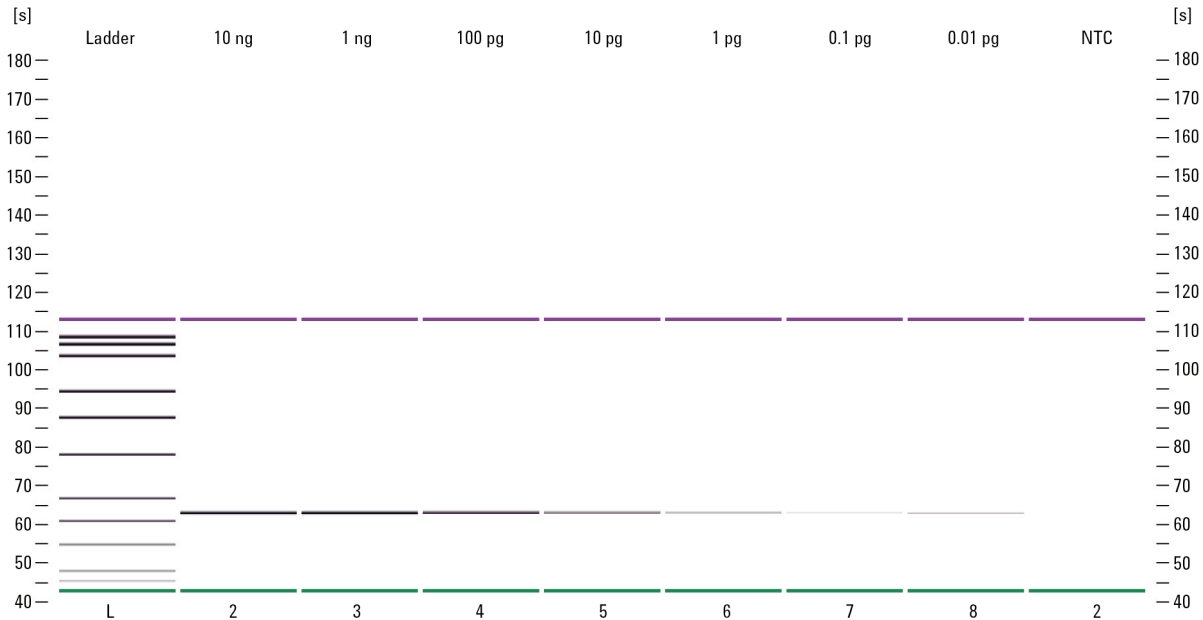


Figure 3. QPCR product on an Agilent 2100 Bioanalyzer. 185 bp band size for different DNA concentrations of *A. flavus* along with NTC.

Conclusions

The probe-based real-time QPCR assay established in this study using the Agilent Mx30005P QPCR system is a sensitive and specific method to detect aflatoxigenic fungal contamination in food samples and can be applied as a standardized protocol for detection of these fungi in food and feed.

References

1. A. Rodríguez, *et al.* "Development of a multiplex real-time PCR to quantify aflatoxin, ochratoxin A and patulin producing molds in foods" *Int. J. of Food Microbiol.* **155** (2012) 10–18.
2. Y. Suanthie, M.A. Cousin, and C.P. Woloshuk, "Multiplex realtime PCR for detection and quantification of mycotoxigenic *Aspergillus*, *Penicillium*, and *Fusarium*" *J. Stored Prod. Res.* **45**:139-145 (2009).

Ordering Information

Agilent Mx30005P QPCR System (p/n 401457),
 Agilent 2100 Bioanalyzer (p/n G2943CA),
 Agilent DNA 1000 kit (p/n 5067-1504),
 2x Brilliant III ultra-fast QPCR Master Mix (p/n 600880)

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