



An Efficient Droplet Digital PCR Approach for Detection DNA at Low Concentrations of Toxigenic Fungi in Food Products

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Abstract

As common toxigenic fungi genera, *Aspergillus*, *Penicillium*, and *Fusarium*, have attracted the attention of governments and people all over the world due to their human carcinogenicity, teratogenicity, and hepatotoxicity. Accordingly, effective and quantitative detection method for toxigenic fungi before mycotoxins produced should be established to ensure food safety. In this paper, two duplex-droplet digital PCR (ddPCR) were developed and optimized for toxigenic fungi. The detection limits of the target genes, including the *AflR*, *Och*, *Pen*, and *Fus* of toxigenic fungi, were 26 copies/reaction, 15 copies/reaction, 161 copies/reaction, and 29 copies/reaction, respectively. Notably, the detection limit of duplex ddPCR was three orders of magnitude higher than that of tradition real-time fluorescence PCR reaction. Moreover the efficiency and sensitivity of the established method were also higher than those of real-time fluorescence PCR. The linear quantitative range of copy number of *AflR* and *Och* genes in *AflR/Och* duplex system and *Pen* and *Fus* genes in *Pen/Fus* duplex system were both 2×10^{-7} – 2×10^{-3} ng/ μ L. From DNA extraction to target gene detection, time consume of 96 samples was within 6 h, achieving the purpose of high-throughput and rapid detection.

Keywords Droplet digital PCR · Toxigenic fungi · Quantitative detection · Rapid detection · Food safety

Introduction

Toxigenic fungi are a class of fungi that can produce stimulating metabolites mycotoxins under the appropriate environmental conditions (Suman 2021). Until now, approximately 400 mycotoxins have been identified (Ülger et al. 2020). Ingestion, inhalation, or skin contact mycotoxins, even trace levels, would result in acute clinical symptom or chronic toxicity to humans and animals (Gallo et al. 2015). Antibiotic treatments have little or no effect on the diseases they caused (Marin et al. 2013). The genera *Aspergillus*, *Fusarium*, and *Penicillium* are the major mycotoxin-producing fungi, and

the most relevant mycotoxins found in foods and feeds are aflatoxins (AFs), ochratoxins A (OTA), deoxynivalenol (DON), zearalenone(ZEN), patulin, and fumonisins (Marin et al. 2013). According to the statistics of the United Nations Food and Agriculture Organization (FAO), about 25% of the world's grain and oil were contaminated by mycotoxins every year (Eskola et al. 2019). Though the methods of chromatography coupled to different detectors (Santos Pereira et al. 2019) and immunoassay-based methods (Pereira et al. 2014), biosensors (Santos Pereira et al. 2019) have been developed to detect mycotoxins in foodstuffs/feedstuffs, the annual costs in association with mycotoxin contamination on foods and feeds are continuing rise in worldwide (Hassan and Zhou 2018). Because mycotoxins are stable in nature and difficult to remove after pollution, it is an important measure to ensure the safety of agricultural products and foods to find fungal contamination as early as possible. Many national and international public health agencies, such as the World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the U.S. Food and Drug Administration (FDA), are concerned about the problem of mycotoxins and have proposed control strategies (Mitchell et al. 2016). However, they have not yet addressed the issue of detecting and controlling those fungi

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with early toxin-producing properties, thus avoiding the risk at the source.

Direct detection of toxigenic fungi can more directly reduce the contamination of foods and the risk to humans compared to detection of mycotoxins (Alshannaq and Yu 2017). Earlier studies have been conducted on the use of PCR technology to detect aflatoxin-producing fungi (Haas et al. 2013; Niessen 2007; Stakheev et al. 2011). Multiplex PCR was also used to detect ochratoxin and *Fusarium* mycotoxin fungi (Sudharsan et al. 2017). However, the number of individual toxin-producing fungi is small in the early stage of food contamination, and the process of fungal enrichment is not only tedious but also time-consuming. Moreover, insufficient of fungal DNA often does not reach the detection limits of ordinary PCR. However, it seems that conventional PCR cannot do more in terms of target gene quantification (Raguseo et al. 2021).

Droplet digital polymerase chain reaction (ddPCR) is a recently proposed method that aims to overcome some of the limitations of qPCR in terms of sensitivity and precision (Yu et al. 2021). As a third-generation PCR technology, precise quantification can be achieved by directly counting the number of positive microdroplets after PCR reactions. The principle of ddPCR is to create a PCR reaction system with a fluorescent probe in oil and dilute it to the single molecule level, producing approximately 20,000 droplets. The detection of positive droplets and negative droplets is performed, and finally, precise quantification can be achieved by directly counting the number of positive microdroplets based on the Poisson distribution (Yu et al. 2020). Studies have been conducted to apply ddPCR technology to detect pathogenic bacteria such as *Vibrio parahaemolyticus* (Lei et al. 2020) and *enterohemorrhagic Escherichia coli* (He et al. 2020) in foods. However, to our knowledge, no quantitative analysis of toxigenic fungi in foods has been performed using a ddPCR method.

Herein, the objective of this work is to establish, optimize, and verify the accurate identification and quantitative methods of toxigenic fungi based on ddPCR. Specific primers and probes for the single-copy nuclear gene were designed for the identification of fungal DNA. In addition, a duplex ddPCR detection system was designed and optimized based on the two fluorescence channels of ddPCR to achieve simultaneous detection of two different toxigenic fungi. Finally, according to the target DNA sequence, the market samples were accurately quantified by ddPCR method.

Materials and Methods

Sample Collection

Escherichia coli TOP10 strain (Ampicillin resistance) and genomic DNA of *Aspergillus flavus*, *Aspergillus parasiticus*,

Aspergillus ochraceus, *Aspergillus nomius*, *Penicillium*, and *Fusarium* were stored in our lab; among them DNA of *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* were from aflatoxigenic strains confirmed by LC–MS. The DNA of fungal strains mentioned above were diluted to 20 ng/mL and stored at $-20\text{ }^{\circ}\text{C}$ until used. DNA of *Aspergillus flavus* NRRL3357, *Aspergillus ochraceus* CBS263.67, *Penicillium* CBS131811, and *Fusarium* NRRL53570 were collected from the School of Food Engineering and Biotechnology, Tianjin University of Science and Technology. Food samples which eaten frequently in our daily lives and are prone to mold were purchased from several local supermarkets and farmers' markets in Beijing, China (Tab. S1).

Primers and Probes

Specific target gene sequences of *aflatoxin-producing fungi*, *Aspergillus ochra*, *Penicillium*, and *Fusarium* were identified through Genbank, and the sequence homology comparison was performed using NCBI BLASTN (Fig. S1). The *Och1* primer–probe pair was drawn from previous study (Patiño et al. 2005). Other primers and probes are designed using DNAMAN and Oligo7.0 software. A total of 8 sets of primer–probe pairs were screened by real-time PCR (data not shown); four sets of them were confirmed (Table 1). The oligonucleotide sequence was synthesized by Sangon Biotech (Shanghai, China) Co., Ltd., and the 5' end of the probe was labeled with 6-carboxyfluorescein (FAM) or VIC, and the 3' end was labeled with Black Hole Quencher 1 (BHQ1).

Construction of Target Genes Recombinant Plasmids

Four PCR products containing target genes, using the primer pairs in Table 1, were cloned into the vector pUC57 (Takara, Japan) according to the instructions of the manufacturer. Plasmids were transformed into *Escherichia coli* TOP10 cells and were purified by using a QIAGEN plasmid purification kit (QIAGEN, Germany) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were determined by spectrophotometry (NanoDrop One, USA) and diluted to 20 ng/ μL and stored at $-20\text{ }^{\circ}\text{C}$ until used.

The Establishment of ddPCR Assay

ddPCR reaction was carried out with QX200 Droplet Digital PCR system (Bio-Rad, USA), according to the manufacturer's instructions. In the establishment of ddPCR assay, three ways to add restriction enzyme *Hind* III (R0104S, NEB, USA) to reaction mixture were test on plasmids in simplex assay (Table 2). Twenty microliters of mixtures contained 10 μL of 2 \times ddPCR Supermix for

Table 1 Primer and probe sequence and target gene information of toxin-producing fungi

Target gene	Genbank number	Primer and probe name and sequence (5'-3')	Length (bp)	Target strain
Protein activation gene: <i>AflR</i>	AY510451.1	<i>AflR</i> 2-F: CGCATAACACGTAACAACGC <i>AflR</i> 2-R: GGAGACGCTACTGCTACCAT <i>AflR</i> 2-P: FAM-CATACGCAGGCCACACTCATGCT-BHQ1	146	Aflatoxin-producing fungi
ITS sequence: <i>Och</i>	KY378947.1	<i>Och</i> 2-F: AGCGAAATGCGATAACTAATGTGA <i>Och</i> 2-R: GCCCATAACGCTCGAGGA <i>Och</i> 2-P: VIC-ATGCTGTCCGAGCGTCATTGCTG-BHQ1	212	<i>Aspergillus ochraceus</i>
β -tubulin: <i>Och</i>	MF588917.1	<i>Pen</i> -F: AGCTCGAGCGTATGAACGCTCTAC <i>Pen</i> -R: ATGGTACCGGGCTCCAAATCGA <i>Pen</i> -P: VIC-AGGCCAGCGGTGACAAGTACGTTCC-BHQ1	161	<i>Penicillium</i> spp.
LS rRNA: <i>Fus</i>	NG_055726.1	<i>Fus</i> -F: TGAGAGCCCCGTCTGGTT <i>Fus</i> -R: TGGCCGGTATTTAGCTTTAGAAGAC <i>Fus</i> -P: FAM-TCCTTCGACGAGTCGAGTAGTTTGG-BHQ1	117	<i>Fusarium</i> spp.

F forward primer, R reverse primer, P probe

probes (No dUTP) (Bio-Rad, USA), primers (10 μ M) 1.8 μ L each, probe (10 μ M) 0.5 μ L, DNA (20 ng/ μ L) 1 μ L, and ddH₂O 6.5 μ L. A volume of 20 μ L reaction mixture and 70 μ L droplet generation oil for probes (Bio-Rad, USA) were used to generate droplets in an 8-channel cartridge with Bio-Rad-automated droplet generator. Then the generated emulsion droplets were transferred to a 96-well PCR plate using a RAININ electronic pipette set to 40 μ L and sealed with foil in a PX1™ PCR plate sealer and then transferred to a T100™ Thermal Cycler for PCR amplification. PCR amplification was initiated by 10 min denaturation at 95 °C followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and 98 °C for 10 min to inactivate the enzyme and then stored at 4 °C. After amplification, PCR plate was transferred to the droplet reader, and data were analyzed by QuantaSoft™ software (version 1.6.6, Bio-Rad). A fluorescence amplitude threshold line was set up manually to distinguish between positive and negative droplets.

Specific of Droplet Digital PCR

The specificity of the primer pairs and probes were confirmed by simplex droplet digital PCR. Four constructed plasmids as mentioned above were tested and evaluated, and ddH₂O was used as blank control. The optimized PCR reactions and parameters were used as described in section of the establishment of ddPCR assay.

Screening and Optimization of Duplex ddPCR Assay

Based on the FAM/VIC (HEX) dual-channel detection of Bio-Rad droplet digital PCR, two duplex ddPCR systems of aflatoxigenic fungi *Aspergillus/Ochraceus* (*AflR/Och* reaction system) and *Penicillium/Fusarium* (*Pen/Fus* reaction system) were established and optimized. For *AflR/Och* reaction system, DNA template mixtures were made by mixing 1 μ L pUC57-*AflR* (20 ng/ μ L) with 1 μ L pUC57-*Och* (20 ng/ μ L); primer–probe premix a was made by mixing *AflR*2 primers (10 μ M) 1.8 μ L each and *AflR*2 probe (10 μ M) 0.5 μ L, and primer–probe premix b was made by mixing *Och*2

Table 2 Different ways to add restriction enzyme in ddPCR reaction system

Component	a		b		c	
	Volume (μ L)	Final concentration	Volume (μ L)	Final concentration	Volume (μ L)	Final concentration
ddPCR™ Supermix for probes	10	1 \times	10	1 \times	10	1 \times
Forward/reverse primer (10 μ M)	1.8/1.8	0.9 nM	1.8/1.8	0.9 nM	1.8/1.8	0.9 nM
Probes (10 μ M)	0.5	0.25 nM	0.5	0.25 nM	0.5	0.25 nM
DNA (0.2 ng/ μ L)	2	0.02 ng/ μ L	2	0.02 ng/ μ L	2 ^d	0.02 ng/ μ L
<i>Hind</i> III	0.2	4 Units	0.2	4 Units	/	/
CutSmart® Buffer	/	/	1.7	/	/	/
ddH ₂ O	3.7		2		3.9	

^dDNA was digested and purified before ddPCR

primers (10 μM) 1.8 μL each and *Och2* probe (10 μM) 0.5 μL ; then five premix a/premix b ratio (3:1, 2:1, 1:1, 1:2, and 1:3) were tested and screened. The *Pen/Fus* reaction system were prepared and tested in a similar way (in this system: premix a was mixture of *Pen* primers and probe, premix b was mixture of *Fus* primers and probe). Duplex ddPCR reaction mixtures contained 10 μL of 2 \times ddPCR Supermix for probes (No dUTP) (Bio-Rad, USA), different ratio primers-probe premix (10 μM) 4.1 μL , mixed template DNA (20 ng/ μL) 1 μL , *Hind* III (20,000 units/mL) 0.2 μL , and ddH₂O 4.7 μL . Five gradient annealing temperatures 60 $^{\circ}\text{C}$, 61 $^{\circ}\text{C}$, 62 $^{\circ}\text{C}$, 63 $^{\circ}\text{C}$, and 64 $^{\circ}\text{C}$ were set to select the best annealing temperature for the duplex ddPCR assay. The experimental data of the duplex system established by adjusting the proportional addition of primers and probes were analyzed by clustering of droplet fluorescence values, and the standard deviation of positive droplet fluorescence values was calculated using SPSS software.

Limit of Detection and the Linear Quantitative Equations of Duplex ddPCR System

Before the limit detection of the duplex ddPCR methods, the four plasmid DNA templates were mixed according to the ratio in the *AflR/Och* and *Pen/Fus* system to make a concentration of 20 ng/ μL . Then the DNA were diluted into 10 gradients of 10^0 – 10^9 and amplified in optimized duplex ddPCR reaction system. Experiments were set up in triplicate, and repeated experiments were carried out among the experimenters. The linear quantitative equations of duplex ddPCR method were also established according to Ren et al (2017).

Selection of DNA Extraction Methods and Analysis of Actual Samples

The quality of DNA has an enormous impact on detection results. Before extraction, all food samples were grind into a fine power by liquid nitrogen; subsequently, one gram of

grinded sample was placed in a 5 mL Axygen tube. For optimum method selection of DNA extraction, three DNA extraction methods were used. Method 1 was a cetyltrimethylammonium bromide (CTAB) method as described by Li et al. (2017), and method 2 and method 3 were commercial kit-based methods with NucleoSpin®Food kit (MP Biomedicals, USA) and FastDNA™ SPIN Kit (Macherey–Nagel, Germany), respectively, used according to the instruction.

To verify the accuracy of the developed double-stranded ddPCR method for target gene quantification, genomic DNA of previously mentioned fungal strains with known concentrations was examined. To further demonstrate the accuracy of the method for the actual food samples, eight food samples including grape, steamed bread, toast, sandwiched bread, northeast rice, vitamin compound nutritious rice noodles, mineral compound nutritious rice noodles, and calcium compound nutritious rice noodles were also detected. DNA extraction of food samples were performed and amplified by optimized duplex ddPCR systems as above-mentioned.

Results

Specificity of Primer Pairs and Probes

The specificity of the four primer pairs and probes were tested with the four recombinant plasmids (pUC57-AflR, pUC57-Och, pUC57-Pen, pUC57-Fus). The results showed that ddPCR amplification of the four plasmid DNA with each set of primer pairs and probe produced a specific signal of expected (Fig. 1). In short, when using *AflR2* primer pairs and probe, specific signals were the recombinant plasmid pUC57-AflR, the other plasmid DNA samples as well as blank control were all negative, and primer pairs and probes of *Och2*, *Pen*, and *Fus* were similar. And the number of droplets generated in each sample was more than 12,000, satisfying the Poisson distribution calculation. Meanwhile, the primers did not cross-react between different samples,

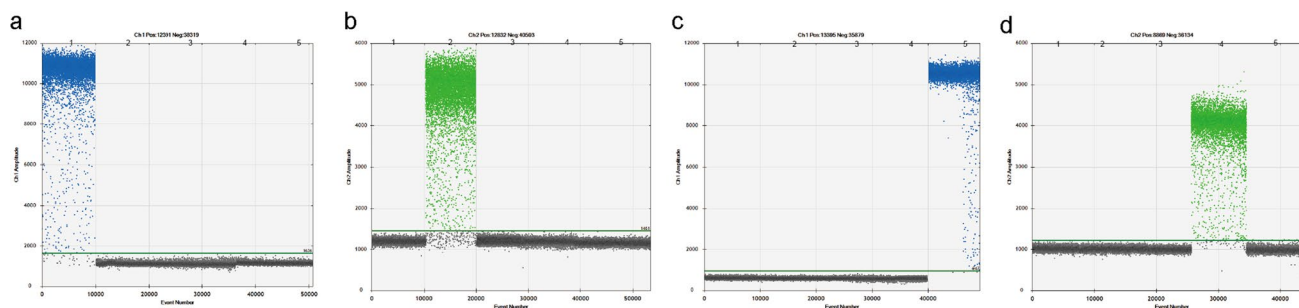


Fig. 1 The specificity of the primer pairs and probes in ddPCR assay. The specific droplets were obtained from four plasmid DNA (pUC57-AflR, pUC57-Och, pUC57-Pen, pUC57-Fus) with different primer pairs and probe: a four recombinant plasmids amplification with

AflR2 primer pairs and probe; b four recombinant plasmids amplification with *Och* primer pairs and probe; c four recombinant plasmids amplification with *Pen* primer pairs and probe; d four recombinant plasmids amplification with *Fus* primer pairs and probe.

indicating that the digital PCR system has good specificity of primers and probes and can be used for subsequent studies.

Establishment of ddPCR Assay

At present study, the ddPCR assay was set up to identified the ways to add restriction enzyme. Figure S2 showed that droplet separation was visibly best in a system than in b and c. And the standard deviation of positive versus negative droplets was also calculated; the smallest value was observed in a system (Fig. 2). Therefore system a was chosen and used in subsequent ddPCR assay.

Optimization of Duplex ddPCR System

The two duplex ddPCR initially optimized the ratio of premix a to premix b in the reaction systems. In *AflR/Och* duplex system, when the ratio of premix a to premix b was 1:3, the positive droplets were better clustered at this time (Fig. 3a), and the value of the standard deviations of the *AflR/Och* duplex system was the smallest (Fig. 3c). In *Pen/Fus* duplex system, the positive droplets clustered better when the ratio of premix a to premix b was 3:1 (Fig. 3b), and also the value of the standard deviations was the smallest (Fig. 3d). Therefore, the ratio of 1:3 and 3:1 of premix a to b in *AflR/Och* and *Pen/Fus*, respectively, were identified as the most appropriate reaction conditions, since the fluorescence reporting values of the two duplex reaction systems were high and with better separate areas between the negative and positive microdroplets cluster.

According to the ddPCR application guidelines, annealing temperature is one of the key parameters affecting specificity. In order to determine the optimum annealing temperature of ddPCR duplex system, five different temperatures

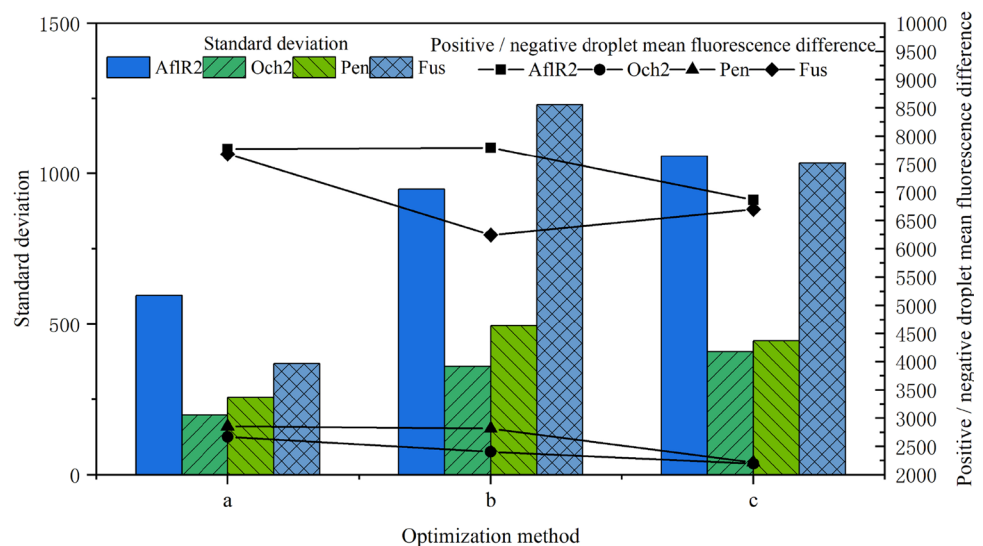
(60°C, 61°C, 62°C, 63°C, and 64°C) were designed and compared. The best separation between positive and negative droplets for the targets in two duplex systems were observed at the lowest tested temperatures, and therefore, 60°C and 61°C were identified as the most appropriate annealing temperatures in *AflR/Och* system (Fig. 4a, b) and *Pen/Fus* system (Fig. 4c, d), respectively, since they presented slightly less droplet rain between positive and negative droplets compared to the other tested temperatures. Similar results were obtained using the standard deviation calculated and analyzed; there were minimum values at 60°C in *AflR/Och* system (Fig. 4e) and 61°C *Pen/Fus* system (Fig. 4f), respectively.

LOD and Quantitative Linear Range of Duplex ddPCR

Ten-fold serial mixed plasmid dilutions were tested and used to construct the linear quantitative curve by putting the plasmid DNA concentration logarithm against the measures copy number logarithm. The LODs of *AflR*, *Och*, *Pen*, and *Fus* genes were 26 copies/reaction, 15 copies/reaction, 161 copies/reaction, and 29 copies/reaction, respectively, calculated by Poisson distribution and eliminating blank aerosol contamination. And after more than two experiments and the replacement of experimental personnel, the experimental results are consistent with expectations, and there is a good repeatability and reproducibility of the experiment (results not show).

The linear quantification range in this study refers to the concentration range in which the assay can stably quantify target genes for toxigenic fungi and is an evaluation of the detection ability of the system. By linear fit analysis, the quantitative ranges of *AflR*, *Och*, *Pen*, and *Fus* gene copy numbers in both *AflR/Och* and *Pen/Fus* duplex systems were

Fig. 2 Comparison of the mean difference and standard deviation of fluorescence of positive/negative droplets of ddPCR systems in different ways to add restriction enzyme *Hind* III. a *Hind* III was added in the reaction mixtures; b *Hind* III and CutSmart® Buffer were added in the reaction mixtures; c plasmid DNA was digested with *Hind* III and purified before ddPCR and then was added in the reaction mixtures



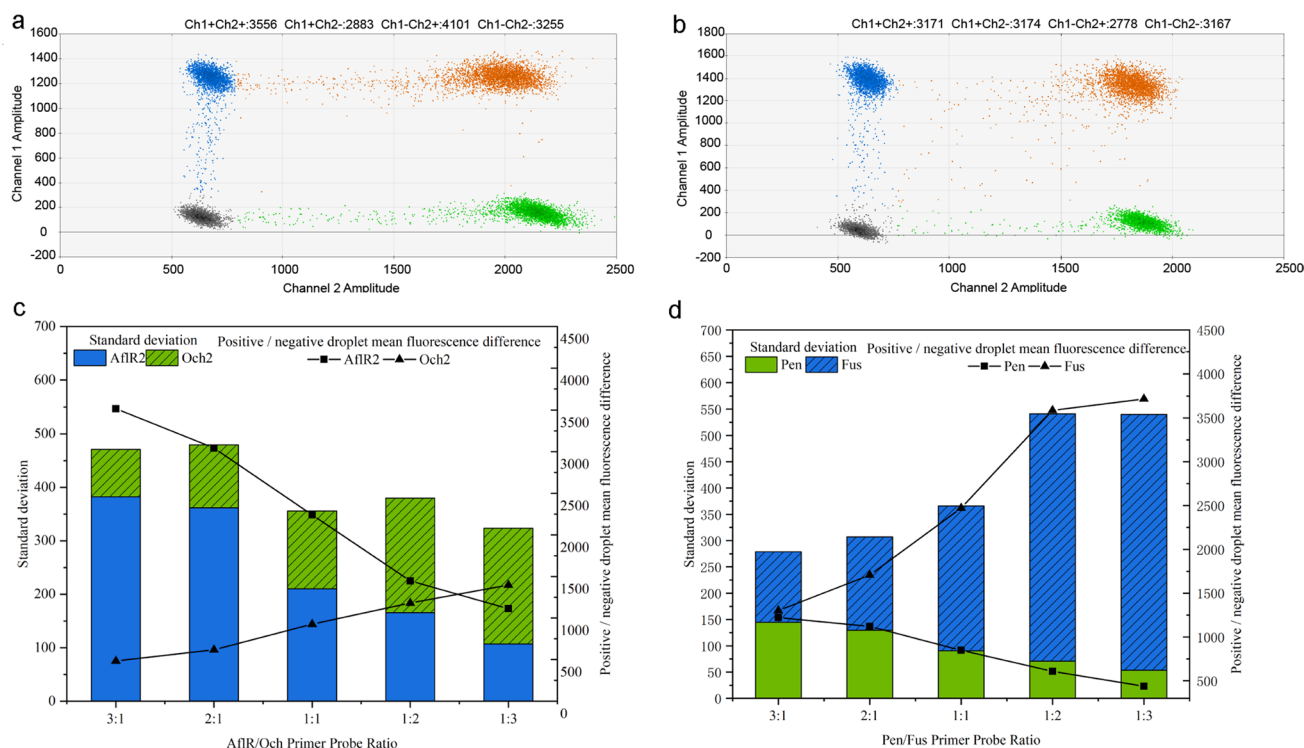


Fig. 3 Optimization of duplex ddPCR primer pairs and probes. a Two-dimensional plots of fluorescence amplitudes with the ratio of premix a to premix b were 1:3 in *AfIR/Och* duplex system; b two-dimensional plots of fluorescence amplitudes with the ratio of premix a to premix b were 3:1 in *Pen/Fus* duplex system; c comparison of the mean difference and standard deviation of fluorescence of posi-

tive/negative droplets in *AfIR/Och* ddPCR system of different ratio of premix a to premix b; d comparison of the mean difference and standard deviation of fluorescence of positive/negative droplets in *Pen/Fus* ddPCR system of different ratio of premix a to premix b. Blue and orange dots represent the positive droplets, and black and green dots represent the negative droplets

2×10^{-7} to 2×10^{-3} ng/ μ L, and the linear quantitative equations for the four genes were shown in Fig. 5.

Selection of Extraction Methods and Detection of DNA in Actual Samples

Eight samples, Jufeng grape, steamed bread, toast, sandwiched bread, northeastern rice, vitamin compound nutritious rice noodles, mineral compound nutritious rice noodles, and calcium compound nutritious rice noodles, were numbered as 1–8, respectively. The results were shown in Table S1, and the concentration of DNA in the table is the concentration extracted from 1-g food samples. The results showed that the extraction rate of DNA extracted by FastDNA™ SPIN Kit is the highest and the intra-group error is low. Sample 6, sample 7, and sample 8 are deeply processed foods, which may be the reason why the yield of DNA is generally low. Then the DNA of food samples extracted by three DNA extraction methods were added to the two established duplex ddPCR reaction systems for amplification. The amplification results of DNA extracted by FastDNA™ SPIN Kit are listed in Table 3, and the amplification results of the other two methods are shown

in Table S2 and Table S3. According to all the quantitative results, the DNA of food samples extracted by FastDNA™ SPIN Kit showed the highest detection rate of 40.63% of toxigenic fungal genes in two duplex ddPCR reaction systems.

Steamed breads and northeastern rice were the food products with the highest rate of detection of toxic fungi, with the detection of toxic fungal genes *AfIR*, *Och*, and *Fus* and *Och*, *Pen*, and *Fus*, respectively. Steamed breads had the highest *AfIR* gene copy number of 3,763 copies/ μ L and *Fus* gene copy number of 4,285 copies/ μ L; toast had the highest *Och* gene copy number of 2,930 copies/ μ L; Jufeng grape had the highest *Pen* gene copy number of 3,123 copies/ μ L.

To determine the accuracy of the duplex ddPCR methods, genomic DNA of fungal strains with known concentration was analyzed. Genomic DNA was extracted from each fungus three times, and three independent replicate assays were performed. The DNA copies were calculated according to the linear quantitative equations for the four genes as shown in Fig. 5. The amounts between 2×10^{-3} and $\sim 2 \times 10^{-7}$ ng DNA per μ L were all quantified. The results showed that for *AfIR* quantification, it was detected in *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus terreus*. The deviation from the theoretical value

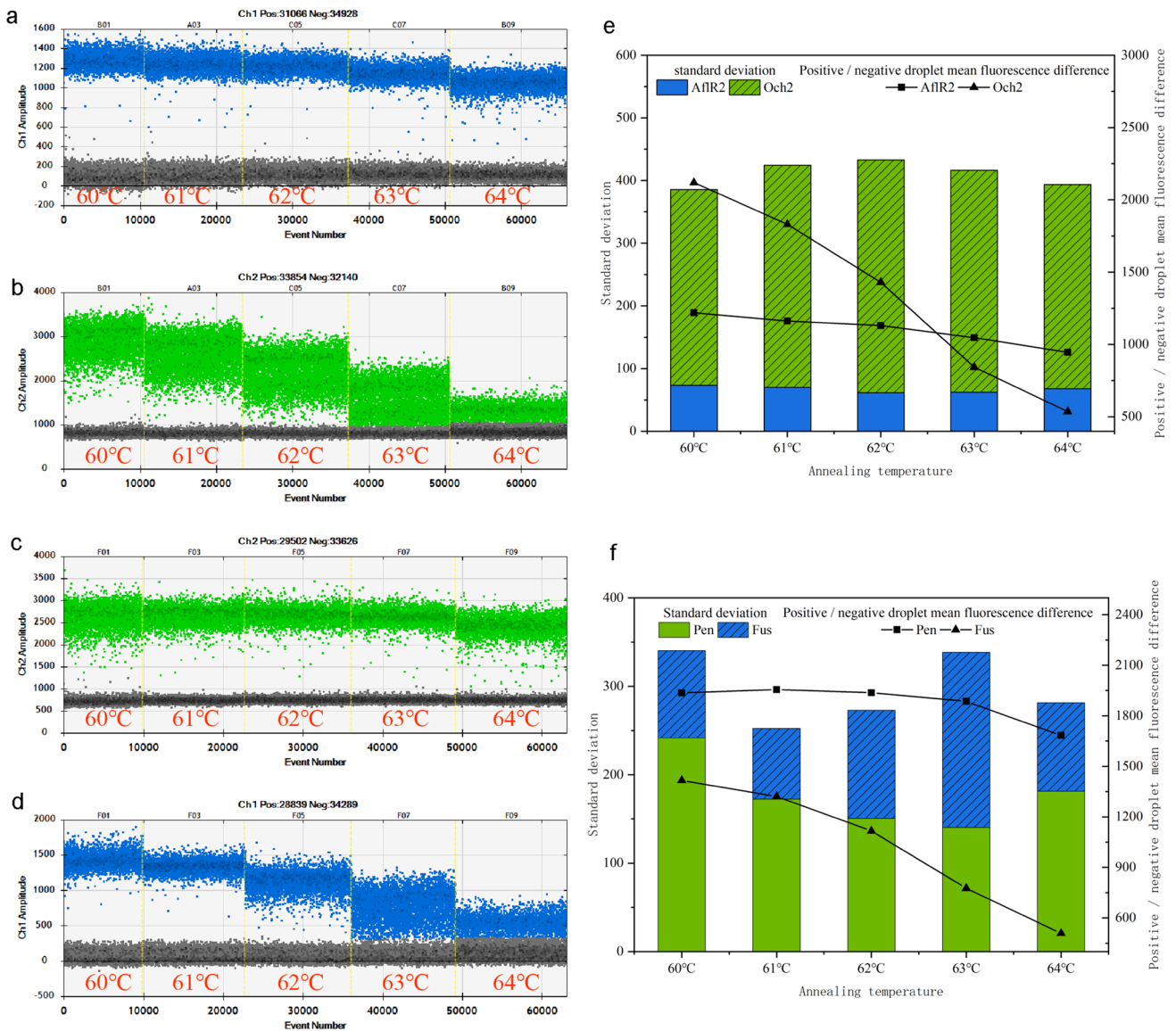


Fig. 4 Optimization of duplex ddPCR annealing temperatures. **a** *AflR* in *AflR/Och* duplex-system, at annealing temperatures 60 °C, 61 °C, 62 °C, 63 °C, and 64 °C; **b** *Och* in *AflR/Och* duplex system, at annealing temperatures 60 °C, 61 °C, 62 °C, 63 °C, and 64 °C; **c** *Pen* in *Pen/Fus* duplex-system, at annealing temperatures 60 °C, 61 °C, 62 °C, 63 °C, and 64 °C; **d** *Fus* in *Pen/Fus* duplex-system, at annealing temperatures 60 °C, 61 °C, 62 °C, 63 °C, and 64 °C; **e** comparison of the mean difference and standard deviation of fluorescence of positive/negative droplets in *AflR/Och* duplex ddPCR system of five annealing temperatures; **f** comparison of the mean difference and standard deviation of fluorescence of positive/negative droplets in *Pen/Fus* duplex ddPCR system of five annealing temperatures. Blue and green dots represent the positive droplets, and black dots represent the negative droplets

was – 89.77 to 0.54 (copies/μL), and the relative standard deviation (RSD) was between 0.32 and 18.78% (Table 4), far less than 25%, following FAO Guidelines for identification and quantification based on specific DNA sequences (Commission 2010). Meanwhile, *Och*, *Pen*, and *Fus* were tested in *Aspergillus ochraceus*, *Penicillium*, and *Fusarium*, respectively. The relative standard deviations ranged from 0.38 to 15.64, which verified the accuracy of the established method.

son of the mean difference and standard deviation of fluorescence of positive/negative droplets in *AflR/Och* duplex ddPCR system of five annealing temperatures; **f** comparison of the mean difference and standard deviation of fluorescence of positive/negative droplets in *Pen/Fus* duplex ddPCR system of five annealing temperatures. Blue and green dots represent the positive droplets, and black dots represent the negative droplets

Discussion

Mycotoxins are widely found in grains (Varzakas 2016), nuts (Rodrigues et al. 2012), fruits, soft drinks, alcoholic beverages (Alshannaq and Yu 2017; Welke 2019), and other agricultural products, which continue to have a great negative impact on food safety, human health, agricultural economy, and so on. The production of mycotoxins can

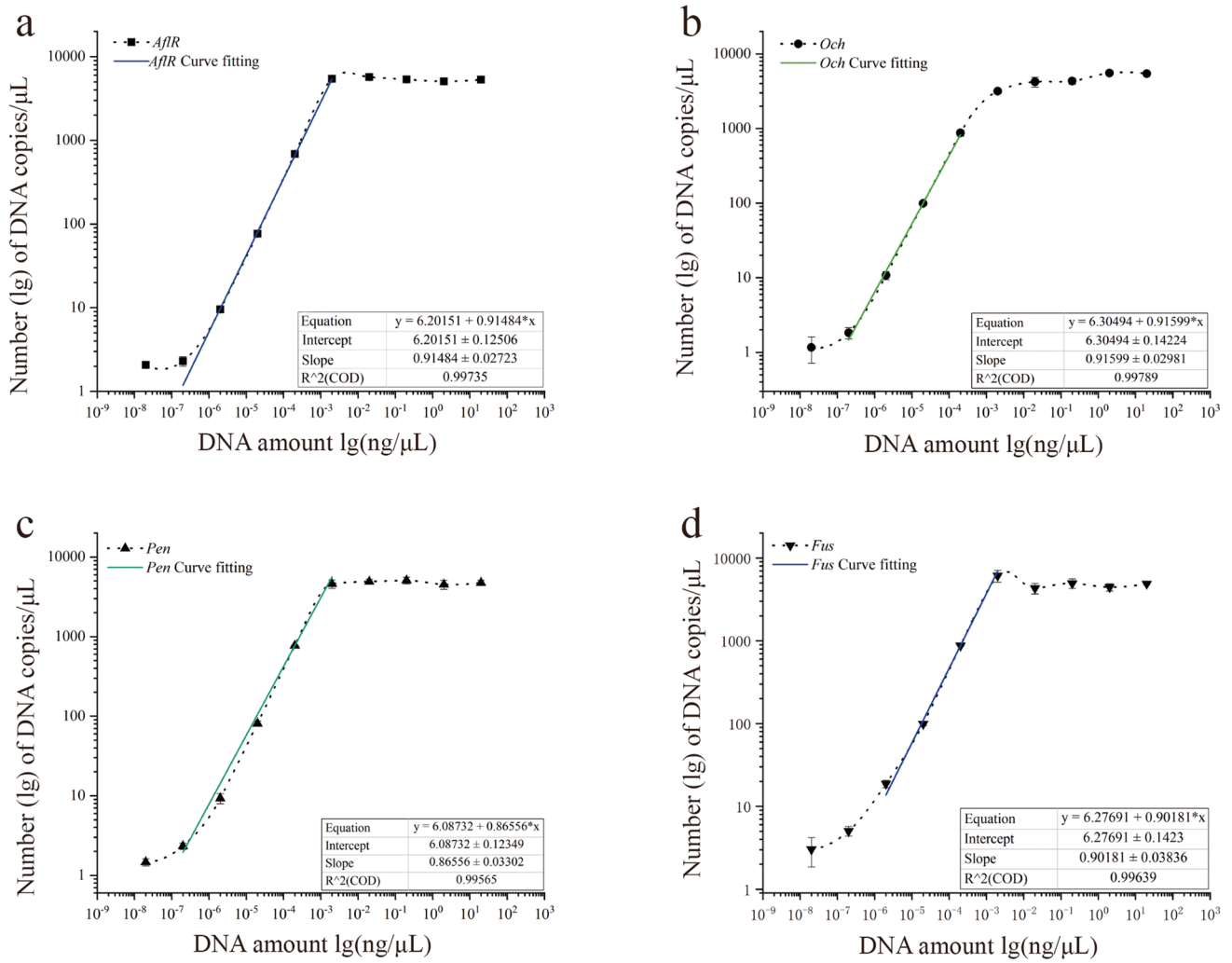


Fig. 5 Linear regression of the duplex ddPCR. a Copy numbers vs. plasmid DNA of *AfIR* in *AfIR/Och* duplex system; b copy numbers vs. plasmid DNA of *Och* in *AfIR/Och* duplex system; c copy numbers vs.

plasmid DNA of *Pen* in *Pen/Fus* duplex system; d copy numbers vs. plasmid DNA of *Fus* in *Pen/Fus* duplex system

Table 3 Quantification results of DNA extracted by FastDNA™ SPIN Kit in duplex ddPCR system

Sample no	<i>AfIR/Och</i> duplex ddPCR system				<i>Pen/Fus</i> duplex ddPCR system			
	<i>AfIR</i> copy number (copies/µL)	DNA concentration (ng/µL)	<i>Och</i> copy number (copies/µL)	DNA concentration (ng/µL)	<i>Pen</i> copy number (copies/µL)	DNA concentration (ng/µL)	<i>Fus</i> copy number (copies/µL)	DNA concentration (ng/µL)
1	-	-	1506.00 ± 116.38	6.57 × 10 ⁻⁵	3123.00 ± 115.49	1.01 × 10 ⁻³	-	-
2	3763.00 ± 143.91	1.35 × 10 ⁻³	2321.00 ± 185.17	1.13 × 10 ⁻³	-	-	4285.01 ± 277.61	1.3 × 10 ⁻³
3	215.00 ± 25.31	5.9 × 10 ⁻⁵	2930.00 ± 197.01	1.52 × 10 ⁻³	-	-	-	-
4	-	-	41.81 ± 2.85	7.91 × 10 ⁻⁶	-	-	-	-
5	-	-	125.00 ± 10.26	2.86 × 10 ⁻⁵	6.80 ± 0.79	8.49 × 10 ⁻⁷	4.00 ± 0.16	3.62 × 10 ⁻⁷
6	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	3.20 ± 0.32	2.78 × 10 ⁻⁷
8	-	-	-	-	-	-	2197.00 ± 103.26	5.9 × 10 ⁻⁴

“-” means not detected

Table 4 Theoretical and measured values and relative standard deviations calculated from quantitative equations after quantification of genomic DNA of fungal strains

	DNA sample (ng/ μ L)	Theoretical values (copies/ μ L)	Measured values 1 (copies/ μ L)	Measured values 2 (copies/ μ L)	Measured values 3 (copies/ μ L)	Average values (copies/ μ L)	SD (copies/ μ L)	Deviation (copies/ μ L)	RSD (%)
<i>Aspergillus flavus</i> (AflR)	2×10^{-3}	5399.54	5312.18	5325.2	5291.92	5309.77	16.77	-89.77	0.32
	2×10^{-4}	656.93	632.58	626.2	619.43	626.07	6.58	-30.86	1.05
	2×10^{-5}	79.92	76.06	70.65	72.22	72.98	2.78	-6.94	3.81
	2×10^{-6}	9.72	11.6	8.95	10.23	10.26	1.33	0.54	12.92
	2×10^{-7}	1.18	1.02	0.94	1.33	1.10	0.21	-0.08	18.78
<i>Aspergillus parasiticus</i> (AflR)	2×10^{-3}	5399.54	5285.35	5300.12	5437.2	5340.89	83.73	-58.65	1.57
	2×10^{-4}	656.93	643.28	650.11	639.18	644.19	5.52	-12.74	0.86
	2×10^{-5}	79.92	83.16	75.23	72.96	77.12	5.36	-2.80	6.94
	2×10^{-6}	9.72	9.05	10.23	9.56	9.61	0.59	-0.11	6.16
	2×10^{-7}	1.18	1.00	1.31	0.98	1.10	0.19	-0.08	16.87
<i>Aspergillus terreus</i> (AflR)	2×10^{-3}	5399.54	5388.76	5402.33	5297.8	5362.96	56.84	-36.58	1.06
	2×10^{-4}	656.93	643.25	650.12	651.28	648.22	4.34	-8.71	0.67
	2×10^{-5}	79.92	82.03	75.69	80.71	79.48	3.35	-0.44	4.21
	2×10^{-6}	9.72	9.56	10.23	9.76	9.85	0.34	0.13	3.49
	2×10^{-7}	1.18	1.03	1.29	1.26	1.19	0.14	0.01	11.92
<i>Aspergillus ochraceus</i> (Och)	2×10^{-3}	3645.83	3712.45	3600.28	3647.11	3653.28	56.34	7.45	1.54
	2×10^{-4}	585.4	580.4	567.31	573.64	573.78	6.55	-11.62	1.14
	2×10^{-5}	93.99	95.11	94.87	96.03	95.34	0.61	1.35	0.64
	2×10^{-6}	15.09	15.11	16.12	13.96	15.06	1.08	-0.03	7.17
	2×10^{-7}	2.42	2.57	2.00	2.62	2.40	0.34	-0.02	14.37
<i>Penicillium</i> (Pen)	2×10^{-3}	5638.42	5620.58	5528.32	5539.4	5562.77	50.37	-75.65	0.91
	2×10^{-4}	768.418	778.33	790.21	758.41	775.65	16.07	7.23	2.07
	2×10^{-5}	104.72	107.32	100.98	108.25	105.52	3.96	0.80	3.75
	2×10^{-6}	14.27	16.35	12.58	15.66	14.86	2.01	0.59	13.51
	2×10^{-7}	1.94	2.01	1.56	2.12	1.90	0.30	-0.04	15.64
<i>Fusarium</i> (Fus)	2×10^{-3}	6217.63	6200.89	6119.58	6197.21	6172.56	45.92	-45.07	0.74
	2×10^{-4}	872.74	890.23	886.72	893.5	890.15	3.39	17.41	0.38
	2×10^{-5}	122.5	130.2	119.58	127.8	125.86	5.57	3.36	4.43
	2×10^{-6}	17.2	20.26	18.69	16.19	18.38	2.05	1.18	11.17
	2×10^{-7}	2.41	3.14	2.58	3.2	2.97	0.34	0.56	11.50

occur at any stage of food production, which makes detection difficult because mycotoxins do not have characteristic odors and do not change the sensory properties of foods (Agriopoulou et al. 2020). Therefore, the accurate and quantitative detection of toxigenic fungi before the occurrence of mycotoxins is the most important part to control mycotoxin pollution. In earlier years, Gil-Serna et al. have described specific PCR methods based on multi-copy ITS regions to detect and identify major ochratoxigenic species of *Aspergillus* in foodstuffs (Gil-Serna et al. 2009). Similarly, Sardiñas et al. (2011) tested the PCR amplification ability of DNA obtained from 93 samples of paprika and pepper by using specific primers for 7 *Aspergillus* species. In some recent work, von Hertwig et al. proposed a real-time PCR method for the detection of *Aspergillus niger/Aspergillus wedii* (von Hertwig et al.

2018). But some mycotoxins are produced by more than one fungus, while others can produce more than one mycotoxin (Zain 2011). Based on this, multiplex PCR technique was applied to the detection of toxigenic fungi. “Multiplex PCR” (mPCR) refers to the simultaneous amplification of multiple targets in a single reaction by using more than one pair of primers. By targeting more than one gene at a time, additional information can be obtained from a single test (Hayat et al. 2012). Sim et al. established two multiplex PCR methods that can sensitively and specifically detect zearalenone-producing fungi in rice (Sim et al. 2018). The two mPCR methods show high sensitivity in detecting ZEA producing species, and the LOD is 1.25 μ g/ μ L of genomic DNA. Rodríguez et al. established a method for quantitative production of aflatoxin-producing mold and ochratoxin A (OTA)-producing mold in foodstuffs based

on TaqMan probe multiple real-time qPCR technology (Rodríguez et al. 2012). And the limit of detection was between 3 and 1 log cfu/g in all inoculated foods depending on the tested mycotoxin producer. In this work, on the basis of predecessors, two duplex droplet digital PCR systems were established to realize the absolute quantitative detection of four kinds of toxigenic fungi. Notably, addition of *Hind* III to the reaction could improve the aggregation of droplets, and the assay could be further optimized by regulating the premix a to b ratio in the two systems. Moreover the optimum annealing temperature was 60°C and 61°C for the *AflR/Och* and the *Pen/Fus* detection system, respectively. The detection limits of *AflR*, *Och*, *Pen*, and *Fus* are 26 copies/reaction, 15 copies/reaction, 161 copies/reaction, and 29 copies/reaction, respectively. The detection limits of duplex ddPCR were two to three orders of magnitude higher than those of our previously established qPCR method, while the detection limits for *Aspergillus ochraceus*, *Penicillium*, and *Fusarium* were 3.37×10^4 , 1.91×10^4 , 1.53×10^4 , and 3.95×10^4 copies/reaction, respectively (Zhang et al. 2020). The linear quantitative range of copy number of *AflR* and *Och* genes in A/O duplex system is 2×10^{-7} – 2×10^{-3} ng/μL, and the linear quantitative range of copy number of *Pen* and *Fus* genes in P/F duplex system is 2×10^{-7} – 2×10^{-3} ng/μL. As shown in Fig. 5, when the amount of target DNA in the sample is higher than the linear quantification range, the DNA concentration in the reaction system is too high, and the recombinant plasmid molecules contained in a single droplet are greater than or equal to 1 during droplet formation, forming a supersaturated state that no longer conforms to the Poisson distribution. Therefore, when the DNA content is high, the copy number of the target gene remains essentially constant. When the DNA content in the sample is below the linear quantification range, the copy number obtained at this point is equal to that of the blank control and can be considered as not amplified.

PCR and qPCR methodologies have been widely used in the detection of toxin-producing fungi. The existence of a variety of substances that inhibit PCR amplification in foods and the environment (Chen et al. 2017). This can lead to a decrease in sensitivity, leading to the emergence of false negative results. The ddPCR can solve part of this problem, because during the process, each template molecule is amplified in a separate reaction chamber, which is produced by droplets emulsified in oil or a microfluidic device (Hindson et al. 2011). The droplet has the same effect as a single test tube. The ddPCR system divides the DNA sample into millions of droplets to support PCR amplification of template molecules (Chen et al. 2017). In our results, positive droplets were clearly distinguished from negative droplets, and there were no false negatives in the ddPCR results. There was also

no cross-reactivity between specific and heterogeneous primers and each sample (Fig. 1). The reason why the blank control produces very few signals is very likely to be the high sensitivity of digital PCR. The false positive results caused by aerosol pollution in the laboratory can be solved by the appropriate threshold line in the quantitative process.

In the existing research, ddPCR was applied to the identification of true properties of foods (Yu et al. 2021) and the detection of pathogenic bacteria in foodstuffs (He et al. 2020; Lei et al. 2020). As far as we know, this is the first time that ddPCR methodology has been applied to the detection of toxigenic fungi. High-quality specific primers and probes were designed, multiple ddPCR reaction conditions were established and optimized, and the detection sensitivity was improved. The toxigenic fungi can be detected accurately and quantitatively during the latent period.

In addition, we used the constructed two duplex ddPCR systems to detect the DNA of 6 kinds of molds (*Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus ochraceus*, *Aspergillus nomius*, *Penicillium*, *Fusarium*) to repeatedly verify the reliability of the system. Among them, *Aspergillus parasiticus* and *Aspergillus nomius* were not detected in our system, which proved the excellent specificity of the two duplex ddPCR (data not shown in the results).

Conclusions

In summary, a method for the detection of potentially toxigenic fungi in foods by two duplex ddPCR systems was established. This method improves the sensitivity for the detection of toxin-producing fungi, has good repeatability, and can complete the detection of no more than 96 samples within 6 h. It meets the requirements of high-throughput and rapid detection. This work provides technical support for predicting the toxin-producing performance of latent toxigenic fungi and pre-intervention of toxin hazards in the early stage of toxin formation and provides a reference for effective prevention and control of toxin-contaminated food and ensuring food safety.

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Data Availability The datasets supporting the conclusions of this article are included within the article and its additional files.

Declarations

Ethics Approval This article does not contain any studies with human or animal subjects.

Informed Consent Not applicable.

Conflict of Interest Ping Wang declares that she has no conflict of interest. Tianming Qu declares that he has no conflict of interest. Xi-aomei Zhao declares that she has no conflict of interest. Yiqiang Ge declares that he has no conflict of interest. Ying Chen declares that she has no conflict of interest.

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