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Development and application of absolute quantitative detection by duplex chamber-based digital PCR of genetically modified maize events without pretreatment steps

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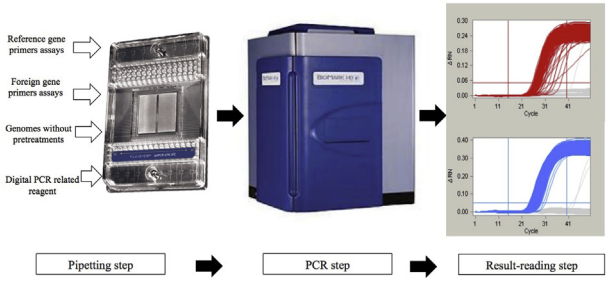
HIGHLIGHTS

- An improved duplex digital PCR independent of pretreatments for detecting GMO.
- Combinations of foreign and reference assays were optimized for duplex digital PCR.
- Duplex digital PCR showed a lower RSD compared with singleplex digital PCR.
- The duplex digital PCR detection system showed good stability and repeatability for all 7 GMO events in our study.

GRAPHICAL ABSTRACT



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ABSTRACT

The possibility of the absolute quantitation of GMO events by digital PCR was recently reported. However, most absolute quantitation methods based on the digital PCR required pretreatment steps. Meanwhile, singleplex detection could not meet the demand of the absolute quantitation of GMO events that is based on the ratio of foreign fragments and reference genes. Thus, to promote the absolute quantitative detection of different GMO events by digital PCR, we developed a quantitative detection method based on duplex digital PCR without pretreatment. Moreover, we tested 7 GMO events in our study to evaluate the fitness of our method. The optimized combination of foreign and reference primers, limit of quantitation (LOQ), limit of detection (LOD) and specificity were validated. The results showed that the LOQ of our method for different GMO events was 0.5%, while the LOD is 0.1%. Additionally, we found that duplex digital PCR could achieve the detection results with lower RSD compared with singleplex digital PCR. In summary, the duplex digital PCR detection system is a simple and stable way to

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achieve the absolute quantitation of different GMO events. Moreover, the LOQ and LOD indicated that this method is suitable for the daily detection and quantitation of GMO events.

1. Introduction

Genetically modified plants (GMPs) have developed rapidly since they were first commercialized in 1996 in the form of the delayed maturity tomato in America. In 2014, the cultivated area of GMPs was 181 million hectares, which represents an increase of 3.4% from 2013 and more than 100 times compared with 1996 [1]. The rapid development of GMPs has raised the public awareness of the uncertainties of their safety. Thus, to protect the consumer's right to know, different labeling laws were put into effect by various countries and groups, such as the 0.9% for European Union (EU), 5% for Japan, the volunteer labeling for America and the qualification labeling law for China. Among all the 357 GMP events, transgenetic maize events cover a significant ratio of 38.1% for 136 events [2], so that the specific quantitative detection methods for the transgenetic maize events take an important status.

To protect the consumers' right to know, different kinds of labeling regulations, which have ruled that the GMO content above a threshold should be labeled, have been published, including the quantitative labeling, qualitative labeling and volunteering labeling. The labeling regulations have raised strict standard to the detection of the GMO contents. To achieve the stable and sensitive detection of GMO content, various methodologies have been developed, including the PCR-based methods [3–7], stripe-based methods [8,9], array-based methods [10–15], sequencing-based method [16–19], biosensor-based methods [20,21], and so on. Currently, the most recommended detection method for labeling GMO content is the quantitative polymerase chain reaction (qPCR) method based on Taqman probes among all the above methodologies. Most of the validated detection standards used for quantitative labeling in, e.g., the EU [22–28] and Japan are based on this method because of its relatively high sensitivity and accuracy. However, for daily detection, qPCR has inevitable drawbacks [3,29] that may exacerbate detection. Quantitative detection based on qPCR relies on the standard curves generated by the serial dilution of certified reference materials [6] (CRMs). Thus, the operational variation of different operators and the lack of CRMs for different GMO events make qPCR a less ideal method for achieving absolute quantitative detection [30]. For the daily detection, the generation of standard curves of different events is a job needing extensive labor and money. According to the validated detection method of EU, the generation of standard curve for a single event should include at least 5 serial dilutions that contain at least 3 parallels. In this manner, simultaneously quantitative detection by qPCR of different events is a very costly and complicated approach.

Digital PCR [31], regarding as an improved quantitative detection method compared to the normal qPCR, is a newly developed absolute quantitative approach based on limiting dilution and Poisson statistical analysis. To achieve truly absolute quantitation, the original PCR reaction volume is separated by the microfluidic chambers [32] or the oil-water structure [33]. Then, the PCR reactions occur in the separated reaction “room”. The fluorescence signals of the separated PCR reactions are finally measured using a specialized device. The absolute copy number of the target sequence in the original sample is calculated according to the ratio of positive reactions to the total reactions. By dividing the original sample into thousands of separated “rooms”, the resistance of PCR inhibitors to the digital PCR reaction can be greatly improved compared with the normal qPCR such that the purity of the original genome is less important in digital PCR than it is in qPCR [34]. Moreover, the “matric effect” of normal PCR [35] that is caused by the unbalanced amplification of different regions in the tube is avoided as the separated digital PCR reaction volumes are around the nL or pL level. Digital PCR has achieved wide usage in the area of clinical research. It has already achieved widely applications among the area of the detection of diagnostic samples [36–38], viral samples [39–41], such as many diseases generated by deletions, insertions or SNP changes [42–44] of genomic DNA. Recent research has shown that digital PCR can be used as an absolute quantitative detection method [45–47]. However, according to different studies, the pretreatments should always be included in all the detection methods based on digital PCR [45–48]. Moreover, the pretreatments used in digital PCR might cause uncertainties in the final detection results as the recycling procedure. Additionally, the pretreatments and recycling procedures can inconvenience the practical detection approach. Thus, to promote the use of digital PCR for practical detection, pretreatment procedures should be excluded.

To develop duplex digital PCR detection methods based on various events of genetically modified maize samples, we evaluated and optimized the digital PCR quantitative detection method according to the event-specific PCR method. Moreover, for the absolute quantitation of GMO content based on the ratio of insertion genes and reference genes, we included duplex detection in our detection system to achieve more accurate results.

2. Materials and methods

2.1. GMO samples

All genetically modified maize samples used in our study were kindly provided by each developing company (Table S1). The negative maize sample was stored in the lab of the Supervision, Inspection and Testing Center of Genetically Modified Organisms, Ministry of Agriculture.

2.2. The preparation of GMO samples with different content

The grains of different GMO events were ground using a Retsch® MM430 mixer. This step could generate a large amount of heat. To protect the genome from heat damage, cooling steps should be included after grinding in each cycle. The final grounding steps involved 10 cycles of agitation for 20 s and cooling for 1 min to room temperature. For the experiments in our study, the relative mass GMO contents of different event samples were set to 100%, 50%, 20%, 10%, 5%, 1%, 0.5% and 0.1%. Samples were mixed according to their mass concentrations. The mixed samples were then placed on the Dynamic CM-200 mixer and shaken overnight for equal distribution.

2.3. Extraction of genomic DNA

The genomic DNA of different samples was extracted with the DNeasy Plant Mini Kit (Qiagen, Germany) using 50 μg of powdered