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Digital PCR as an effective tool for GMO quantification in complex matrices

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ABSTRACT

The increased use of genetically modified organisms (GMOs) is accompanied by increased complexity of the matrices that contain GMOs. The most common DNA-based approach for GMO detection and quantification is real-time quantitative polymerase chain reaction (qPCR). However, as qPCR is sensitive to inhibitors and relies on standard curves for quantification, it has limited application in GMO quantification for complex matrices. To overcome this hurdle in DNA quantification, we present droplet digital PCR (ddPCR) assays that were designed to target 'Roundup Ready' soybean and the soybean reference gene. Three ddPCR assays were transferred from qPCR to QX100/QX200 ddPCR platforms and characterised. Together, the fitness-for-purpose study on four real-life samples and the use of a chamber-based PCR system, showed that dPCR has great potential to improve such measurements in GMO testing and monitoring of food authenticity.

1. Introduction

The first genetically modified (GM) plant came to market in 1994 (U.S. Food & Drug administration, 1994) and, since then, the use of GM organisms (GMOs) in food and feed products has increased several fold. This has influenced legislation and, today, most countries regulate the cultivation and trade of GMOs through authorisation systems and mandatory labelling of products above a certain threshold (Gruère, 2007; 'Strategic Plan for the Cartagena Protocol on Biosafety for Period 2011–2020 (BS-V/16 Annex I)' 2014; Vigani, Raimondi, & Olper, 2012; Žel et al., 2012). In the European Union, the labelling threshold for food and feed products that contain, consist of or are produced from authorised GMOs is set at 0.9% per ingredient (Regulation [EC] 1829/2003; European Commission, 2003). Thus, rigorous control of both the presence and quantities of GMOs in food and feed supply chains is needed. To ensure such control, sensitive and specific detection and quantification approaches have been, and are still being, developed.

Real-time quantitative polymerase chain reaction (qPCR) has been the gold standard for detection and quantification of GMOs, due to its sensitivity and robustness, for almost two decades. It is by far the most widely used DNA-based approach (Mazzara et al., 2013; Žel et al., 2012). Nonetheless, qPCR methods have some drawbacks, not least being sensitive to inhibitors and reliant upon standard curves for quantification. These drawbacks are, therefore, most obvious when there is the need for quantification of GMOs in complex matrices.

Digital PCR (dPCR) is less sensitive to inhibitors present in complex samples and does not rely on reference material for standard curves. The concept of dPCR was first introduced in 1992 by Sykes et al. (1992) and was named later by Vogelstein and Kinzler (1999). For dPCR, the reaction mixture is divided into many individual reactions, called partitions, whereby each contains none, one or more target copies. Partitions are read as negative or positive at the end-point and DNA concentration calculated using the Poisson distribution. The division of reaction volumes into partitions is achieved using either wells on a chip in microfluid/chip-based dPCR (cdPCR) or droplets in emulsion/droplet-based dPCR (ddPCR). This approach enables quantification without the need for any standard curves.

The objective of the present study was to overcome the issue of GMO quantification in complex samples. dPCR is optimal for quantification of GMOs in complex samples, because it is less sensitive to inhibitors. Additionally, previous studies have shown that direct transferability of qPCR validated methods in GMOMETHODS (European Union database of reference methods for analysis of GMOs, http://gmo-crl.jrc.ec.europa.eu/gmomethods/) to a dPCR system is possible (Corbisier, Bhat, Partis, Xie, & Emslie, 2010; Dalmira et al., 2016; Dobnik, Spilsberg, Bogožalec Košir, Holst-Jensen, & Žel, 2015; Dobnik, Štebih, Blejec, Morisset, & Žel, 2016; Košir, Spilsberg, Holst-Jensen, Žel, & Dobnik, 2017; Morisset, Štebih, Milavec, Gruden, & Žel, 2013; Wan et al., 2016).

We considered two simplex dPCR assays that target the soybean reference lectin gene (*Le1*) and one of the most abundant soybean lines on the world market, MON40-3-2 (MON-Ø4Ø32-6), which is known

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commercially as the 'Roundup Ready' soybean. We merged these two simplex assays in one duplex dPCR assay that targeted both *Le1* and MON40-3-2 in a single reaction. We used ddPCR platforms with high numbers of partitions per well to allow for a wide linear range of responses (QX100/QX200; BioRad). This characterisation of both simplex and duplex assays was followed by a fitness-for-purpose study, conducted using four complex real-life samples, which had previously been shown to result in qPCR inhibition and, thus, difficult or even impossible to quantify. This fitness-for-purpose study was conducted on DNA extracted using two common DNA extraction techniques. Additionally, the transferability of these assays to a cdPCR platform was assessed (Fluidigm HD system; Biomark).

2. Materials and methods

2.1. Test materials and methods

The certified reference material ERM-BF410gn (European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials Reference Materials Unit, Retieseweg 111, B-2440 Geel, Belgium) that contains 10% (mass/mass) MON40-3-2 GM soybean was used for characterisation (intermediate precision, robustness) of the simplex and duplex ddPCR assays, as the positive control in the fitness-for-purpose and transferability studies, and standard curves for qPCR. Four samples (samples A-D) from routine studies were chosen as test materials in the fitness-for-purpose and transferability studies. Samples A-C were pet foods while sample D was chicken feed. Samples A-D contained the MON40-3-2 GM soybean. For sample D, the MON40-3-2 content was known, as this sample had been analysed previously in a EURL proficiency test scheme.

2.2. DNA extraction and purification

Two different DNA extraction protocols were used. First, DNA was extracted from 2 g real-life samples A-D and 200 mg certified reference material using a cetyltrimethyl-ammonium bromide (CTAB) protocol (as described in Annex A.3 of ISO21571; International Organization for Standards, 2005). DNA was also extracted and purified from 200 mg certified reference material using NucleoSpin Food kits (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Each extraction was conducted in two extraction parallels. Dilutions of the extracted DNA were prepared in nuclease- and protease-free water (Sigma-Aldrich Chemie GmbH, Munich, Germany). The DNA extracts were stored at less than −15 °C until further use.

2.3. Primers, probes and PCR methods

The primer and probe sequences were taken from GMOMETHODS (http://gmo-crl.jrc.ec.europa.eu/gmomethods/)), as the entries for QT-TAX-GM-002 and QT-EVE-GM-005 for *Le1* and MON40-3-2, respectively. The final concentrations of *Le1* and MON40-3-2 primers were 650 nM and 600 nM, respectively. The probe concentrations were 180 nM and 200 nM, respectively. The final primer and probe concentrations were the same for the simplex and duplex assays for qPCR, ddPCR and cdPCR. The probes that targeted *Le1* and MON40-3-2 were labelled with 6-carboxyfluorescein and 2′-chloro-7′-phenyl-1, 4-dichloro-6-carboxyfluorescein, respectively, for both the simplex and duplex assays.

2.4. Real-time quantitative PCR

qPCR reactions (total volume, 10 or 20 µL) contained (respectively) 5 µL or 10 µL 2× TaqMan Universal PCR Master Mix (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA), 3 µL or 6 µL primers and probe mix, and 2 µL or 4 µL DNA. These were carried out in a real-time PCR system (Viia 7; Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA). The amplification used MicroAmp EnduraPlate Optical 384-Well Clear Reaction Plates with Barcode (Applied Biosystems by Thermo Fisher Scientific). The reactions were conducted under universal conditions, i.e. 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Analysis of the data was performed using the QuantStudio Real-Time PCR software, version 1.3. For both extraction methods, DNA from the samples A-D was tested for the presence of soybean (*Le1* assay) and MON40-3-2 in 10 µL reactions. The *Le1* assay used two dilutions of extraction parallel one and one dilution of extraction parallel two, and the MON40-3-2 assay was tested on one dilution of both extraction parallels. All of the reactions were performed as two technical repeats. After identification, quantification was performed in 20 µL reactions, using a standard curve that comprised six dilutions of the MON40-3-2 certified reference material, where applicable. Both extraction parallels were tested in three dilutions, each as two technical repeats for both the *Le1* and MON40-3-2 assays. The positive control contained a known amount of MON40-3-2 and was tested at two concentrations and as two technical repeats.

2.5. Droplet digital PCR

The ddPCR reactions (total volume, 20 µL) contained 10 µL ddPCR Supermix for probes (No dUTP) (BioRad, Pleasanton, CA, USA), 6 µL primers and probe mix, and 4 µL DNA, for both the simplex and duplex assays. For droplet generation, DG8 droplet generator cartridges (BioRad) were combined with the droplet digital system (QX100/QX200; BioRad). The generated droplets were transferred to 96-well plates, and the PCR reactions carried out using a thermal cycler (C1000 or T100; BioRad, USA) under the following amplification conditions: 10 min DNA polymerase activation at 95 °C; followed by 40 cycles of a two-step thermal profile of 30 s at 94 °C for denaturation, and 60 s at 60 °C for annealing and extension; followed by 10 min at 98 °C; and then cooled to 4 °C. After thermal cycling, the 96-well plates were transferred to a droplet reader (QX100/QX200; BioRad) and data were gathered and analysed using the software package provided with the ddPCR system (QuantaSoft 1.7.4.0917; BioRad). The rejection criterion was set to exclude reactions from subsequent analysis when/if the number of accepted droplets was below 10,000 per 20 µL PCR.

For the purpose of characterisation of the ddPCR assays, an intermediate precision study was performed for at least eight dilutions of the certified reference material. The DNA dilutions contained from 50,400 to 2.19 copies of *Le1* per reaction, and from 5040 to 0.2 copies of MON40-3-2 per reaction and were tested as five technical repeats over two consecutive days. The limits of detection (LOD) and quantification (LOQ) were determined from these data as well as the dynamic range. LOQ was determined as the lowest concentration in a series of concentrations that yielded consistently a relative standard deviation (RSD) < 25%, and the LOD was determined as the lowest concentration that yielded positive signals with all replicates (Decathlon Project, 2015; ENGL et al., 2015).

Robustness was assessed by varying the concentrations of the primers and the probe. For both simplex assays, four additional reaction mixtures were prepared with primers concentrations either 20% higher or 20% lower, or with probe concentrations either 20% higher or 20% lower, compared to the original assays. Two dilutions of the certified reference material were tested, as five technical repeats, one within the dynamic range and one close to the LOD. For the duplex assays, four additional reaction mixtures were prepared: 20% higher concentrations of the *Le1* probe and 20% lower concentration of the MON40-3-2 probe; 20% lower concentration of the *Le1* probe and 20% higher concentrations of the MON40-3-2 probe; and 20% higher concentrations of both probes and 20% lower concentrations of both probes. Again, two dilutions of the certified reference material were tested, as five technical repeats, with the first in the dynamic range for both targets and the second chosen to be close to the LOD for MON40-3-2, while still in the

dynamic range for *Le1*.

To test fitness-for-purpose, DNA extracted from samples A-D was used. For each DNA extraction method, two extraction parallels were tested using the simplex and duplex assays, each as two dilutions and with each dilution as two technical repeats. The GM% was compared to the qPCR data where applicable and compared between the simplex and duplex assays by calculating the bias.

2.6. Microfluidics based digital PCR

To determine the transferability of the assays to a chamber-based platform (BioMark HD; Fluidigm Europe, Amsterdam, The Netherlands), the 12.765 Digital Array integrated fluidic circuit (IFC) was used, where the reaction mixtures (total volume, 8 μL) contained 1.2 μL sample, 2.4 μL primers and probe mix, 0.4 μL 20× GE sample loading reagent (Fluidigm Europe), and 4 μL 2× TaqMan Universal PCR Master Mix (Applied Biosystems by Thermo Fisher Scientific). After loading, the arrays were transferred to the cdPCR system (BioMark HD; Fluidigm Europe). The reactions were performed under the same universal conditions as for the qPCR. Data analysis was carried out using the Biomark HD data collection software version 4.1.2, with manual determination of the fluorescence threshold, quality threshold (0.2), and accepted quantification cycle (Cq) range (15–45 Cq).

For each of the extraction methods, two extraction parallels were tested for both the simplex and duplex assays, as one dilution and two technical repeats. The GM% and copy numbers per reaction were compared to data from the QX100/QX200 systems by calculating the bias.

3. Results and discussion

dPCR assays in this study were introduced for the purpose of quantifying GMOs in complex matrices, where high concentrations of potential inhibitors in qPCR require the use of more diluted samples, raising Cq values and, consequently, generating data that are below the practical LOQ (pLOQ).

Currently, there are 19 GM soybean lines authorised in the European Union, 14 of which are so-called single transformations (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm). Out of these, MON40-3-2 is the GM soybean line that is most frequently used in food and feed. The assays reported in the present study were transferred from the qPCR methods validated by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) in collaboration with the European Network of GMO Laboratories (ENGL) in an inter-laboratory study (Mazzara et al., 2007). The last revision of the guidelines for performance assessment, and acceptance of methods for legal compliance with European Union GMO legislation, was issued in 2015 (written by EURL-GMFF together with the ENGL laboratories) (ENGL et al., 2015). That document contains the specific performance criteria that were adopted in the present study.

3.1. Limits of detection and quantification, and dynamic range

The first step in our assay evaluation was to determine the LOD and LOQ and dynamic range, using DNA extracted from the certified reference material that contained 10% (mass/mass) MON40-3-2. With dPCR, the GM% was determined in copies not mass (i.e. copy/copy instead of mass/mass) and, thus, a conversion factor was needed. Due to soybean biology, this conversion factor is one and the mass/mass GM % is directly comparable to copy/copy (Corbisier et al., 2017; Mazzara, Plan, Savini, Van den Bulcke, and Van den Eede, 2011).

To determine LOD/LOQ for the simplex assay, which targeted the soy reference gene *Le1*, a total of eight dilutions were tested as five technical repeats on two consecutive days and, for the simplex assay that targeted MON40-3-2, nine dilutions were tested (see Methods). Nine DNA dilutions were also tested using the duplex assay. Copy numbers were assigned based on a ddPCR pre-run that targeted *Le1*, which was performed as three dilutions (3×, 9×, 27 ×), each as two technical repeats. The assigned copy numbers were determined as 111,186 copies/μL for *Le1* and as 11,119 copies/μL for MON40-3-2 (Supplementary Table S1). As the certified reference material contained 10% MON40-3-2, the copy numbers for MON40-3-2 were calculated as one tenth of the *Le1* copy numbers.

The LOD was defined as the minimum copy number concentration that can be reliably detected. For *Le1*, the LOD was estimated to be 21 copies/reaction in the simplex assay and 22 copies/reaction in the duplex assay (Supplementary Tables S2, S3). For MON40-3-2, the LOD was estimated to be 20 copies/reaction for the simplex assay and 24 copies/reaction for the duplex assays (Supplementary Tables S4 and S5).

The LOQ was defined as the minimum copy number concentration where the observed RSD between technical repeats was consistently below 25%, which is the acceptance limit (Decathlon Project, 2015; ENGL et al., 2015). For *Le1* and MON40-3-2, using the simplex assays, LOQ was estimated to be 46 and 44 copies/reaction, respectively, and, using the duplex assays, as 51 and 46 copies/reaction, respectively (Supplementary Tables S2-S5). For both *Le1* and MON40-3-2 targets in the duplex assay, RSD was a little over 25% for the second repeat (25.77%, 27.14%, respectively; Supplementary Tables S3, S5). However, as the mean RSD calculated from all of 10 technical repeats (i.e., five from day 1, five from day 2) was less than 25% (Supplementary Tables S3, S5), the LOQ was deemed compliant with the acceptance limit.

To comply with the minimal performance parameters (Decathlon Project, 2015; ENGL et al., 2015), LOD should be at or below 25 copies/reaction and the LOQ at or below 50 copies/reaction. All of the assays here had LODs below 25 copies/reaction and both the *Le1* and MON40-3-2 simplex assays and the MON40-3-2 duplex assay had LOQs at or below 50 copies/reaction. For *Le1* in the duplex assay, overall, LOQs were a little above 50 copies/reaction, but values were above 50 copies/reaction in repeat one but not repeat two (Supplementary Table S3). Closer investigation of these data indicated that one of the five technical repeats on day 1 gave a high copy number, which might have been the result of a pipetting error. Thus, all of these assays were deemed compliant with the minimal performance parameters.

The dynamic range lower limit includes the LOQ and the upper limit includes the highest concentration of DNA tested. The dynamic range for the *Le1* simplex assay was, thus, 8153 to 50 copies/reaction and, for the MON40-3-2 simplex assay, 5,172 to 46 copies/reaction (Fig. 1). For the *Le1* duplex assay, the dynamic range was 50,450 to 52 copies/reaction and, for the MON40-3-2 duplex assay, 5101 to 48 copies/reaction (Fig. 2). The *Le1* simplex assay dynamic range can, however, be further extended when data from the pre-run were also considered, where the mean copy number/reaction were 142,784, 49,970 and 16,894 (Supplementary Table S1). Regardless, the dynamic range of these assays complied with the minimum performance parameters (Decathlon Project, 2015; ENGL et al., 2015).

3.2. Robustness

Robustness was tested at two concentrations: one in the dynamic range and one close to the LOD. For the *Le1* simplex assay, these were 8400 and 26 copies/reaction and, for the MON40-3-2 simplex assay, 840 and 21 copies/reaction. For the duplex assay, the dilutions tested for *Le1* contained 8400 and 210 copies and, for MON40-3-2, 840 and 21 copies. Here, one of the concentrations was in the dynamic range for both targets and one at the LOD for MON40-3-2. Robustness was tested by varying the primers and probes concentrations (see Methods). A ± 30% bias is allowed between copy number determined by the original and modified assays for the approach to be considered robust. For both the simplex and duplex assays, the bias was below ± 30% for the concentrations within the dynamic range (Tables 1 and 2). The bias

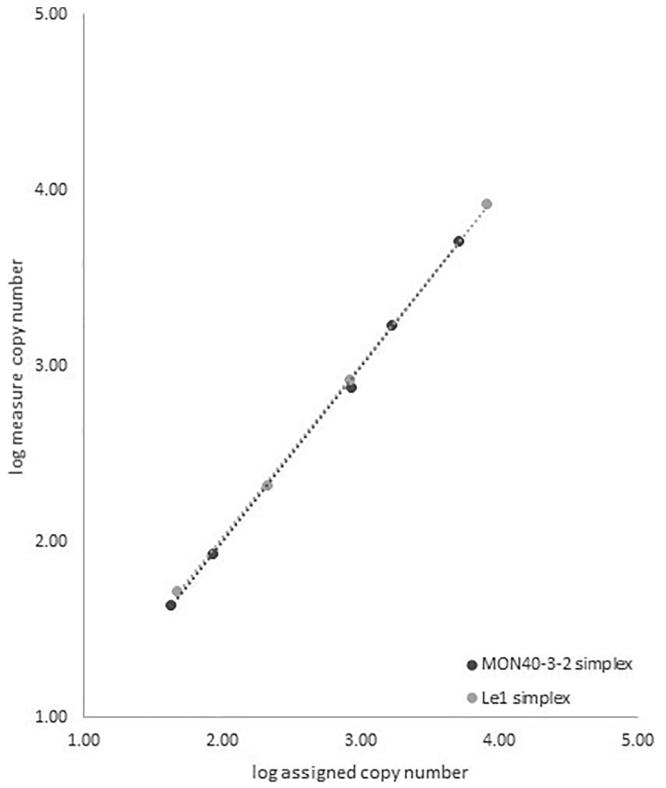

Fig. 1. Dynamic range of the targets in the *Le1* and MON40-3-2 simplex assays. Both assays show high degree of linearity (R^2) above the concentration estimated as the limit of quantification (R^2 = 0.9996, 0.9992, respectively). Data are means from two independent experiments, each as five technical repeats, conducted on a dilution series prepared from the certified reference material.

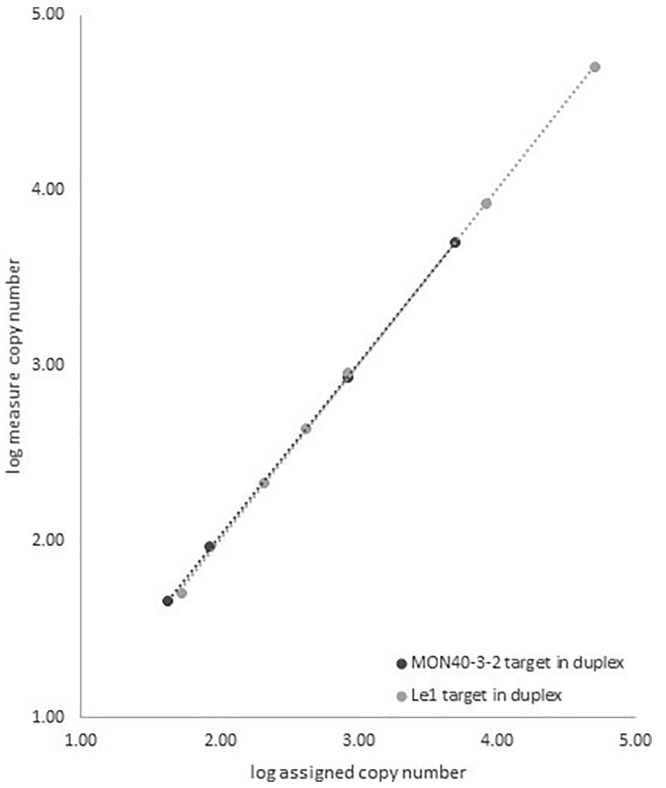

Fig. 2. Dynamic range of the targets in the *Le1* and MON40-3-2 duplex assays. Both assays show high degree of linearity (R^2 = 0.9998, 0.9999, respectively). Data are means from two independent experiments, each as five technical repeats, conducted on a dilution series prepared from the certified reference material.

between GM% measured using the original and modified duplex assays was also calculated (Supplementary Table S6), and did not exceed 25%. As different DNA concentrations were used for the simplex assays, calculation of the GM% was not applicable. All of the assays (i.e., the original and modified assays) also showed reliable amplification at concentrations near the LOD (Tables 1 and 2). Here, all of the technical repeats need to be positive and the bias may exceed ± 30%. All three assays, thus, showed satisfactory robustness.

3.3. Trueness

To test trueness of the assays, samples A-D were included. Samples A-C represented complex feed matrices. The fourth sample (D) was designed to test precision, as it was known to contain a low level of MON40-3-2. In parallel with trueness, performance of two DNA extraction methods (i.e., CTAB, NucleoSpin Food kits) was tested. DNA extraction with the CTAB method required more time and the use of potentially harmful chemicals in comparison with the NucleoSpin kits. However, the CTAB method was limited by the capacity of laboratory equipment (e.g., centrifuges) while the NucleoSpin kits were limited to 200 mg starting material. In principle, this leads to different DNA yields, although more starting material usually also provides greater homogeneity.

In the present study, extractions were carried out on the maximum amounts of starting material, which was 2 g for the CTAB method (in our laboratory) and 200 mg for the NucleoSpin Food kits. For the CTAB extraction, samples B and D could be quantified using qPCR, whereas sample A was below the pLOQ and, in sample C, the amount of soybean DNA obtained was not sufficient for determination of the pLOQ. For the NucleoSpin Food kit extraction, for samples A-C, the amounts of soybean DNA were not sufficient to determine pLOQ while sample D was below the pLOQ (Table 3).

Table 1
Robustness of the simplex assays.

Protocol	DNA conc.[a]	*Le1* simplex Copies/ reaction	*Le1* simplex RSD%	*Le1* simplex Bias to original %	MON40-3-2 simplex Copies/ reaction	MON40-3-2 simplex RSD%	MON40-3-2 simplex Bias to original %
Original	High	8142	3.67	/	917	5.14	/
	Low	9	47.53	/	17	25.30	/
Probe + 20%	High	6032	13.70	−25.92	944	1.89	2.91
	Low	13	46.31	40.01	22	16.76	30.30
Probe −20%	High	6426	7.80	−21.07	922	5.60	0.51
	Low	7	35.52	−21.54	22	34.09	32.05
Primer + 20%	High	5598	13.28	−31.24	915	5.66	−0.20
	Low	9	34.75	−1.26	24	49.94	42.06
Primer −20%	High	8182	3.22	0.49	906	3.82	−1.18
	Low	11	36.05	25.45	25	8.16	50.25

[a] High, within the dynamic range; Low, at the limit of detection.

Quantification was carried out using both the simplex and duplex ddPCR assays. Using the CTAB extraction meant all of samples could be quantified while, for the NucleoSpin Food kit extraction, quantification was only possible for samples A and B (Table 3). The bias between GM % from qPCR and ddPCR was calculated, where applicable, and was < ± 25% in both cases (Table 3). As these samples had high levels of inhibition, they needed to be diluted, which limited quantification. Digital PCR is in general less sensitive to inhibitors and, thus, higher concentrations could be used for ddPCR than for qPCR. This made quantification with ddPCR feasible for samples where it was not possible using qPCR. For sample B, a 180-fold dilution was needed for qPCR, whereas quantification was performed on 10-fold diluted

Table 2
Robustness of the duplex assays.

Protocol		DNA	*Le1*			MON40-3-2		
Le1	MON40-3-2	conc.[a]	Copies/reaction	RSD%	Bias to original%	Copies/reaction	RSD%	Bias to original%
Original	Original	high	8613	1.45		894	3.65	
		low	222	9.97		21	16.03	
Probe −20%	Probe + 20%	high	8712	1.08	−25.92	893	4.14	−0.20
		low	202	6.72	40.01	20	9.19	−1.63
Probe + 20%	Probe + 20%	high	8738	1.57	−21.07	867	7.53	−3.06
		low	196	12.09	−21.54	21	33.34	2.63
Probe + 20%	Probe + 20%	high	8448	4.36	−31.24	851	2.38	−4.83
		low	201	10.51	−1.26	21	11.01	−0.31
Probe + 20%	Probe + 20%	high	9006	9.26	0.49	932	12.09	4.25
		low	209	10.42	25.45	20	31.17	−4.55

[a] High, within the dynamic range; Low, at the limit of detection.

Table 3
Comparison of GM% measured for real-life samples either with qPCR or ddPCR.

Sample	Mean GM%					
	CTAB method			NucleoSpin Food kits		
	qPCR	ddPCR [a]	Bias%	qPCR	ddPCR [a]	Bias%
A	< pLOQ [b]	33.54	*Na* [e]	*Na* [c]	38.21	*Na* [e]
B	41	35.87	14.30	*Na* [c]	35.57	*Na* [e]
C	*Na* [c]	45.12	*Na* [e]	*Na* [c]	< LOQ	*Na* [e]
D	0.65	0.62	4.84	< pLOQ[d]	< LOQ	*Na* [e]

[a] means from simplex and duplex assays.
[b] practical LOQ with qPCR was 49%.
[c] Amount of soybean DNA not sufficient for determination of practical LOQ.
[d] practical LOQ with qPCR was 2.2%.
[e] *Na* – not applicable.

samples using ddPCR (data not shown). However, ddPCR can also experience inhibition. Although copy numbers did not exceed the upper LOQ, quantification of the MON40-3-2 target was inhibited in the undiluted samples using both the simplex and duplex ddPCR assays (data not shown).

Performance of the simplex and duplex assays was tested using both these extraction methods (Supplementary Tables S7–11). The simplex and duplex assays gave comparable GM% (bias < ± 19%) with both CTAB and NucleoSpin Food extracts (Supplementary Table S7) and comparable copy numbers (bias < ± 23%) (Supplementary Tables S8, S9). The second CTAB extraction parallel for sample C was an exception, since the duplex assays generated a lower copy number than the first parallel duplex assay and either of the extraction parallels using the simplex assays (Supplementary Table S8). The reason for this discrepancy remains unclear.

In general, the copy numbers were lower with NucleoSpin Food kits, which is not surprising as the amount of starting material was one tenth that of the CTAB method. This is usually not a problem, as higher concentrations can be tested. However, when samples are complex and show inhibition, use of more concentrated DNA is not an option. In these cases, the extraction needed to be repeated with a different method.

3.4. Transfer of the method to cdPCR

Once both the simplex and duplex ddPCR assays had been characterised, they were transferred directly to another dPCR platform, namely cdPCR (BioMark HD cdPCR). As the array (12.765 Digital Arra IFC) contained a relatively small number of partitions (i.e., 765 chambers), the duplex assays were less suitable for quantification of targets with large differences in copy numbers. For this reason, only the three samples with relatively high GM% were tested in the duplex reactions (i.e., A-C).

Firstly, the duplex assays were tested on sample A but quantification could not be performed. As cdPCR records the amplitude of fluorescence at the end-point, as well as after every PCR cycle, it is possible to check amplification curves for individual chambers. From the shape of the MON40-3-2 amplification curves, it was evident that amplification efficiency was very low. The MON40-3-2 target was inhibited in both the CTAB and NucleoSpin Food extractions. As the same primers and probes were used for all the platforms (i.e., qPCR, ddPCR, cdPCR), a possible explanation for this low efficiency may lie in the mastermix. The mastermix used was TaqMan Universal PCR Master Mix. Although validated for qPCR simplex assays, this mastermix is not optimal for multi-target assays. As the mastermix for cdPCR is not proprietary, it can be changed in order to determine whether direct transfers between the platforms are possible, but testing carried on for the simplex assays. Transfer of the simplex assays was successful, and quantification was possible for both the CTAB and NucleoSpin Food extractions, although there were large differences in GM% between the extraction parallels for sample B extracted with CTAB (28.5%, 65.04%; Supplementary Table S12). Such variation was not seen for ddPCR, neither for the simplex nor for the duplex assays. One possibility is that the sample was inhibited. However, this cannot be stated for certain, as only one dilution was tested. Nonetheless, as data for the cdPCR were comparable to those for the ddPCR (bias between ddPCR and cdPCR < ± 25%, except for CTAB extraction sample C where bias was slightly higher −27.36%, Table 4), transfer of the simplex assays to the cdPCR platform was deemed successful (Table 4, Supplementary Tables S13, S14).

4. Conclusions

The purpose of the present study was to develop a DNA-based method for quantification of GMOs in complex samples. This goal was achieved through the transfer of qPCR assays, which targeted the soybean endogene and GM-soybean line MON40-3-2, to a ddPCR platform. Both the simplex and duplex ddPCR assays performed within the required parameters (Decathlon Project, 2015; ENGL et al., 2015) and

Table 4
Comparison of mean GM% gains with ddPCR and cdPCR.

Sample	Mean GM%					
	CTAB method			NucloSpin Food kits		
	ddPCR [a]	cdPCR	Bias%	ddPCR [a]	cdPCR	Bias%
A	35.45	31.07	14.09	36.27	43.50	−16.62
B	36.79	46.77	−21.33	34.72	35.12	−1.13
C	40.50	55.76	−27.36	*Na* [b]	52.42	*Na*

[a] means from simplex and duplex assays.
[b] not applicable < LOQ.

quantified MON40-3-2 in the complex samples, even where quantification with qPCR failed. Additionally, direct transfer of the qPCR methods to a cdPCR platform was assessed. Although the transfer from qPCR to ddPCR was relatively straightforward, this was not the case for transfer to cdPCR. Indeed, transfer of the simplex assays was successful for both the ddPCR and cdPCR platforms. However, transfer of the duplex assay was not possible using the cdPCR platform. Nonetheless, further optimisation, namely a change in the mastermix to one that is more appropriate for multi-target analysis, would probably make transfer possible. The present study showed there is great potential for use of dPCR to improve GMO testing and qPCR methods can be transferred directly to alternative platforms. However, as the transfer is not always simple, the assays would need to be verified before implementation in any GMO testing scheme.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.05.029.

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