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Application of Digital PCR in the Analysis of Transgenic Soybean Plants

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Abstract

Detection and quantification of transgenes are important in analyzing genetically modified organisms (GMOs). Quantitative polymerase chain reaction (qPCR) is commonly utilized for such purposes. However, qPCR has certain limitations in detecting and quantifying transgenes in GMOs, such as the need of certified reference materials, a standard curve, and possible affection by inhibitors. Therefore, alternative and possibly better methods are needed. Recent advances in digital PCR technologies have promised to allow accurate quantification of nucleic acids and therefore provided another useful technique to analyze GMOs. Thermo Fisher Scientific™ has recently commercialized the Applied Biosystems™ QuantStudio™ 3D digital PCR system that can be used for a wide range of applications involving nucleic acids. It will be beneficial to the scientific community to show the applicability of this digital PCR system in detecting and quantifying transgenes in GMOs. In the present study, the transgenes present in the Roundup® Ready Soybean (RR1, event 40-3-2) and Roundup Ready Soybean 2 (RR2, event MON89788) developed by Monsanto Corporation were analyzed by using this digital PCR system. The qPCR analysis results were included for comparison. Using specifically designed TaqMan assays, as low as 1% of the RR1 or RR2 soybean material was reliably detected and quantified on the dPCR platform. Therefore, digital PCR is a sensitive and reliable method to analyze the RR transgenic soybeans, and should be another useful tool for analyzing other transgenic plants.

Keywords

Digital PCR, GMO, qPCR, Soybean, TaqMan

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1. Introduction

Nowadays, genetically modified crops are commonly grown in the United States. According to the recent report from the United States Department of Agriculture, genetically modified crops (mainly corn, cotton, and soybean) were planted on approximately half of the U.S. land used for crops in the year 2013 (http://www.ers.usda.gov/media/1282246/err162.pdf). Other countries, such as China and Brazil, are catching up fast in adopting and growing genetically modified crops due to the benefits of engineered traits, such as herbicide tolerance and insect resistance. However, the general public is still very concerned about the potential risks of genetically modified organisms (GMOs) to humans, animals, and the environment [1]-[6]. Strict regulations have been adopted by several countries to control GMOs, for example, the European Union (EU). Detection and quantification of GMOs are needed to implement such regulations. Currently, various DNA-based polymerase chain reaction (PCR) approaches are commonly used for detecting and/or quantifying transgenes in GMOs due to its sensitivity, for example, regular end-point PCR and real-time quantitative PCR (qPCR) methods, especially the latter [5] [7]-[16]. Occasionally, other methods, such as biosensors and immunoassays, are also used in analyzing GMOs [5] [7]-[13]. Additionally, some new technologies, such as next-generation sequencing and digital PCR (dPCR), have also been employed in analyzing GMOs, e.g., [15] [17] [18] [19] [20] [21].

dPCR reactions are based on the following principles: a DNA template is sufficiently diluted and subsequently partitioned into many independent small equal volume reactions (in wells or droplets). And the number of template molecules in these reactions follows a Poisson distribution. Therefore, an absolute quantification can be achieved by comparing the positive and negative reactions [14] [22] [23] [24] [25] [26]. Thus, dPCR possesses the following potential advantages over qPCR. dPCR can be used to accurately determine the number of nucleic acid molecules in a sample without certified reference material and a standard curve; the dilution of a template also correspondingly dilutes inhibitors possibly present in the template to make dPCR less sensitive to inhibitors [27] [28], therefore further improving accuracy and efficiency. These properties and advantages make dPCR an excellent tool for many applications where sensitivity or precise quantification of nucleic acids is needed, such as identifying mutations or copy number variations in tumor cells, detection of low copy number nucleic acid targets, or examining gene expression at the single-cell level [26] [29] [30] [31] [32]. Such properties of digital PCR should also be useful for analyzing GMOs. A number of studies have already been reported using digital PCR in analyzing GMOs, e.g., [17] [18] [20] [21] [28] [30] [32] [33] [34].

Currently, several digital PCR systems are available from the following companies: Bio-Rad Laboratories, RainDance, and Stilla Technologies [droplet-based], and Fluidigm Corporation and Thermo Fisher Scientific™ Applied Biosystems™ (chip-based) [25] [35] [36]. The digital PCR system released by Thermo Fisher Scientific™ Applied Biosystems™ in June, 2013 is called QuantStudio™ 3D Digital PCR System

(http://www.thermofisher.com/us/en/home/life-science/pcr/digital-pcr/quantstudio-3d-digital-pcr-system.html). This system allows up to 20,000 reactions to be run in parallel on a single, enclosed chip. The whole system, which includes a thermo cycler, an automatic chip loader, and a chip reader, is compact and can be easily fitted on a lab bench. The system is affordable (under $50,000). The system is easy to use and allows absolute quantification of nucleic acids (as copies of a nucleic acid per μl). Because the chip is enclosed, potential cross contamination can be avoided. Additionally, multiple chips can be combined to achieve necessary partitions and/or quantification of a DNA target in a sample (https://apps.thermofisher.com/quantstudio3d/). To test its usefulness in analyzing GMOs, the transgenic soybeans called Roundup® Ready (RR) 1 and 2 developed by the Monsanto Corporation were analyzed by using this QuantStudio™ 3D Digital PCR System.

Many studies have been conducted on the RR1 soybean, mainly using PCR-based methods to detect and/or quantify the transgene, e.g., [37]-[44]. Recently, detection and quantification of RR2 using digital PCR were also reported [34]. In the present study, the applicability of the Quant Studio™ 3D Digital PCR system in analyzing both RR1 and RR2 soybeans was examined. qPCR was also incorporated in the study as a comparison. In this study, the RR1 and RR2 soybean event-specific TaqMan assays were designed and shown that they were specific in detecting such transgenic events. As low as 1% of the RR1 or RR2 soybean material could reliably be detected and quantified by using these assays on the dPCR platform. Furthermore, dPCR results were not affected by DNA integrity, concentration, and purity. Overall, our work demonstrated the application of dPCR in detecting and quantifying the RR1 and RR2 soybeans. Obviously, dPCR should also be very useful in analyzing other GMOs, especially in the practical implementation of the EC Regulation 1830/2003, which states that "the results of quantitative analysis should be expressed as the number of target DNA sequences per target taxon specific sequences calculated in terms of haploid genomes" [45].

2. Materials and Methods

2.1. Plant Materials

S08-6201RR1 (Roundup® Ready 1 soybean; RR1 soybean) and S10-2635RR2 (Roundup® Ready 2 soybean; RR2 soybean) and Jake soybeans were used in the present study. These materials were provided by Dr. J. Grover Shannon (Division of Plant Sciences, University of Missouri, Columbia, MO 65211). Both RR1 and RR2 soybeans were developed by Monsanto through expressing the glyphosate-tolerant 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase. This shikimate pathway enzymeis absolutely required for the survival of plants. Therefore, plants (including RR1 and RR2 soybeans) expressing this glyphosate-tolerant EPSP are tolerant to Roundup® agricultural herbicides with glyphosate as the active ingredient, but plants, such as weeds, without this glyphosate-tolerant EPSP enzyme, are instead sensitive to these herbicides. Jake is a released soybean cultivar with the assigned plant introduction ID PI 643912, and does not contain the transgene for resistance to glyphosate.

2.2. DNA Extraction

Dry seeds were ground to fine powder using a coffee grinder, and then DNA was extracted from the powder using two methods: the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA, Cat# 69104) (http://www.qiagen.com/resources/resourcedetail?id=95dec8a9-ec37-4457-8884-5dedd8ba9448&lang=en) and the method developed by the Directorate General-Joint Research Centre Institute for Health and Consumer Protection Biotechnology & GMOs Unit (http://gmo-crl.jrc.ec.europa.eu/summaries/A2704-12_soybean_DNAExtr_report.pdf). We called the latter the traditional method for simplicity and also for distinguishing it from the Qiagen DNeasy Plant Mini Kit.

2.3. Initial Measurement of DNA Concentrations

DNA concentrations and quality were initially estimated using QuantiT dsDNA HS Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) with Qubit and a Nanodrop spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively. The concentrations from the Qubit assays were used to estimate the DNA input amount in each PCR reaction.

2.4. TaqMan Assays

TaqMan assays were modified from the original assays designed by the European Commission (Joint Research Centre) for these transgenic events using qPCR (Please refer to: "Event-specific method for the quantification of soybean lines 40 - 3 - 2 and MON89788 using real-time PCR") (http://gmo-crl.jrc.ec.europa.eu/summaries/40-3-2_validated_Method.pdf; http://gmo-crl.jrc.ec.europa.eu/summaries/MON89788_validated_Method.pdf). The assays were designed using the bioinformatic power of the TaqMan® Assay design pipeline, which uses proprietary algorithms of Thermo Fischer Scientific (https://www.thermofisher.com/order/custom-genomic-products/tools/gene-expression/). The primer sequences used in the present work were listed in **Table 1**. The differences between our modified assays and those original ones were also included in **Table 1**.

2.5. Digital PCR Experiments

For a 20 µl reaction: add 10 µl 2x master reaction mix, 1 µl 20 x assay mix, y µl DNA, and 9-y µl dd H_2O. Mix well and span briefly. Load 14.5 µl of the PCR mixture onto a Quant Studio™ 3D Digital PCR 20K Chip, cover the chip with immersion fluid, apply a lid, fill the assembly with immersion fluid, and then seal the loading port according to the manufacturer's instructions (User guide, Quant Studio™ 3D Digital PCR System, AppliedBiosystems, Thermo Fisher Scientific, Waltham, MA, USA). Perform the PCR using the AppliedBiosystems™ Dual Flat Block GeneAmpR PCR System 9700 with the following conditions: 96˚C for 10 minutes; 60˚C for 2 minutes and 98˚C for 30 seconds,

for 39 cycles; 60˚C for 2 minutes; 10˚C hold. Reading of the Digital PCR 20K Chip was performed using the Quant Studio™ 3D Digital PCR Chip Reader. The data analysis, including statistical analysis, was conducted using the Quant Studio™ 3D Analysis Suite™ Cloud Software (https://apps.thermofisher.com/quantstudio3d/). The following method was used to calculate the percentage of the RR1 or RR2 soybean material added in the non-transgenic soybean material: (copies/μl of the RR1 or RR2 transgene)/(copies/μl of the Lectin gene) in the same sample.

2.6. qPCR Experiments

For comparison, DNA samples were also analyzed using the same primers and probes on a 7900 HT Fast Real-Time PCR System (ABI Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), with the following PCR conditions: 95˚C for 10 minutes; 95˚C for 15 seconds, 60˚C for 1 minute, for 40 cycles. Three technical replicates were conducted for each sample. The following method was used to calculate the percentage of the RR1 or RR2 soybean material added in the non-transgenic soybean material: 2^{deltaCT} [%]. DeltaCT = CT value of the RR1 or RR2 transgene-CT value of the Lectin gene in the same sample.

3. Results

3.1. Design of TaqMan Assays for Detecting and Quantifying the RR1 and RR2 Transgenes

To specifically detect and quantify the RR transgenes in the RR1 and RR2 soybeans, respectively, TaqMan assays that target their specific transgenic events (RR1/40-3-2 and RR2/MON 89788) were designed (Table 1). As a reference and control, the TaqMan assay was also designed for the soybean endogenous gene, Lectin (Glyma02g01260) (Table 1), a single copy gene in the soybean genome [46].

Table 1. TaqMan assays.

Name		Sequence	Note
For Roundup Ready Soybean event 40-3-2 [RR1]	40-3-2A-F	TTCATTCAAAATAAGATCATACATACAGGTT	Forward primer; sameas the original primer
	40-3-2A-R	GCATTTGTAGGAGCCACCTT	Reverse primer; 1-nt [nucleotide] shorter than the original primer
	40-3-2A-PFAM	6-FAM-CCTTTTCCATTTGGG-MGB/NFQ	Probe; same as the original probe
For Roundup Ready Soybean event MON 89788 [RR2]	MON89788-F	CCGCTCTAGCGCTTCAAT	Forward primer; 1-nt shorter than the original primer
	MON89788-R	GAGCAGGACCTGCAGAA	Reverse primer; 2-nt shorter than the original primer
	MON89788-PFAM	6-FAM-CTGAAGGCGGGAAAC-MGB/NFQ	Probe; shorter than the original probe
For the Lectin gene	Lec-F	GCTTCGCCGCTTCCTTC	Forward primer; 3-nt shorter than the original primer
	Lec-R	AAGCCCATCTGCAAGCC	Reverse primer; 1-nt shorter than the original primer
	Lec-PFAM	6-FAM-CTTCACCTTCTATGCCCCTG-MGB/NFQ	Probe [FAM]; 4-nt shorter than the original probe
	Lec-PVIC	VIC-CTTCACCTTCTATGCCCCTG-MGB/NFQ	Probe [VIC]; 4-nt shorter than the original probe

3.2. The Designed TaqMan Assays Were Specific for Detecting and Quantifying the RR1 and RR2 Soybeans Using dPCR

To test the assay specificity in detecting the event-specific RR soybeans, the designed TaqMan assays were employed to analyze both RR1 and RR2 soybeans, together with the conventional non-transgenic soybean control (Jake), using dPCR. For this purpose, the intact DNA was used, which was isolated via the method developed by the Directorate General-Joint Research Centre Institute for Health and Consumer Protection Biotechnology & GMOs Unit (http://gmo-crl.jrc.ec.europa.eu/summaries/A2704-12_soybean_DNAExtr_report.pdf), the so-called traditional method designated in the present study. In this first experiment, the target and reference gene reactions were conducted separately. Equal amounts (40 ng) of genomic DNA from the RR1, RR2 and Jake soybeans were used as the template in each reaction, leading to a final concentration of 2 ng/microliter (μl) of genomic DNA. After TaqMan PCR reactions, the chips were scanned using the chip reader, and the obtained data were analyzed using the QuantStudio™ 3D AnalysisSuite™ Cloud Software (https://apps.thermofisher.com/quantstudio3d/).

After the data analysis, the copies of the target [RR1 or RR2 transgene] or reference gene per μl in each reaction were obtained (**Table 2**): 1198, 1403.3, and 1416 copies/μl of the Lectin gene; 0.5, 1443.7, and 0.948 copies/μl of the RR1 transgene; and 0.4, 0.4,

Table 2. The TaqMan assays were specific for RR soybean events using digital PCR.

Target	Sample	Copies/μl	Confidence interval of copies/μl
Lectin	Jake soybean	1198.0	1175.7 - 1220.7
RR1	Jake soybean	0.5	0.3 - 1.1
RR2	Jake soybean	0.4	0.2 - 0.8
Lectin	RR1 soybean	1403.3	1377.9 - 1429.1
RR1	RR1 soybean	1443.7	1417.0 - 1470.9
RR2	RR1 soybean	0.4	0.2 - 0.8
Lectin	RR2 soybean	1416.0	1390.3 - 1442.2
RR1	RR2 soybean	0.9	0.6 - 1.5
RR2	RR2 soybean	1482.5	1455.3 - 1510.3
RR1	RR Maize	0.9	0.5 - 1.5
adh1	RR Maize	372.5	362.1 - 383.2
RR2	RR Maize	0	NA
adh1	RR Maize	419.1	408.1 - 430.3
RR1	Non-GMO Maize	1.2	0.8 - 1.8
adh1	Non-GMO Maize	460.7	449.3 - 472.5
RR2	Non-GMO Maize	0.1	0 - 0.5
adh1	Non-GMO Maize	469.0	457.6 - 480.6

and 1482.5 copies/μl of the RR2 transgene were detected in the Jake, RR1, and RR2 reactions, respectively. Because only 0.948 copies of the RR1 transgene in the RR2 soybean and 0.4 copies of the RR2 transgene in the RR1 soybean, and only 0.5 copies of the RR1 transgene and 0.4 copies of the RR2 transgene in the non-transgenic Jake soybean were detected, the assays designed for the RR1 and RR2 soybeans are thus very specific for each of the two transgenic events.

Notably, the copies of a RR transgene per μl was very close to those of the reference Lectin gene in the same RR soybean sample: There were 1443.7 copies of the RR1 transgene and 1403.3 copies of the reference Lectin gene in each μl of the RR1 PCR reaction, and 1482.5 copies of the RR2 transgene and 1416 copies of the reference Lectin gene in each μl of the RR2 PCR reaction. No RR transgene was detected in the non-transgenic Jake soybean. Therefore, both TaqMan assays worked very well for detecting and quantifying the RR transgenes in the RR soybeans on the dPCR platform.

The DNA concentration in the reaction mix as well as in the original DNA stock can easily be calculated using these data. Because the soybean genome (haploid) size is 1.1 Gbp (1.1×10^9 bp), the soybean genome mass is estimated at 1.21×10^{-12} g or 1.21 pg/genome, using the formula: $m = (n)\ (1.096 \times 10^{-21}$ g/bp), where m is the genome mass in grams, and n is the genome size [haploid] in base pairs. Because the Lectin gene is only one copy in the soybean haploid genome, the measured copies of the Lectin gene in each reaction should represent the copies of the soybean genome. Therefore, there were 1198, 1403.3, and 1416 copies of the soybean genome/μl in the Jake, RR1, and RR2 reactions, respectively, and the DNA concentrations should be $1.21 \times 1198 = 1449.6$ pg/μl (or 1.45 ng/μl), $1.21 \times 1403.3 = 1698$ pg/μl (or 1.70 ng/μl), $1.21 \times 1416 = 1713.4$ pg/μl (or 1.71 ng/μl), respectively. Initially, 40 ng of DNA was input in each of these 20 μl reactions based on the measurements using Qubit™ assays, leading to 2 ng/μl DNA in these reactions. Therefore, Qubit™ assays over-estimated these DNA concentrations by 18% - 33%. However, we noticed that comparable Lectin copies were observed in different samples, suggesting that Qubit assays provided comparable estimations of DNA concentrations in different samples, although such concentrations were over-estimated. The DNA concentrations can also be calculated using the copy numbers of the RR transgenes, and they should be very similar to those calculated using the copy numbers of the Lectin gene.

To further test the specificity of the assays designed for the RR1 and RR2 soybeans, these assays were also used to analyze the RR maize. In this experiment, 60 ng of the RR maize DNA (based on the concentration measured using a Nanodrop spectrometer) was input in each reaction. After the data analysis, only 0.85 copies/μl of the RR1 transgene and 0 copies/μl of the RR2 transgene were detected in the RR maize sample using 40-3-2A (for the RR1 soybean) assay and MON89788 (for the RR2 soybean) assay, respectively. As expected, only 1.1 copies/μl of the RR1 transgene and 0.1 copies/ μl of the RR2 transgene were detected in the non-transgenic maize DNA sample using 40-3-2A (for the RR1 soybean) assay and MON89788 (for the RR2 soybean] assay, respectively. Meanwhile, the maize adh1 (alcohol dehydrogenase 1) reference assay de-

tected 372.5 to 469.7 copies of this reference gene, adh1, or the genome per µl in these reactions (Table 2). Therefore, the assays designed for the RR1 and RR2 soybeans are indeed specific, and can be used to detect whether the RR transgene comes from the RR soybeans in a mixed transgenic sample.

Overall, the designed TaqMan assays were excellent for detecting the RR soybean specific transgenic events and measuring DNA concentrations in these samples using the dPCR platform.

3.3. Sheared Genomic DNA Had No Effect on Detecting and Quantifying the RR Transgenes Using dPCR

To test whether sheared genomic DNA affects the accurate detection and quantification of the transgene and copies of the genome, and whether the results can be repeated using different DNA formats and concentrations, genomic DNA was extracted using the Qiagen DNeasy Plant Mini Kit (http://www.qiagen.com/resources/resourcedetail?id=95dec8a9-ec37-4457-8884-5dedd8ba9448&lang=en). It took much less time to extract DNA using this kit than the lengthy traditional method used for isolating the intact genomic DNA as described afore. This kit is supposed to generate sheared and pure DNA predominating with fragments of 20 - 25 kb (http://gmo-crl.jrc.ec.europa.eu/summaries/A2704-12_soybean_DNAExtr_report.pdf). In this experiment, 20 ng, instead of 40 ng, of sheared genomic DNA was input in each 20 µl reaction, leading to 1 ng/µl of DNA in the reaction mix. The duplex assays were conducted in the same PCR reaction to detect both target and reference genes. In this case, the target probe [for RR1 or RR2] was labeled with 6-FAM, and the reference Lectin gene probe was labeled with VIC.

Data analysis has revealed that 644.3 - 754.3, 603, and 555.5 copies/µl of the Lectin gene were present in the Jake, RR1, and RR2 reactions, respectively. Notably, these numbers were roughly half of those revealed in the first experiment, in which twice the amount of intact genomic DNA [40 ng] was input in each reaction. For the RR targets, 0.7 copies of the RR1 transgene/µl and 0.8 copies of RR2 transgene/µl were detected in Jake, 625.9 copies of RR1 transgene/µl were detected in the RR1 reaction, and 688.6 copies of RR2/µl were detected in the RR2 reaction (Table 3). Once again, similar copy

Table 3. Sheared genomic DNA produced results similar to those from intact genomic DNA using digital PCR.

Target	Sample	Copies/µl	Confidence interval of copies/µl
RR1	Jake soybean	0.7	0.4 - 1.2
Lectin	Jake soybean	754.4	738.0 - 771.1
RR2	Jake soybean	0.8	0.5 - 1.4
Lectin	Jake soybean	644.3	630.2 - 658.8
RR1	RR1 soybean	625.9	612.0 - 640.0
Lectin	RR1 soybean	603.0	589.5 - 616.8
RR2	RR2 soybean	688.7	673.8 - 703.8
Lectin	RR2 soybean	555.5	542.6 - 568.7

numbers were detected for both the Lectin gene and RR transgene in the same RR sample. There were 625.9 copies of the RR1 transgene and 603 copies of the Lectin gene in each μl of the RR1 PCR reaction, and 688.6 copies of the RR2 transgene and 555.5 copies of the Lectin gene in each μl of the RR2 PCR reaction. No RR transgene was detected in the non-transgenic Jake soybean. These numbers were also roughly half of those revealed in the first experiment. Additionally, the duplex assays in this experiment produced results similar to those obtained with the simplex assays in the first experiment. Therefore, the Qiagen DNeasy Plant Mini Kit can be used to isolate DNA for digital PCR, even though sheared DNA is generated. Overall, the above two experiments demonstrated that dPCR was not affected by DNA integrity, concentration and purity, and whether simplex or duplex assays were used.

3.4. dPCR Was Accurate and Sensitive in Detecting and Quantifying the RR1 and RR2 Soybeans

To test the accuracy and sensitivity of digital PCR in detecting and quantifying the RR transgenes in a soybean mixture, the RR soybean fine powder was spiked in the non-transgenic Jake powder at 100%, 10%, 1%, 0.1%, and 0% (Weight/Weight), and then DNA was isolated using the Qiagen DNeasy Plant Mini Kit. The isolated DNA (20 ng each) was assayed using dPCR. After data analysis, 603, 618.6, 637.8, 571.5, and 754.4 copies of the Lectin gene/μl were detected in each of the RR1 dilution series, indicating the input of comparable amounts of DNA in each of the PCR reactions. Similar numbers of the Lectin gene copies/μl, 555.5, 548, 673.4, 598.3, and 644.3, were also detected in each of the RR2 dilution series. Impressively, 625.9, 61.1, 5.1, 0.9, and 0.7 copies of the RR1 transgene/μl were detected respectively in the 100%, 10%, 1%, 0.1%, and 0% of the RR1 soybean samples, very similar to the expected dilutions; and 688.7, 83.1, 4.1, 1.3, and 0.8 copies of the RR2 transgene/μl were respectively detected in the 100%, 10%, 1%, 0.1%, and 0% of the RR2 soybean samples, also very similar to the expected dilutions (Table 4). Furthermore, the RR materials could easily be detected and quantified in these mixed samples.

To compare the accuracy and sensitivity of dPCR and qPCR in measuring RR transgenes, the above DNA samples were also tested using qPCR. As shown in Figure 1, dPCR appeared to perform slightly better than qPCR in quantifying high levels of the RR1 DNA, while qPCR appeared to perform slightly better than dPCR in quantifying high levels of the RR2 DNA. But both appeared to be equally good at quantifying low levels of the RR1 and RR2 transgenes.

Therefore, dPCR showed good accuracy in measuring DNA in a series of diluted samples and sensitivity in quantifying as low as 1% of the RR transgenic materials in a sample, comparable to that provided by qPCR.

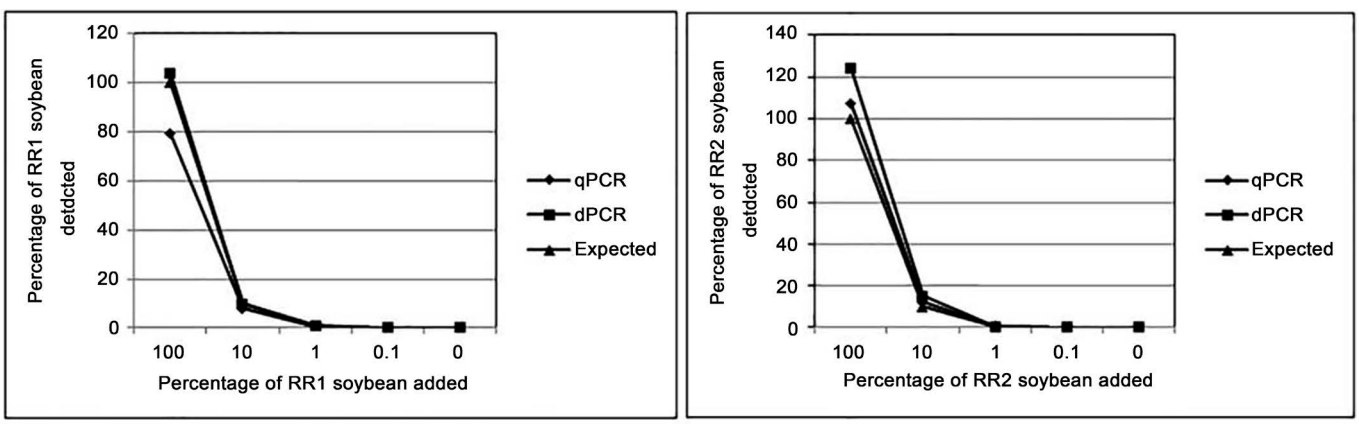
4. Discussion

4.1. Assay Specificity

In the present study, the previous TaqMan assays designed for analyzing the RR1 and RR2 soybean events, 40-3-2 and MON89788, respectively, using qPCR, were modified

Table 4. Digital PCR was accurate and sensitive in detecting RR soybeans.

Target	Sample [RR soybean mixed with Jake soybean]	Copies/μl	Confidence interval of copies/μl
RR1	100% RR1 + 0% Jake	625.9	612.0 - 640.0
Lectin	100% RR1 + 0% Jake	603.0	589.5 - 616.8
RR1	10% RR1 + 90% Jake	61.1	57.3 - 65.1
Lectin	10% RR1 + 90% Jake	618.6	604.7 - 632.8
RR1	1% RR1 + 99% Jake	5.1	4.1 - 6.3
Lectin	1% RR1 + 99% Jake	637.8	623.9 - 652.0
RR1	0.1% RR1 + 99.9% Jake	0.9	0.5 - 1.4
Lectin	0.1% RR1 + 99.9% Jake	571.5	558.5 - 584.8
RR1	0% RR1 + 100% Jake	0.7	0.4 - 1.2
Lectin	0% RR1 + 100% Jake	754.4	738.0 - 771.0
RR2	100% RR2 + 0% Jake	688.7	673.8 - 703.8
Lectin	100% RR2 + 0% Jake	555.5	542.6 - 568.7
RR2	10% RR2 + 90% Jake	83.1	78.7 - 87.8
Lectin	10% RR2 + 90% Jake	548.0	535.3 - 560.9
RR2	1% RR2 + 99% Jake	4.6	3.6 - 5.7
Lectin	1% RR2 + 99% Jake	673.4	658.7 - 688.4
RR2	0.1% RR2 + 99.9% Jake	1.3	0.8 - 2.0
Lectin	0.1% RR2 + 99.9% Jake	598.3	584.3 - 612.6
RR2	0% RR2 + 100% Jake	0.8	0.5 - 1.4
Lectin	0% RR2 + 100% Jake	644.3	630.2 - 658.8



(a) (b)

Figure 1. Comparison of quantitative PCR and digital PCR in quantifying roundup ready soybeans. X-axis: Percentage of roundup ready soybeans [RR1 or RR2 added in a mixed sample; Y-axis: Percentage of roundup ready soybean RR1 or RR2 transgenic material detected in a mixed sample. (a) roundup ready soybean RR1; (b) roundup ready soybean RR2.

and shown to work very well in both dPCR and qPCR reactions in detecting and quantifying their own target sequences. No cross reactions were found between each other and with any other soybean or maize DNA sequence. Therefore, these assays can be used by others in detecting and quantifying the RR1 and RR2 soybean materials in GMOs.

4.2. Accuracy and Sensitivity of dPCR

In the present work, dPCR was shown to be capable of detecting and measuring DNA copy numbers consistently under various conditions: different DNA formats [intact *vs.* sheared, isolated using different methods], different input amounts, different purities, and simplex *vs.* duplex assays. Furthermore, dPCR can easily and confidently measure 1% of the RR soybean material in a mixed sample.

4.3. Digital PCR *vs.* qPCR

In the present work, dPCR and qPCR provided comparable results in analyzing the RR soybeans by using the TaqMan assays in terms of accuracy and sensitivity. However, dPCR is a simple, straightforward method for the absolute quantitation of DNA. Therefore, dPCR should be another good approach to be considered when it comes to analyzing GMOs. It is a particularly useful tool for the practical implementation of EC Regulation 1830/2003, which states that "the results of quantitative analysis should be expressed as the number of target DNA sequences per target taxon specific sequences calculated in terms of haploid genomes" [45].

4.4. Effect of Intact and Sheared Genomic DNA on DNA Quantification Using dPCR

In the present work, the effect of DNA samples extracted from the RR soybean seeds using two different methods was examined: the traditional, lengthy method that was supposed to produce intact genomic DNA, and the column-based Qiagen DNeasy Plant Mini Kit that was supposed to produce sheared DNA ranging from 20-40 Kb. Additionally, the latter method is supposed to yield DNA with less contaminants. Notably, DNA samples from these two methods have generated very comparable results, indicating that dPCR was less sensitive to DNA integrity and purity in contrast to qPCR, which tended to be affected by such factors [47] [48].

4.5. DNA Concentration Measurements

In the present work, both Qubit assays and Nanodrop spectrometer was shown to significantly over-estimate all DNA samples used in the study, especially the Nanodrop spectrometer: a DNA sample can be over-estimated by over 10 times by a Nanodrop spectrometer and 30% by the Qubit assay. One possible cause for such over-estimation was likely from contaminants in DNA samples. Overall, Qubit assays appeared to be a good, dependable assay in the initial estimation of DNA concentrations for dPCR experiments.

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Conflict of Interest

The authors hereby declare no conflict of interest.

References

[1] Paparini, A. and Romano-Spica, V. (2004) Public Health Issues Related with the Consumption of Food Obtained from Genetically Modified Organisms. *Biotechnology Annual Review*, **10**, 85-122. http://dx.doi.org/10.1016/S1387-2656(04)10004-5

[2] Varzakas, T.H., Arvanitoyannis, I.S. and Baltas, H. (2007) The Politics and Science behind GMO Acceptance. *Critical Reviews in Food Science and Nutrition*, **47**, 335-361. http://dx.doi.org/10.1080/10408390600762696

[3] Hug, K. (2008) Genetically Modified Organisms: Do the Benefits Outweigh the Risks? *Medicina* (*Kaunas*), **44**, 87-99.

[4] Rajan, S.R. and Letourneau, D.K. (2012) What Risk Assessments of Genetically Modified Organisms Can Learn from Institutional Analyses of Public Health Risks. *Journal of Biomedicine and Biotechnology*, **2012**, Article ID: 203093. http://dx.doi.org/10.1155/2012/203093

[5] Kamle, S. and Ali, S. (2013) Genetically Modified Crops: Detection Strategies and Biosafety Issues. *Gene*, **522**, 123-132. http://dx.doi.org/10.1016/j.gene.2013.03.107

[6] Lucht, J.M. (2015) Public Acceptance of Plant Biotechnology and GM Crops. *Viruses*, **7**, 4254-4281. http://dx.doi.org/10.3390/v7082819

[7] Ahmed, F.E. (2002) Detection of Genetically Modified Organisms in Foods. *Trends in Biotechnology*, **20**, 215-223. http://dx.doi.org/10.1016/S0167-7799(01)01920-5

[8] Elenis, D.S., Kalogianni, D.P., Glynou, K., Ioannou, P.C. and Christopoulos, T.K. (2008) Advances in Molecular Techniques for the Detection and Quantification of Genetically Modified Organisms. *Analytical and Bioanalytical Chemistry*, **392**, 347-354. http://dx.doi.org/10.1007/s00216-008-1868-4

[9] Michelini, E., Simoni, P., Cevenini, L., Mezzanotte, L. and Roda, A. (2008) New Trends in Bioanalytical Tools for the Detection of Genetically Modified Organisms: An Update. *Analytical and Bioanalytical Chemistry*, **392**, 355-367. http://dx.doi.org/10.1007/s00216-008-2193-7

[10] Holst-Jensen, A. (2009) Testing for Genetically Modified Organisms (GMOs): Past, Present and Future Perspectives. *Biotechnology Advances*, **27**, 1071-1082. http://dx.doi.org/10.1016/j.biotechadv.2009.05.025

[11] Querci, M., Van den Bulcke, M., Zel, J., Van den Eede, G. and Broll, H. (2010) New Approaches in GMO Detection. *Analytical and Bioanalytical Chemistry*, **396**, 1991-2002. http://dx.doi.org/10.1007/s00216-009-3237-3

[12] Broeders, S.R., De Keersmaecker, S.C. and Roosens, N.H. (2012) How to Deal with the Upcoming Challenges in GMO Detection in Food and Feed. *Journal of Biomedicine and Biotechnology*, **2012**, Article ID: 402418. http://dx.doi.org/10.1155/2012/402418

[13] Holst-Jensen, A., Bertheau, Y., de Loose, M., Grohmann, L., Hamels, S., Hougs, L., *et al*.

(2012) Detecting Unauthorized Genetically Modified Organisms (GMOs) and Derived Materials. *Biotechnology Advances*, **30**, 1318-1335. http://dx.doi.org/10.1016/j.biotechadv.2012.01.024

[14] Milavec, M., Dobnik, D., Yang, L., Zhang, D., Gruden, K., and Zel, J. (2014) GMO Quantification: Valuable Experience and Insights for the Future. *Analytical and Bioanalytical Chemistry*, **406**, 6485-6497. http://dx.doi.org/10.1007/s00216-014-8077-0

[15] Fraiture, M.A., Herman, P., Taverniers, I., De Loose, M., Deforce, D. and Roosens, N.H. (2015) Current and New Approaches in GMO Detection: Challenges and Solutions. *BioMed Research International*, **2015**, Article ID: 392872. http://dx.doi.org/10.1155/2015/392872

[16] Salihah, N.T., Hossain, M.M., Lubis, H. and Ahmed, M.U. (2016) Trends and Advances in Food Analysis by Real-Time Polymerase Chain Reaction. *Journal of Food Science and Technology*, **53**, 2196-1209. http://dx.doi.org/10.1007/s13197-016-2205-0

[17] Dobnik, D., Spilsberg, B., Bogožalec Košir, A., Holst-Jensen, A. and Žel, J. (2015) Multiplex Quantification of 12 European Union Authorized Genetically Modified Maize Lines with Droplet Digital Polymerase Chain Reaction. *Analytical Chemistry*, **87**, 8218-8226. http://dx.doi.org/10.1021/acs.analchem.5b01208

[18] Fu, W., Zhu, P., Wang, C., Huang, K., Du, Z., Tian, W., *et al*. (2015) A Highly Sensitive and Specific Method for the Screening Detection of Genetically Modified Organisms Based on Digital PCR without Pretreatment. *Scientific Reports*, **5**, 12715. http://dx.doi.org/10.1038/srep12715

[19] Holst-Jensen, A., Spilsberg, B., Arulandhu, A.J., Kok, E., Shi, J. and Zel, J. (2016) Application of Whole Genome Shotgun Sequencing for Detection and Characterization of Genetically Modified Organisms and Derived Products. *Analytical and Bioanalytical Chemistry*, **408**, 4595-4614. http://dx.doi.org/10.1007/s00216-016-9549-1

[20] Gerdes, L., Iwobi, A., Busch, U. and Pecoraro, S. (2016) Optimization of Digital Droplet Polymerase Chain Reaction for Quantification of Genetically Modified Organisms. *Biomolecular Detection and Quantification*, **7**, 9-20. http://dx.doi.org/10.1016/j.bdq.2015.12.003

[21] Zhu, P., Wang, C., Huang, K., Luo, Y. and Xu, W. (2016) A Novel Pretreatment-Free Duplex Chamber Digital PCR Detection System for the Absolute Quantitation of GMO Samples. *International Journal of Molecular Sciences*, **17**, 402. http://dx.doi.org/10.3390/ijms17030402

[22] Sykes, P.J., Neoh, S.H., Brisco, M.J., Hughes, E., Condon, J. and Morley, A.A. (1992) Quantitation of Targets for PCR by Use of Limiting Dilution. *Biotechniques*, **13**, 444-449.

[23] Vogelstein, B. and Kinzler, K.W. (1999) Digital PCR. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 9236-9241. http://dx.doi.org/10.1073/pnas.96.16.9236

[24] Bhat, S., Herrmann, J., Armishaw, P., Corbisier, P. and Emslie, K.R. (2009) Single Molecule Detection in Nanofluidic Digital Array Enables Accurate Measurement of DNA Copy Number. *Analytical and Bioanalytical Chemistry*, **394**, 457-467. http://dx.doi.org/10.1007/s00216-009-2729-5

[25] Baker, M. (2012) Digital PCR Hits Its Stride. *Nature Methods*, **9**, 541-544. http://dx.doi.org/10.1038/nmeth.2027

[26] Day, E., Dear, P.H. and McCaughan, F. (2013) Digital PCR Strategies in the Development and Analysis of Molecular Biomarkers for Personalized Medicine. *Methods*, **59**, 101-107. http://dx.doi.org/10.1016/j.ymeth.2012.08.001

[27] Rački, N., Dreo, T., Gutierrez-Aguirre, I., Blejec, A. and Ravnikar, M. (2014) Reverse Tran-

scriptase Droplet Digital PCR Shows High Resilience to PCR Inhibitors from Plant, Soil and Water Samples. *Plant Methods*, **10**, 42. http://dx.doi.org/10.1186/s13007-014-0042-6

[28] Iwobi, A., Gerdes, L., Busch, U. and Pecoraro, S. (2016) Droplet Digital PCR for Routine Analysis of Genetically Modified Foods (GMO)—A Comparison with Real-Time Quantitative PCR. *Food Control*, **69**, 205-213. http://dx.doi.org/10.1016/j.foodcont.2016.04.048

[29] Pohl, G. and Shih, I.M. (2004) Principle and Applications of Digital PCR. *Expert Review of Molecular Diagnostics*, **4**, 41-47. http://dx.doi.org/10.1586/14737159.4.1.41

[30] Burns, M.J., Burrell, A.M. and Foy, C. (2010) The Applicability of Digital PCR for the Assessment of Detection Limits in GMO Analysis. *European Food Research and Technology*, **231**, 353-362. http://dx.doi.org/10.1007/s00217-010-1277-8

[31] Devonshire, A.S., Sanders, R., Wilkes, T.M., Taylor, M.S., Foy, C.A. and Huggett, J.F. (2013) Application of Next Generation qPCR and Sequencing Platforms to mRNA Biomarker Analysis. *Methods*, **59**, 89-100. http://dx.doi.org/10.1016/j.ymeth.2012.07.021

[32] Morisset, D., Štebih, D., Milavec, M., Gruden, K. and Žel, J. (2013) Quantitative Analysis of Food and Feed Samples with Droplet Digital PCR. *PLoS ONE*, **8**, e62583. http://dx.doi.org/10.1371/journal.pone.0062583

[33] Corbisier, P., Bhat, S., Partis, L., Xie, V. and Emslie, K. (2010) Absolute Quantification of Genetically Modified MON810 Maize (*Zea mays* L.) by Digital Polymerase Chain Reaction. *Analytical and Bioanalytical Chemistry*, **396**, 2143-2150. http://dx.doi.org/10.1007/s00216-009-3200-3

[34] Lievens, A., Jacchia, S., Kagkli, D., Savini, C. and Querci, M. (2016) Measuring Digital PCR Quality: Performance Parameters and Their Optimization. *PLoS ONE*, **11**, e0153317. http://dx.doi.org/10.1371/journal.pone.0153317

[35] Miliaras, N. (2014) Digital PCR Comes of Age. *Genetic Engineering & Biotechnology News*, **34**, 14-16. http://dx.doi.org/10.1089/gen.34.04.08

[36] Dong, L., Meng, Y., Sui, Z., Wang, J., Wu, L. and Fu, B. (2015) Comparison of Four Digital PCR Platforms for Accurate Quantification of DNA Copy Number of a Certified Plasmid DNA Reference Material. *Scientific Reports*, **5**, Article Number: 13174. http://dx.doi.org/10.1038/srep13174

[37] Vaïtilingom, M., Pijnenburg, H., Gendre, F. and Brignon, P. (1999) Real Time of Genetically Modified Maximizer Maize and Roundup Ready Soybean in Some Representative Foods. *Journal of Agricultural and Food Chemistry*, **47**, 5261-5266. http://dx.doi.org/10.1021/jf981208v

[38] Nair, R.S., Fuchs, R.L. and Schuette, S.A. (2002) Current Methods for Assessing Safety of Genetically Modified Crops as Exemplified by Data on Roundup Ready Soybeans. *Toxicologic Pathology*, **30**, 117-125. http://dx.doi.org/10.1080/01926230252824806

[39] Terry, C.F., Harris, N. and Parkes, H. (2002) Detection of Genetically Modified Crops and Their Derivatives: Critical Steps in Sample Preparation and Extraction. *Journal of AOAC International*, **85**, 768-774.

[40] Rott, M.E., Lawrence, T.S., Wall, E.M. and Green, M.J. (2004) Detection and Quantification of Roundup Ready Soy in Foods by Conventional and Real-Time Polymerase Chain Reaction. *Journal of Agricultural and Food Chemistry*, **52**, 5223-5232. http://dx.doi.org/10.1021/jf030803g

[41] Andersen, C.B., Holst-Jensen, A., Berdal, K.G., Thorstensen, T. and Tengs, T. (2006) Equal Performance of TaqMan, MGB, Molecular Beacon, and SYBR Green-Based Detection Assays in Detection and Quantification of Roundup Ready Soybean. *Journal of Agricultural and Food Chemistry*, **54**, 9658-9663. http://dx.doi.org/10.1021/jf061987c

[42] Samson, M.C., Gullì, M. and Marmiroli, N. (2010) Quantitative Detection Method for Roundup Ready Soybean in Food Using Duplex Real-time PCR MGB Chemistry. *Journal of the Science of Food and Agriculture*, **90**, 1437-1444. http://dx.doi.org/10.1002/jsfa.3961

[43] Kodama, T., Kasahara, M., Minegishi, Y., Futo, S., Sawada, C., Watai, M., *et al.* (2011) Qualitative PCR Method for Roundup Ready Soybean: Interlaboratory Study. *Journal of AOAC International*, **94**, 224-231.

[44] Xiao, X., Wu, H., Zhou, X., Xu, S., He, J., Shen, W., *et al.* (2012) The Combination of Quantitative PCR and Western Blot Detecting CP4-EPSPS Component in Roundup Ready Soy Plant Tissues and Commercial Soy-Related Foodstuffs. *Journal Food Science*, **77**, C603-C608. http://dx.doi.org/10.1111/j.1750-3841.2012.02718.x

[45] European Commission (EC) (2003) Regulation No 1830/2003 of the European Parliament and of the Council of 22 September 2003 Concerning the Traceability and Labelling of Genetically Modified Organisms and the Traceability of Food and Feed Products Produced from GMOs and Amending Directive 2001/18/EC. *Official Journal of the European Union*, **268**, 24-28.

[46] Barbosa, E.G.G., Leite, J.P., Marin, S.R.R., Marinho, J.P., de Fátima Corrêa Carvalho, J., Fuganti-Pagliarini, R., *et al.* (2013) Overexpression of the ABA-Dependent AREB1 Transcription Factor from Arabidopsis Thaliana Improves Soybean Tolerance to Water Deficit. *Plant Molecular Biology Reporter*, **31**, 719-730. http://dx.doi.org/10.1007/s11105-012-0541-4

[47] Demeke, T. and Jenkins, G.R. (2010) Influence of DNA Extraction Methods, PCR Inhibitors and Quantification Methods on Real-Time PCR Assay of Biotechnology-Derived Traits. *Analytical and Bioanalytical Chemistry*, **396**, 1977-1990. http://dx.doi.org/10.1007/s00216-009-3150-9

[48] Turkec, A., Kazan, H., Baykut, A. and Lucas, S.J. (2014) Evaluation of DNA Extraction Methods in Order to Monitor GM Materials in Soy Foodstuffs and Feeds Commercialised in Turkey by Multiplex Real-Time PCR. *Journal of the Science of Food and Agriculture*, **95**, 386-392. http://dx.doi.org/10.1002/jsfa.6731