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ORIGINAL PAPER

Absolute quantification of genetically modified MON810 maize (*Zea mays* L.) by digital polymerase chain reaction

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Abstract Quantitative analysis of genetically modified (GM) foods requires estimation of the amount of the transgenic event relative to an endogenous gene. Regulatory authorities in the European Union (EU) have defined the labelling threshold for GM food on the copy number ratio between the transgenic event and an endogenous gene. Real-time polymerase chain reaction (PCR) is currently being used for quantification of GM organisms (GMOs). Limitations in real-time PCR applications to detect very low number of DNA targets has led to new developments such as the digital PCR (dPCR) which allows accurate measurement of DNA copies without the need for a reference calibrator. In this paper, the amount of maize MON810 and *hmg* copies present in a DNA extract from seed powders certified for their mass content and for their copy number ratio was measured by dPCR. The ratio of these absolute copy numbers determined by dPCR was found to be identical to the ratios measured by real-time quantitative PCR (qPCR) using a plasmid DNA calibrator. These results indicate that both methods could be applied to determine the copy number ratio in MON810. The reported values were in agreement with estimations from a model elaborated to convert mass fractions into copy number fractions in MON810 varieties. This model was challenged on two MON810 varieties used for the production of MON810 certified reference materials (CRMs) which differ in the parental origin of the introduced GM trait. We conclude that dPCR has a high metrological quality and can be used for certifying GM CRMs in terms of DNA copy number ratio.

Keywords PCR · Maize · *Zea mays* · Genetically modified organism · Biochips high-throughput screening · Nucleic acids (DNA|RNA)

Abbreviations

CRM	Certified reference material
DNA	Deoxyribonucleic acid
dPCR	Digital polymerase chain reaction
EC	European Commission
ERM	European reference material
EU	European Union
gDNA	Genomic DNA
GM	Genetically modified
GMO	Genetically modified organism
HG	Haploid genome
hmg	High-mobility-group protein A gene
MON810	MON810 gene
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
SD	Standard deviation
RSD	Relative standard deviation

Introduction

GM content in food and feed is strictly regulated in most countries around the world. Regulations (EC) No 1829/2003 and (EC) No 1830/2003 form the basis for GMO food

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and feed use and labelling in the EU [1]. The presence of GMOs must be declared unless the presence of authorised GM material can be proven to be adventitious or technically unavoidable and levels are below the threshold specified for non-declaration of GMO presence. The threshold for declaration is 0.9% for approved GMOs and 0.5% for unapproved GMOs that are under assessment and have passed the safety review step of the approval process. The current interpretations of "adventitious" and "technically unavoidable" are generally explained by guidelines within the respective member states. The unit of measurement for the threshold mentioned in the European Regulation has been specified in a Commission Recommendation defining the GM content as "the percentage of GM DNA copy number in relation to target taxon specific DNA copy numbers, calculated in terms of haploid genomes (HG)" [2].

The ratio in terms of GM copies per HG differs from the GM percentage in mass ratio. A unique mathematical relationship between the two units does exist but depends on the species and the tissue analysed. An empirical relationship between GM mass percentage and the GM content expressed per HG estimated by real-time PCR has been discussed recently taking the experimental data from MON810 maize seeds as a practical example [3].

Detection and quantification methods have been developed based on the amplification of DNA copies extracted from the sample to be analysed. Real-time qPCR utilises optical measurement of generated amplicons to assess PCR amplifications. The initial template concentration is derived from the number of amplification cycles (Ct value) required for the optical signal to pass a threshold chosen for the measurement. In theory, PCR exponentially amplifies nucleic acids, and the number of amplification cycles and the amount of PCR amplicons should allow the computation of the starting quantity of the initial template. That starting quantity is calculated using a calibrator for which the initial amount of copies is ideally known and to which the signal of the unknown sample is compared.

For GM analysis, the copy number ratio between the amount of transgene copies and the amount of a reference gene which is present once per HG is calculated. Dilution series of purified plasmid DNA certified to contain one copy of each target per plasmid can be generated to produce a calibration curve for each of the two copies [4]. Following this approach, demonstration must be made that the amplification kinetics of each target is the same in the material analysed as for the calibrator used. However, many factors complicate this approach, creating uncertainties and inaccuracies in the measurement. Indeed, (1) the initial amplification cycles may not be exponential [5], (2) the PCR amplification eventually plateaus after an unknown number of cycles, (3) the low initial concentrations of target DNA molecules may not allow amplification to detectable levels, (4) the PCR amplification efficiency in a sample of interest may be different from that of the calibrator and finally (5) the PCR amplification may be less efficient for one target DNA fragment compared to another target. These differences are mainly observed when the assays are not fully optimised or when the DNA solution still contains co-extracted impurities that affect the kinetics of the amplification process [6] or the DNA is degraded [7, 8].

Digital PCR overcomes several of these difficulties by transforming exponential data from conventional PCR to digital signals that simply indicate whether or not amplification has occurred after a defined number of cycles. dPCR involves distributing the PCR solution containing template nucleic acid molecules across a very large number of individual partitions prior to amplification. Following PCR amplification, a count of the proportion of partitions containing a detectable number of PCR amplicons can be used to estimate the total number of template DNA copies in the original DNA extract. Accurate quantification relies on the fact that after 40 to 45 amplification cycles, the number of false negatives (single DNA templates present in a partition which are not detected) is very low. The sources of uncertainties related to DNA quantification by dPCR have recently been studied [9].

In this paper, we used dPCR to quantify the amount of MON810 DNA copies in seeds, and in powders that were certified for their mass fraction as well as for their MON810 copy number ratio. MON810 was chosen as an example as it is the only reference material that has been certified for its mass fraction as well as for the ratio of MON810 copies per *hmg* copies to date. MON810 represents also the only GM crop currently cultivated in the EU making the choice of this GM variety even more relevant in a European context.

Materials and methods

Plant materials

Seeds from two MON810 hybrids, DK 513 and DKC57-84, which differ only in the transgenic locus originating from the female and male parents, respectively, were provided by Monsanto for the production of the of CRMs ERM-BF418 and ERM-BF418k series, respectively. All other materials tested were seed-powder-based CRMs (ERM-BF413a, ERM-BF413b, ERM-BF413d, ERM-BF413e, ERM-BF413f) [10] from the Institute for Reference Material and Measurements (IRMM, Geel, Belgium). The seeds were rinsed in water, and dried under vacuum at 30 °C. The dried seeds were then milled using a high-impact mill with a triangular ribbed open grinding track in order to obtain

the ground base material. The high-impact mill was flushed with nitrogen gas throughout the milling process and milling was interrupted if the temperature rose above 40 °C. An additional vacuum drying at 30 °C was carried out to further reduce the water content of the once ground base material. The powders were ground a second time under the same conditions, followed by a second drying step under vacuum at 30 °C. For the second grinding step, a sieve insert with a mesh size of 0.5 mm was used. Slow feeding of the mill ensured that the whole base material passed the sieve, thus excluding selection during grinding. Each ground base material was mixed in a Dynamic CM-200 mixer (WAB, Basel, CH) for 30 min to improve equal distribution of the different parts of the maize tissues separated by the milling process.

Extraction of genomic DNA

Genomic DNA (gDNA) was extracted using the Wizard genomic DNA extraction protocol (Promega) from five individual 20 mg portions of GM maize (MON810) CRM, ERM-BF413 series (IRMM, Geel, Belgium) and the resultant DNA was pooled together. Six hundred microlitres of nuclei lysis solution was added to 20 mg of powder in a 1.7-mL Eppendorf tube and vortexed for 30 s. The tubes were incubated at 65 °C for 20 min. Four microlitres of RNase A solution was added and incubated at 37 °C for 15 min. Two hundred fifty microlitres of protein precipitation solution was then added and the contents were vigorously vortexed for 20 s followed by centrifugation at 14,000 rpm for 10 min. Six hundred microlitres of the supernatant containing DNA was transferred to a clean 1.7-mL Eppendorf tube containing 600 μL of isopropanol placed on ice. The contents were gently mixed by inverting the tubes for several times followed by centrifugation at 13,000 rpm for 5 min. The supernatant was discarded and the DNA pellet was washed with 70% ice cold ethanol by gently inverting the tubes several times followed by centrifugation at 13,000 rpm for 1 min (unless indicated all centrifugation steps were performed at room temperature). Excess ethanol was removed and the pellet was air-dried and dissolved in 30 μL of $1\times TE_{0.1}$ (10 mM Tris, 0.1 mM EDTA, pH 8.0). The tubes were incubated overnight at 4 °C to rehydrate the DNA. The five fractions were pooled and the quality and purity of the extracted gDNA was estimated by absorbance at 260 nm and by the PicoGreen® dsDNA quantification kit (Invitrogen).

Digestion of DNA

For enzyme digestion, 30 to 50 ng of MON810 gDNA was used in a total volume of 50 μL of the restriction digestion reaction mix containing the appropriate restriction enzyme buffer, 0.02 mg/mL RNase A and 40 unit of *Hin*P1I (NEB, Arundel, Australia). The final volume was made up with nuclease-free water (Promega, Sydney, Australia) and incubated for 2 h at 65 °C for *Hin*P1I digestion. The *Hin*P1I enzyme was inactivated by incubating at 80 °C for 10 min. 5 μL of digested gDNA was analysed on a 1% agarose gel to confirm complete digestion.

Digital PCR analysis

Digital PCR was performed on the BioMark System (Fluidigm, South San Francisco) using the 12.765 digital arrays (Fluidigm). The digital array comprises twelve panels and each panel contains 765 individual partitions of approximately 6 nL volume each with a total volume per panel of approximately 4.6 μL (6 nL×765) [11, 12]. The instrument software generates PCR amplification curves and real-time cycle threshold (Ct) values for each of the 9,180 chambers (765×12). Following amplification, digital raw data was processed by the BioMark dPCR Analysis software using a manually set threshold of 0.03 and target Ct range of 23 to 43.

For analysis of dPCR response, gravimetric dilutions of *Hin*P1I-digested gDNA were prepared in $TE_{0.1}$ using a Genius ME215S balance (Sartorius, Göttingen, Germany) starting with an initial nominal DNA concentration of 18,350 copies of *hmg*/μL in the undigested gDNA preparation. The number of copies was estimated by using PicoGreen® method assuming an average *Zea mays* genome mass of 2.73×10^{-9} g [13]. The final reaction mix for each digital panel comprised of 1× Taqman Universal mastermix without UNG® AmpErase (Applied Biosystems, Melbourne, Australia), 1× sample loading reagent (Fluidigm, South San Francisco), relevant forward and reverse primers at final concentration of 300 nM and relevant probe at final concentration of 180 nM and DNA (nominally, 147 copies of endogenous target/μL resulting in 674 copies endogenous target per panel for all *hmg* assays and for MON810-specific assay of seed samples; nominally, $(0.73–1.58)\times10^4$ copies of endogenous target/μL resulting in $(1.3–2.9)\times10^4$ copies endogenous target per panel for MON810-specific assays on ERM-BF413 CRM series; see Table 1 for primer and probe details). The nominal copy number of target DNA per panel for all *hmg* assays and for MON810-specific assays, where feasible, was adjusted to minimise the relative uncertainty in the estimate of target DNA concentration using digital PCR [8]. The primer and probe sequences were the same as those published in the standard ISO 21570:2005 [14]. However, the TAMRA quencher was replaced by a Black Hole Quencher 1 (BHQ-1) which has the advantage of exhibiting no native fluorescence, additionally, the concentration of both probes were optimised to 180 nM. No template

Table 1 Primers and probes used in this study

Target DNA	Primer/probe sequence	Final PCR concentration (nM)
hmg	(F) TTGGACTAGAAATCTCGTGCTGA	300
	(R) GCTACATAGGGAGCCTTGTCCT	300
	(P) 6-FAM-CAATCCACACAAACGCACGCGTA-BHQ-1	180
MON810 event	(F) TCGAAGGACGAAGGACTCTAACGT	300
	(R) GCCACCTTCCTTTTCCACTATCTT	300
	(P) 6-FAM- AACATCCTTTGCCATTGCCCAGC - BHQ-1	180

(F) forward primer, *(R)* reverse primer, *(P)* probe, *6-FAM* 6-carboxyfluorescein, *BHQ-1* black hole quencher 1

controls (NTC) contained $TE_{0.1}$ in place of DNA. To minimise the uncertainty from pipetting, all components excluding DNA were pre-mixed and then the final reaction mix was prepared gravimetrically by combining the DNA solution and the pre-mixed solution. 10 µL of reaction mix was dispensed into each sample inlet and approximately 4.6 µL of this reaction mix was distributed throughout the partitions within each panel using an automated NanoFlex IFC Controller (Fluidigm, South San Francisco). In general, replicates of five assays were analysed for each reaction mix apart from controls which were analysed in single or duplicate panels.

The digital array thermocycling conditions on the Bio-Mark System PCR consisted of a 10 min activation step at 95 °C, followed by 45 cycles of a two-step thermal profile involving 15 s at 95 °C for denaturation, and 60 s at 60 °C, for annealing and extension.

Results and discussion

The ERM-BF418k CRM series has been produced to replace the existing ERM-BF418 CRM series and will be released by IRMM. For the new ERM-BF418k CRM series, the MON810 transformation event was introduced from the male parent whereas for the ERM-BF418 CRM series the MON810 transformation event was introduced from the female parent. It was therefore necessary to test the MON810 copy number ratio in the two varieties DK 513 and DKC57-84 used for the production of the ERM-BF418 and ERM-BF418k, respectively.

Genomic DNA was extracted from both varieties and the DNA concentration estimated using the PicoGreen® dsDNA quantification kit before being diluted to 50 ng/µL in $TE_{0.1}$ corresponding to 18,350 copies of the genome/µL. The gDNA was further digested with *Hin*P1I to produce smaller fragments of DNA to optimise the PCR amplification in the digital arrays as recommended previously [9]. The *Hin*P1I endonuclease was chosen as in silico analysis indicated no *Hin*P1I restriction sites within the MON810 or the *hmg* regions. The gDNA digestion resulted in a dilution of the initial DNA concentration by a factor of 1.25. The *Hin*P1I digested gDNA from each variety was further diluted by a factor of 40 to achieve approximately 367 copies gDNA/µL and this diluted gDNA solution was analysed by dPCR using the 12.765 digital arrays. Typical amplification plots for the MON810 and *hmg* are presented in Fig. 1. Partitions are marked as positive (shown in red) if amplification of the DNA target molecule took place and a fluorescent signal is detected above a manually set threshold. A stronger relative fluorescent signal (ΔRN) was amplified for the *hmg* targets compared to the MON810 targets but this difference in signal intensities does not affect the number of positive partitions counted between 23 and 43 cycles. Most positive partitions had Ct values between 24 and 30 for the *hmg*, and between 27 and 32 for the MON810.

The number of target template molecules per panel (M) following analysis of the diluted solution of sample was estimated using binomial approximation in relation to the number of partitions containing amplified product (H) and the total partitions (C) according to [9–15]. As the DNA molecules are randomly partitioned into the chambers it is possible that two or more molecules are present in the same chamber. The probability increases as the number of molecules per panel increases. Therefore, a mathematical correction is performed using the formula below:

$$M = \frac{\log\left(1 - \frac{H}{C}\right)}{\log\left(1 - \frac{1}{C}\right)}$$

The number of positive partitions and the number of estimated *hmg* and MON810 copies determined in the two MON810 varieties are summarised in Table 2. In the same diluted DNA extract, the estimated number of MON810 copies is clearly lower than the number of *hmg* copies. The mean MON810/*hmg* copy number ratios were 57.1% and 41.1% for the DK 513 and DKC57-84 maize varieties, respectively, with a corresponding standard deviation (SD)

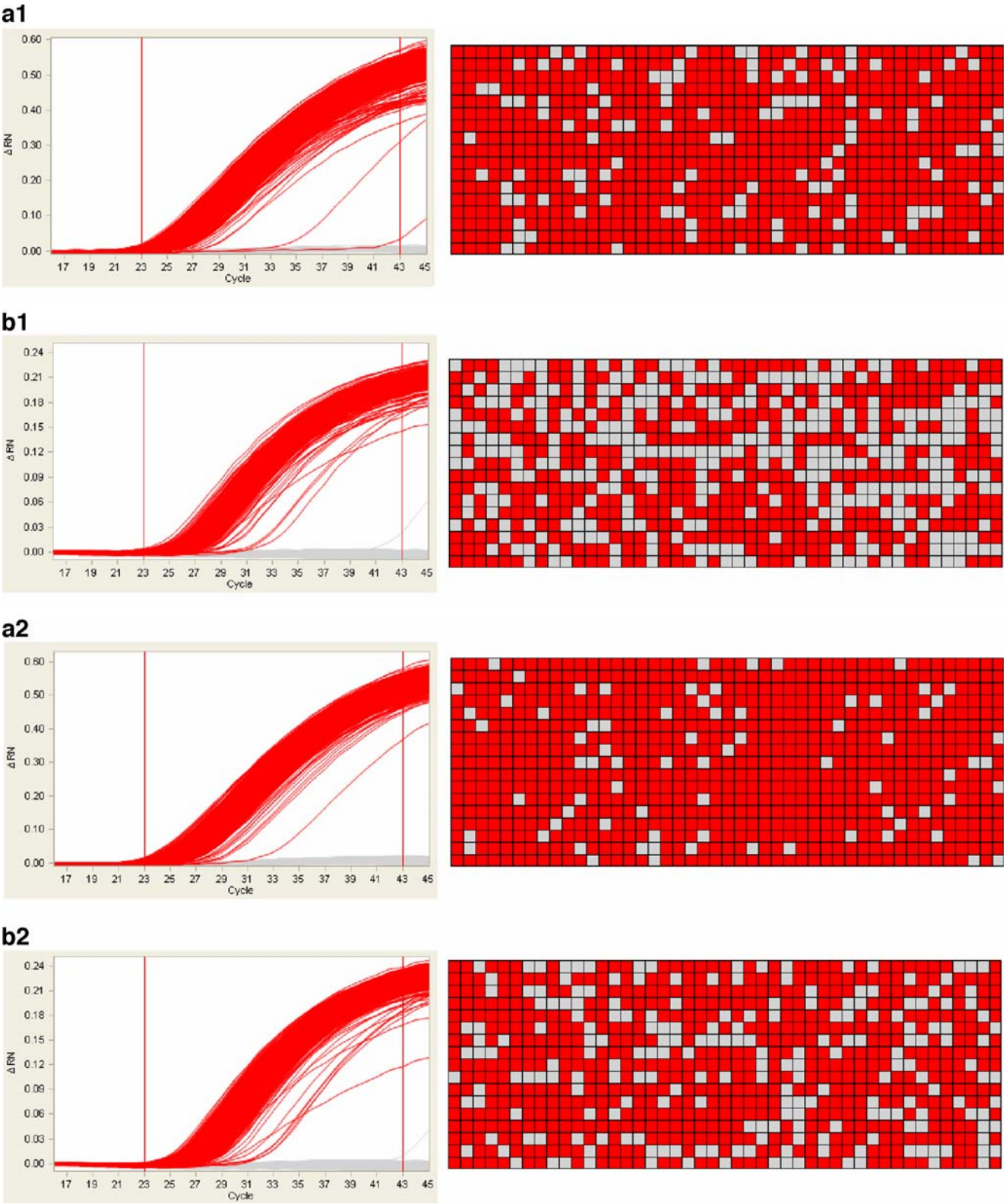

Fig. 1 Digital PCR amplification plots (*left*) and panel readouts (*right*) of *hmg* (**a**) and MON810 (**b**) assays in MON810 DK 513 (female donor) (*1*) and DKC57-84 (male donor) (*2*) varieties. The *red colour* corresponds to positive hits in the partitions, while *grey colour* signifies partitions with no amplification

Table 2 Number of positive partitions for MON810 and *hmg*, estimated number of copies per panel and copy number ratio measured in two MON810 varieties in which the MON810 event originated from the female (DK 513) or from the male (DKC57-84) parent

	MON810 event			*hmg*			
MON810 variety	Number positive partitions	Estimated number of copies per panel	Average number of copies per panel	Number positive partitions	Estimated number of copies per panel	Average number of copies per panel	Average MON810/*hmg* ratio (%)
DK 513	526	889		689	1,765		
	534	915		701	1,897		
	552	977	964	672	1,611	1,706	57.1
	562	1,014	(6.2)	676	1,645	(7.3)	(9.6)
	565	1,026		672	1,611		
DKC57-84	409	585		657	1,497		
	433	638		633	1,343		
	398	562	588	640	1,385	1,408	41.1
	399	564	(5.3)	642	1,397	(4.0)	(6.6)
	413	593		645	1,416		

The average MON810/*hmg* ratio is calculated on the basis of five replicate measurements (n= 5) and has been adjusted to account for the gravimetrically prepared PCR solutions for the MON810 and *hmg* assays. The relative standard deviation is provided in parenthesis

of 5.5% and 2.7%, respectively. The absolute difference between the two average ratios is larger than the expanded uncertainty associated with this difference, showing that dPCR has the necessary precision to differentiate between the two maize varieties. Both copy number ratios are in agreement with the theoretical [3] and other experimental values obtained by qPCR calibrated with a dual target plasmid [16].

In the paper of Zhang [3], the ratio of transgenic HGs to the total HG in maize seeds is calculated taking into account the parental origin of the transgenic allele as well as the impact of the maize endosperm DNA content which was reported earlier to vary between 36.3% and 59.4% in maize seeds according to varieties [17]. The average relative DNA ratio of the endosperm to total gDNA measured on four independent seeds was found to be 36.3% (SD of 2.4%) and 42.1% (SD of 1.9%) for the varieties DK 512 and DK 585, respectively [3]. Despite the fact that the relative DNA ratio of the endosperm to total gDNA proportion was not measured for the varieties DK 513 and DKC57-84 in the above study, one can calculate a theoretical MON810 copy number ratio of 57.1% and 41.1% for the female and male parent, respectively, under the assumption of a 40% DNA ratio of the endosperm to total gDNA. These two predicted values fit perfectly with the ratios measured by dPCR (Table 2).

The MON810 copy number ratio measured for the MON810 variety DK 513 can also be compared to the certified copy number ratio for the CRM ERM-BF413d. This 10 g/kg (1% m/m) powder mixture has been produced from whole seeds of non-modified maize (variety DK 512) and GM MON810 maize (variety DK 513). To support EU legislation, CRM ERM-BF413d was also certified for the DNA copy number ratio of the MON810 event-specific plant/P35S junction region and a single copy target of the *hmg* measured by qPCR using the same PCR targets as those in this study but calibrated with a MON810 maize plasmid DNA ERM-AD413 [18]. This DNA copy number ratio was certified to be 0.57%±0.17% (expanded uncertainty using a coverage factor k = 2) which is entirely consistent with the expected ratio since the pure MON810 powder (variety DK 513) used to prepare the 1% m/m CRM contained an average of 57 copies (SD of six copies) of MON810 per 100 copies of *hmg* when measured by dPCR (Table 2).

To further demonstrate the validity of using dPCR to determine the MON810 copy number measurement by absolute quantification of the number of MON810 and *hmg* molecules present in a DNA extract, the MON810 copy number ratio was determined for the following CRMs (ERM-BF413a, ERM-BF413b, ERM-BF413d, ERM-BF413e, ERM-BF413f) containing <0.2, 1.00, 10.0, 20.0, and 50.0 g/kg MON810, respectively. The DNA was extracted using the Wizard genomic DNA extraction method, digested with the *Hin*P1I endonuclease and diluted where necessary to obtain an optimal nominal number of positive partitions before being analysed on the 12.765 digital arrays. The estimated number of copies of MON810 and *hmg* measured from five replicate panels as well as the resulting copy number ratio for the different materials was determined (Table 3). The fourteen 'No Template Control' digital panels comprising a total of 10,710 individual partitions in which the DNA template had been replaced by $TE_{0.1}$ contained a total of only nine positive partitions

Table 3 Estimated number of MON810 and *hmg* copies and MON810/*hmg* copy number ratio measured in the ERM-BF413 series by dPCR (n=5)

CRM		Mass fraction of DK 513		Target DNA concentration in DNA extract (copies per µL)				MON810/hmg copy number ratio (%)	
				MON810		*hmg*			
		Certified value (g/kg)	Expanded uncertainty (g/kg)	Average	RSD	Average	RSD	Average	RSD
ERM-BF413a	Blank	<0.2	–	5	26.6	29,052	3.6	0.018	26.8
ERM-BF413b	Level 1	1.00	0.26	16	21.4	23,650	7.2	0.067	22.6
ERM-BF413d	Level 3	10.0	0.5	194	12.1	49,287	9.7	0.395	15.5
ERM-BF413e	Level 4	20.0	0.6	379	5.5	34,666	4.3	1.09	7.0
ERM-BF413f	Level 5	50.0	1.1	891	4.9	31,532	10.3	2.83	11.3
Not certified	DK 513			26,904	6.2	47,115	7.3	57.1	9.6

The average MON810/*hmg* ratio is calculated on the basis of five replicate measurements (n=5) and has been adjusted to account for the gravimetrically prepared PCR solutions for the MON810 and *hmg* assays. The expanded uncertainty U uses a coverage factor k=2 [9]

(data not shown) demonstrating the high specificity of the assays in the digital format**.** The presence of traces of MON810 in the blank material (ERM-BF413a) was confirmed by dPCR as an average of 5 (SD of one copy)/µL extracted DNA compared to the *hmg* target that was present at (2.91×10^4 copies (SD of 0.10×10^4 copies) per µL. The resulting copy number ratio of 0.018% (SD of 0.005%) is comparable to the certified level of the blank material estimated by qPCR. However, one should take the ratio estimated with caution as these are low numbers of counts with a large variability observed. The analysis of traces of DNA molecules remains a difficult exercise even with dPCR and a higher number of samples would be required to quantify such traces with a better precision.

A good proportionality between the copy number ratio and the mass fraction is observed for all tested materials (Fig. 2) within the ERM-BF413 series and for the DK 513 variety used as MON810 source in those materials. The slightly lower copy number ratio of 0.39% (SD of 0.06%; Table 3) observed for the ERM-BF413d is still in agreement with the 0.57%±0.17% (expanded uncertainty using a coverage factor of 2) certified value when taking into consideration the expanded uncertainty. The conserved ratio among the different MON810 CRMs is an additional indication that the material was correctly homogenised and mixed to prepare the different mass fractions.

The level of repeatability of positive partitions among the five replicate panels within the same digital arrays appears to be very adequate. The relative standard deviation (RSD) of the MON810/*hmg* ratio as shown in Tables 2 and 3 is reported as the square root of the sum of the squares of the relative standard deviations obtained for each estimated target. The ERM-BF413a and ERM-BF413b samples contained only low amounts of MON810 with an average of five and 16 copies MON810/µL DNA extract, respectively. Consequently, these two samples produced the highest RSDs for the copy number ratio of 26.8% and 22.6%, respectively. The RSDs for the copy number ratio in the remaining samples were between 6.8% and 15.5%.

Conclusion

This paper describes for the first time the absolute quantification of GM target using a dPCR apparatus and digital arrays. This technology relies on the spatial randomness of the positive partitions as demonstrated earlier [9] and on the ability to reliably detect DNA

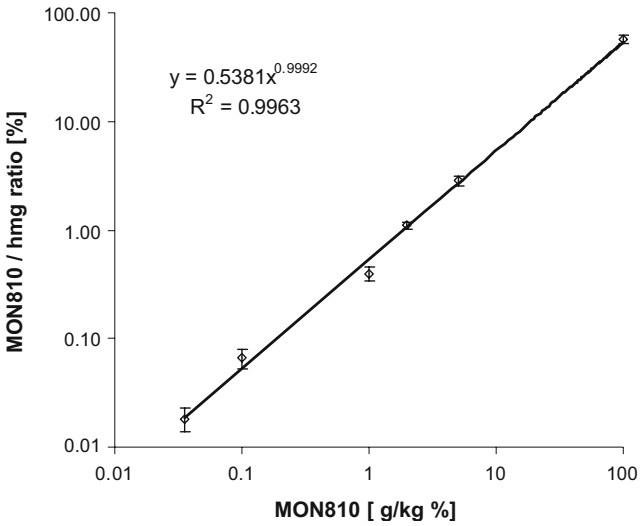

Fig. 2 Relationship between the MON810 mass fraction and the corresponding MON810 copy number ratio measured by dPCR on the ERM-BF413 series as well as in the DK 513 variety. Each *point* represents the average of five measurements with the observed standard deviation

molecules present at a very low number of copies as was the case for the blank material tested in this study. Digital PCR is an alternative technique for quantifying gene copy number which may provide more accurate measurements than other approaches currently available as it is not dependant on amplification efficiency. Of particular importance, dPCR measurements are made independent of any calibrator and, therefore, this technique has the potential to be considered as a primary method for use in certification of nucleic acid reference materials. The measurement principle has a high metrological quality. However, the absolute number of DNA molecules estimated is a measure of those molecules that can be amplified during the PCR. The number of amplifiable DNA target sequences is not necessarily equal to the total number of target molecules initially present in the reaction solution as it is difficult to prove that all target DNA sequences have been amplified after 40 or 45 cycles. However, this limitation also exists with real-time qPCR analysis. Taking into account its specific uncertainties, dPCR has the potential to be used for the assignment of copy number ratios to reference materials during certification. Current costs of the digital arrays may however hinder the use of dPCR for routine analysis of GM samples.

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