

Validation of 13 duplex droplet digital PCR systems for quantitative GMO analysis of most prevalent GMO traits

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Abstract Digital PCR methods were recently introduced in food analysis. To run these methods within the scope of ISO17025, they have to be validated. Although several guidelines are available, each laboratory has to implement these guidelines in an adapted validation scheme. We present here one possible implementation. We chose 13 GMO traits which were predominantly detected in the past. The results show that in the range of 1% GMO content, the digital PCR has a little superior performance compared to real time PCR. In the range of the detection limit, measurement uncertainty remains comparable to real time PCR. During validation, a conversion factor was determined for each trait suggesting that the calculation from % copies/copies to % weight/weight may be possible. This shows that GMO contents can be measured without the use of reference material by the validation described here and determination of a conversion factor, which is a great improvement in terms of expense and storage capacity.

Keywords Droplet digital PCR · GMO traits · Quantification · Validation · Conversion factor

Introduction

Establishment of digital PCR can be done in a straight forward process and within reasonable time [1–3]. Duplex [4] and even 12-plex assays were recently successfully realized [5]. The optimization of primers and probes as well as the

other conditions were investigated extensively [6]. In consequence the quantification of GMO contents without the use of reference material may be possible. The previously gained results seem to exhibit a lower measurement uncertainty than when applying real time PCR [7, 8]. The main advantage seems to be a better precision. To convert measured copy number and their percentage to percentage by weight to weight [9] a so called conversion factor (earlier called zygosity factor) has to be applied. This factor can be determined using a certified reference material. For some new traits, this was already done by the European Joint Research Center (JRC), Ispra, Italy (<http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). These factors are available in the appropriate validation report of the transgene traits, published after successful validation by the JRC. Unfortunately, these factors are not available for the older GMO traits we used in this study.

To assess specificity, e.g., false positive signals, in silico investigations can give hints but remain incomplete. Extensive testing of DNA from different GMO traits and other plant and animal species is required. The specificity of digital PCR can be higher as mostly only one target DNA copy is enclosed per reaction chamber (droplet). This reduces the chance of amplification of not targeted primer sites or of unexpected DNA-hybrids.

For routine application in the scope of ISO 17025 full validation has to be done to show the suitability of such methods. Several guidelines were released for the validation of PCR systems already [10–12]. Although they are an important help to resolve the different aspects during each validation step, they remain unclear in the concrete steps and details and do not address digital PCR specifically. This gives space for interpretation. In this work we focussed on a validation scheme to comply with the guidelines with reasonable input. The final goal was to assess the lower limit of

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quantification and the measurement uncertainty. In addition, conversion factors were also determined and were compared with previously determined ones.

Results from real time PCR suggested, that below 0.1% the quantification has a high measurement uncertainty (often in the range of $\pm 30\%$) and becomes rather arbitrary. We expected that the measurement uncertainty to be smaller with digital PCR.

The selection of GMO traits and duplex droplet digital PCR (ddPCRTM) systems were made by identified positive samples from the last 10 years of GMO) analysis at our laboratory.

Materials and methods

Reference material

Genetically modified reference material was purchased from the following source: Sigma-Aldrich, St. Louis, USA: RoundupReady (RR, ERM 410dk), Bt176 (ERM BF 411f), Bt11 (ERM BF 412f), Mon810 (ERM BF 413ck), GA21 (ERM BF 414f), NK603 (ERM BF 415f), Mon863 (ERM BF 416d), 59122 (ERM BF424d), Mir604 (ERM BF423d), TC1507 (ERM BF 418d). AgrEvo GmbH Berlin, Germany: T25 (Reference C/F/95/12-07). AOCS, Urbana, USA: Mon89788 (Cat. No. 0906 B), 2704 (Cat. No. 0707 B6).

Plant and animal material

Plant and animal material was purchased from local retailer as whole recognizable material. Animal tissue was sequenced to ensure correct species identification. From this material DNA was extracted and this DNA was used as non-target template to assess the specificity of the here validated assays.

Equipment

The QX200 Droplet Digital PCR (ddPCRTM) system of Bio-Rad Laboratories Inc. USA was used to perform the droplet digital PCR in this work. For pipetting we used manual Gilson Pipetman of 20, 100 and 200 μl volume. To transfer the Droplets from the droplet generating cartridge to the PCR-tubes we used ART 100 μl filter tips (Cat No. 713116) or Biosphere plus tips 200 μl (Sarstedt, Cat. No. 70.760.211) or Clear Line low binding tips 100 μl (Milian, Cat. No. 713116) filter tips in combination with a Gilson 8 channel manual Pipetman L P8 \times 200L. All combinations lead to successful generation of 10,000 droplets or more.

DNA extraction

DNA extraction from all sample matrices was performed using the column based Wizard Plus Miniprep DNA purification system (Promega, Madison, USA). 200 mg of ground sample material were extracted and the DNA was eluted into 50 μl elution buffer according to the supplier's manual. The concentration was determined spectrophotometrically (denatured single strand DNA, 320/260 nm) and adjusted by dilution to 20 ng/ μl using PCR-grade water.

Primers and probes

Primers and hydrolysis-probes were published earlier or published by the European Union Reference Laboratory for Genetically Modified Food & Feed (EURL-GMFF, <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>, see Table 1). Primer concentrations were optimised by titration. All primers and hydrolysis-probes were synthesized by Microsynth AG, Balgach, Switzerland. The sequences of primers and probes as well as the labelling are listed in Table 1.

Other material

All plastic material was of PCR-grade quality. To avoid cross-contamination, all pipetting steps were performed using filter tips. Solutions were prepared with DNA/RNA free water. To diminish contaminations, a three room concept was applied.

Droplet digital PCR procedure (ddPCRTM)

5 μl DNA extracts were added to 17 μl of reaction mix containing 11 μl Supermix for Probes (Cat No. 186-3024) and the primers and probes. 20 μl of these final 22 μl were used to generate a water/oil emulsion. The final primer concentrations for the applied ddPCR systems are listed in Table 1, the concentration of the probes was always 250 nM. The setup was according to the manual of the QX200 ddPCRTM system. To generate water in oil emulsion microfluidic cartridges for the ddPCRTM were used. After pipetting the 40 μl emulsion to the 96 multiwell-plates, they were sealed using the heat sealer PX1 from Bio-Rad. The emulsion-PCR was performed on a Master-Cycler Nexus gradient from Eppendorf. The cycling was done according to the manual except that 50 cycles were performed (see below). The ramp rate was fixed to 2.5 $^{\circ}\text{C}/\text{s}$, the annealing temperature was set at 55 $^{\circ}\text{C}$ for all systems. The whole cycling required approximately 2.5 h. The reading of the droplets was then performed using the droplet reader of the QX200-system. All steps were performed according to manual of the QX200 ddPCRTM system of Bio-Rad Laboratories Inc. USA.

Table 1 Compilation of primer/probe sequences and optimised concentrations

Target/label and f-primer/r-primer/probe sequences (5'–3')	Final conc. primer nM	Origin
Lectin TCC ACC CCC ATC CAC ATT T GGC ATA GAA GGT GAA GTT GAA GGA Hex-AAC CGG TAG CGT TGC CAG CTT CG-BHQ1	400	[16]
Mon89788 TCC CGC TCT AGC GCT TCA AT TCG AGC AGG ACC TGC AGA A Joe-CTG AAG GCG GGA AAC GAC AAT CTG -BHQ1	200	EU-RL GMFF
RR TTC ATT CAA AAT AAG ATC ATA CAT ACA GGT T GGC ATT TGT AGG AGC CAC CTT FAM-CCT TTT CCA TTT GGG-BHQ1	800	EU-RL GMFF
2704 GCA AAA AAG CGG TTA GCT CCT ATT CAG GCT GCG CAA CTG TT Hex-CGG TCC TCC GAT CGC CCT TCC -BHQ1	800	EU-RL GMFF
mhmG TTG GAC TAG AAA TCT CGT GCT GA GCT ACA TAG GGA GCC TTG TCC T Fam or Hex-CAA TCC ACA CAA ACG CAC GCG TA -BHQ1	400	EU-RL GMFF
Bt176 GGC CGT GAA CGA GCT GTT GGG AAG CCT ACA TGT TTT CTA A FAM-AGC AAC CAG ATC GGC CGA CAC C-BHQ1	230	EU-RL GMFF
Bt11 GCG GAA CCC CTA TTT GTT TA TCC AAG AAT CCC TCC ATG AG Fam-AAA TAC ATT CAA ATA TGT ATC CGC TCA -BHQ1	800	[17]
Mon810 TCG AAG GAC GAA GGA CTC TAA CGT GCC ACC TTC CTT TTC CAC TAT CTT Fam-AAC ATC CTT TGC CAT TGC CCA GC-BHQ1	800	EU-RL GMFF
NK603 CTG CTC GGC CAG CAA GCC T AAG CAT ATG AAT GAC CTC GAG TA Joe-TAG CGG CCC ACG CGT GGT AC -BHQ1	400	[18]
Mon863 GTA GGA TCG GAA AGC TTG GTA C TGT TAC GGC CTA AAT GCT GAA CT Joe-TGA ACA CCC ATC CGA ACA AGT AGG GTC A -BHQ1	400	EU-RL GMFF
esGA21 CGT TAT GCT ATT TGC AAC TTT AGA ACA GCG ATC CTC CTC GCG TT Fam-TTT CTC AAC AGC AGG TGG GTC CGG GT-BHQ1	450	EU-RL GMFF
59122 TGA GTC GTA TTA CAA TCG TAC GCA AAA CGG GAC CAT AGA AGG GAA Joe-CCG CAA TGT GTT ATT AAG TTG TCT AAG CGT CAA TT -BHQ1	400	[19]
Mir604 GCGCGGTGTCATCTATGTT CCGTACCACCAACTCTTAAT Joe-CATGGCCGTATCCGCAATGT -BHQ1	400	[20]
TC1507 GAC GTC TCA ATG TAA TGG TTA ACG A GGG TAA CCG CTC TTC CAG TTG T Fam-ATC ACA AAC CGA GAG AAG AGG GAT CTC GA -BHQ1	400	[21]

Table 1 (continued)

Target/label and f-primer/r-primer/probe sequences (5'–3')	Final conc. primer nM	Origin
T25 ACA AGC GTG TCG TGC TCC AC GAC ATG ATA CTC CTT CCA CCG Fam-TCA TTG AGT CGT TCC GCC ATT GTC G -BHQ1	400	EU-RL GMFF

Nine of the PCR systems originated from the European Joint Research Center, European reference laboratory for GM Food and Feed (EU-RL GMFF): <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>. The others were taken from other publications. All probe concentrations were 250 nM as recommended by Bio-Rad

Calculation of % GMO content

The percentage of GMO content was calculated as following:

$$\%GMO = cf \times 100 \times \frac{\text{target}}{\text{housekeeping}}$$

cf: conversion factor (no unit); target (droplet/ μ l); house-keeping (droplet/ μ l)

Determination of the optimal primer concentration and annealing temperature

Cycling numbers must be enough to amplify a signal also in cases where the sample matrix is inhibiting the polymerase. To assure this, we decided to cycle 50 times in all cases. The optimization of the primer concentration was done according to a previous publication [4]. All ddPCR™ systems were run in duplex format using lectin (for soy traits) or mhmg (for maize traits) as housekeeping gene. For some of the PCR systems negative and positive droplet populations were difficult to distinguish due to “rain”. Using an annealing temperature of 55 °C for all systems leads to sufficient signal separation to set a clear threshold. This finding corresponds to observations during extensive optimizations previously described [6]. Optimised primer concentrations are listed in Table 1.

Results and discussion

Problems during validation

During validation the following issues had to be assayed: optimal template concentration, achievable lower limit of quantification (LOQ), no template control (ntc) and conversion factor to convert percent values of droplets to weight percent values. In a previous establishment we experienced rapid first results and were, therefore, confident to finish validation in a reasonable time. Optimal template concentration was at 100 ng total DNA per assay. For two (GA21 and Bt176) of the total 13 Systems we faced serious problems when assaying the ntc samples.

A slight but resistant rain of false positive droplets were observed making distinction of positive to negative samples in the low range impossible. These two systems [15] and an unpublished in house system served in multiplex real time PCR faithfully since years. We therefore think that these false positive results are due to difference in cycling parameters, annealing temperature and the DNA-polymerase used in digital PCR. We decided to exchange these PCR systems by systems from the JRC.

Specificity

All digital PCR systems were tested for cross reactions to a wide selection of gm traits, plant and animal DNA. To reduce the reactions and costs we used pooled templates DNA's of eight species (100 ng of total DNA per assay, 12.5 ng per species). DNA of the following organisms were tested (100 ng total DNA per assay): genetically modified traits: Soybean MON87769 100%, Soybean MON87708 100%, Soybean MON87705 100%, Soybean FG72 100%, Maize MON810 5%, Maize MON863 9.9%, Maize T25 100%, Maize CBH351 1%, Maize NK603 5%, Maize Bt176 5%, Maize Ly038 E-7, Maize Bt11 5%, Maize GA21 4.3%, Maize 59122 10%, Maize MON98034 100%, Maize MON88017 100%, Maize ES3272 10%, Maize Mir604 10%, Maize TC1507 10%, Maize DP98140 10%, Maize DAS40278-9 1%, Maize Mir162 100%, Maize MON87460 100%, Maize Bt10 100%, Soybean RRS 5%, Soybean A2704 100%, Soybean MON89788 100%, Soybean A5547 100%, Soybean DP356 10%, Soybean DP305 10%, Soybean MON87701 100%, Soybean CV127 100%, Rice KMD1

The following cross reactions were observed: The GA21-system exhibit cross reaction when applying DNA from Starlink (2%) and Mon89034 (0.1%). These cross reactions are unlikely (Starlink, cultivation stopped) or may come from contaminated reference material (Mon 89034) and have to be considered when examining such samples.

Bt10 Maize can not be distinguished from Bt11 by the Bt11-system. This was expected as Bt10 and Bt11 are very close relatives. However, Bt10 maize is very unlikely to be detected as cultivation was stopped years ago.

Animals Cattle (*Bos taurus*), turkey (*Meleagris gallopavo*), horse (*Equus caballus*), porc (*Sus scrofa*), chicken (*Gallus gallus*), swordfish (*Xiphias gladius*).

No cross reactions were observed.

Plants Almond (*Prunus dulcis*), apricot (*Prunus armeniaca*), walnut (*Juglans regia*), hazelnut (*Corylus avellana*), peanut (*Arachis hypogaea*), cinnamon (*Cinnamomum verum*), beans (*Phaseolus vulgaris*), rice (*Oryza* spp.), wheat (*Triticum* spp.), sesame (*Sesamum indicum*), lentil (*Lens culinaris*), lupin (*Lupinus*), maize (*Zea mays*), cauliflower (*Brassica oleracea*), peas (*Pisum sativum*), tomato (*Solanum lycopersicum*), rape (*Brassica napus*), mango (*Mangifera indica*), banana (*Musa*), broccoli (*Brassica oleracea*), rye (*Secale cereale*) durum wheat (*Triticum durum*), avocado (*Persea americana*), lupine (*Lupinus albus*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*)

The following cross reactions were observed: The 2704-system detects 100% cinnamon with 0.2%. This has to be considered when assaying samples containing high level of cinnamon. Radish (0.3%) and rye (1.4%) lead to cross reactions in the Mon863-system. This has also to be considered. No other unspecific signal was observed. Cross-contamination for these findings could be an explanation, however, this was considered to be very unlikely. For all traits only one batch was tested.

Measurement range

For the housekeeping assays, the lambda factor was below 0.5 positive droplets/total droplets. For the transgene target systems, the lambda factor was below 0.05.

Most transgene target systems showed a consistent positive signals, at a level of 11 positive droplets (0.5 copies/ μ l) or more. We consider that the limit of detection (LOD) is close to this value. Expressed in %, 11 or more positive droplets were gained at a level of about 0.1% (prerequisite: 100 ng of total template DNA, spectrophotometrically determined) The system for the corresponding housekeeping gene exhibited about 1500 copies/ μ l positive droplets of housekeeping gene, e.g., mhmng, if the reference material contained 100% transgene DNA). In practice the upper limit was limited by the maximal amount of template DNA used and the quantification range of the QX200 system. Considering the average droplet number of 10 000, the upper quantification limit was experienced to be at about 5000 copies/ μ l. In our hands the lower limit of quantification was, therefore, not lower than when using real time PCR (also at 0.1%). In many cases, the available reference material does not consist of 100% GMO, in consequence, the tested quantification range became smaller, e.g., 0.1–1%. Values higher than 1% have to be determined by extrapolation or dilution. This was not investigated here

as usually the most samples contain low amounts of GMO. The labelling threshold for authorized traits in Switzerland and EU is at 0.9% and is covered by this measurement range. And in cases of unauthorized gm traits the product has to be withdrawn from the market, when a reproducible positive signal is measured, a quantification is not required.

Accuracy, precision, measurement uncertainty (MU) and robustness

Limit of quantification (LOQ), precision and measurement uncertainty of the ddPCRTM was tested at three levels of DNA concentration: e.g., if the content was 100% GMO: 2 ng/ μ l corresponds to 10%, 0.2 ng/ μ l corresponds to 1% and 0.02 ng/ μ l corresponds to 0.1%. But the proportion of GMO relative to the housekeeping lectin (for soy) or mhmng (for maize) was not always 100%. In cases where the level was, e.g., only 5% (depending on the available reference material), this material could only be diluted 1: 50 to reach the lower LOQ of 0.1% for the GMO marker gene, which had to be reached. This low level of 0.1% was estimated to be close to the limit of quantification usually obtained for GMO quantification methods using real time PCR methods [12]. We therefore determined measurement uncertainty performing 12 duplex experiments for each transgene soy trait. These experiments were performed at different days by 3 persons to assess the robustness of the systems. Further robustness testing was not done. Recommendations of varying annealing temperature and primer concentration is not helpful for ddPCRTM as this is an endpoint method and such variations have only minor impact, which was shown during optimization experiments [6]. To assay false positive rate, e.g., by possible cross-contamination during the droplet procedure, no template controls (ntc) were run during four different assays. For the systems that were finally selected, no false positive droplets were observed. However, from a total of 13 systems, two systems showed consistent false positive signals during development and had to be replaced (GA21 and Bt176).

At the level of 0.1% GMO, all assays always showed positive results and we conclude, therefore, that 0.1% GMO content is covered by the quantification range.

The determined measurement uncertainty (MU), including the precision and accuracy, ranged from 9 to 43%. The guidance document for method performance requirements of the European network of GMO Laboratories sets a limit of maximal $\pm 50\%$ [12] for the MU. The determined MU is, therefore, acceptable at this low concentration level. For the higher concentration level of 1% GMO, data for precision and measurement uncertainty are also acceptable (measurement uncertainty ranging from 7.5 to 26%).

Comparing to internal validation data, in six cases the ddPCRTM exhibited a lower measurement uncertainty in the low range than real time PCR. But in other six cases the real time PCR exhibited lower measurement uncertainty. We estimate, therefore, that ddPCRTM and real time PCR have comparable measurement uncertainty in general in the low range. In the higher range of 1% GMO we observed for ddPCRTM in 8 cases superior MU and for real time PCR only 4 times. This suggests that ddPCRTM can exhibit lower measurement uncertainty comparing to real time PCR, only in cases where the template concentration is high. The high expectations for ddPCR were, therefore, not fulfilled in this case.

Zygoty/conversion factor

Reference material for GMO analysis is often a mixture of flour with and without GMO trait by weight to weight, leading to a certain percentage of GMO. Digital PCR determines copy numbers leading to percentage of GMO as well but not weight to weight. Surprisingly, for all tested soy traits only minor difference were observed [13, 14]. The zygoty or conversion factor for the tested soy traits was always close to 1. For maize traits this seems not to be the case. For GMO maize traits the factor is often higher than 1. Usually the PCR systems to determine the genetic modification use a single integration site. The second gene, often called housekeeping, determines the plant species, in this case the major high mobility group gene (mhmg) of maize. This gene may have multiple sites and the number of sites may be different from one trait to the other. Earlier publications report that the mhmg used here should be present in all maize traits at comparable amounts and is therefore widely used as a housekeeping gene [15]. In principle the difference between percentage measured by copy numbers and the reference material weight by weight may also come from use of not appropriate traits or different weights of the kernels or zygoty in the F1 generation. But these arguments also apply to GM soy. But for soy the conversion factor seems to be close to 1 for all investigated traits [8]), see Table 2. We suspect therefore the different conversion factors of GM maize traits to be mainly a result from difference in copy number of the mhmg housekeeping gene in maize traits. An additional explanation may be the fact that maize is not self-pollinating, in contrast to soy. In addition, maize is mostly sold as hybrid trait. As a consequence, first generation kernels are not 100% homozygous and the zygoty may be therefore affected.

We determined the conversion factors using the actually available reference material described above (section reference material). Once determined, the conversion factor can be used for the appropriate primer system without

using reference material for each analysis. However, when changing the PCR system for the housekeeping gene or the reference material, we expect that this factor has to be determined again.

Results from proficiency studies

Earlier experiences showed that ddPCRTM may be superior to real time PCR results. To assess this, we analysed proficiency test samples in parallel using real time PCR and ddPCRTM (applying the determined conversion factors). The results are compiled in Table 3. In 25 cases ddPCR was superior to real time PCR, in 23 cases the real time PCR was superior. We consider this as not significant. Earlier results showed comparable MU at low concentrations, confirming this result. Looking at the performance of individual PCR systems, e.g., Mon863 and 59122, the general finding of equal MU and closeness to the true value seem to depend mainly on the PCR-system applied. This could be a hint for future ameliorations.

Conclusion

We validated 13 droplet digital PCR systems according to guidelines [10–12], fulfilling ISO17025 requirements using certified reference material. The lower range of quantification could be confirmed for all systems at a level of 0.1% GMO. At this level total positive droplets were in the range of 10–20 per assay which is statistically sufficient. At a level of 1%, measurement uncertainty was significantly lower than when using real time PCR in the majority of cases. The accuracy for maize samples had to be corrected by a conversion factor to get the true value of the GMO content by weight to weight. For soy samples this factor was close to one. Published values for soy traits are until now are also close to one, confirming our findings. This seems to be a principal difference between maize and soy traits. Earlier work attempted to use a sequence for the housekeeping gene, which should lead to comparable signals for all maize traits. This mhmg-gene, however, seem to vary significantly. This effect is not observed when analysing soy traits. All effects discussed concerning mixing and selection of the material may therefore not have an impact on the zygoty and conversion factor as they apply to both traits, soy and maize. We therefore consider the difference in mhmg as the major impact for the different conversion factors in maize traits. The presented conversion factors only apply to the PCR systems and reference material in this study. And they may need to be confirmed by a greater effort including more defined reference material and statistical calculations as described by the European Joint Research Center [23].

Table 2 Conversion factor, accuracy, precision and measurement uncertainty (MU)

GMO trait	Plant	Conversion factor	Level %	Accuracy ± %	Precision %	MU ddPCR %	MU real time PCR %
T25	Maize	1.1	0.1	-0.2	32	32	15
			1	-14	7.5	15	9.3
GA21	Maize	1.55	0.1	+6	64	35	45
			1	-24	26	26	24
Bt176	Maize	1.5	0.1	+5	7.8	13	15
			1	-0.9	13	9.1	21
Mon863	Maize	1.6	0.1	+9	22	24	21
			1	-5	7.7	9	24
TC1507	Maize	2.1	0.1	+0.17	31	31	22
			1	+0.2	4.4	21	15
NK603	Maize	2.3	0.1	+4.7	6.6	8	22
			1	+4.6	7.3	9	12
MIR604	Maize	2.3	0.1	+0.2	14.8	15	25
			1	+1.5	7.3	7.5	17
MON810	Maize	2.6 ^a	0.1	+17	30	34	25
			1	+13	6	16	33
Bt11	Maize	2.8	0.1	-3.4	14.7	15	43
			1	+8.4	8.8	12	16
59122	Maize	3.6	0.1	-16.5	8.1	18	31
			1	+11.4	2.6	12	26
RR	Soy	1.1	0.1	+7.1	37	38	33
			1	-2.6	11.4	12	9.3
Mon89788	Soy	1	0.1	-4	42	43	30
			1	+2.6	14.6	15	14
2704	Soy	1.1	0.1	-18.4	13	22	21
			1	+3.1	8.9	9	17

To estimate the precision we used relative standard deviation RSD, for the accuracy the relative difference from the measured to the assigned value which was taken after applying a factor to maximise the accuracy for all measured levels. This maximising factor was assigned as zygosity factor. As observed earlier [22], the conversion factor for soy samples was always close to 1. For maize this factor varied significantly. The measurement uncertainty was calculated by geometrical addition of SD and accuracy [11]. MU for the ddPCRTM and real time PCR are compiled, MU with Bold were superior (difference >1%)

^aAn earlier publication [7] observed for a 1% reference material a copy number ratio of 0.395. This corresponds well to a conversion factor of 2.6 ($1/2.6 = 0.38$)

The results presented here were generated without enzymatic cutting of the template DNA suggesting that this step is not required. As real samples in general have degraded template DNA (therefore sensitive PCR systems usually amplify amplicons below 150 bp) and make such an enzymatic degrading step unnecessary. The guidelines to perform robustness testing should be adapted for digital PCR as the recommendations don't apply for digital PCR as the

amplification is intrinsically less dependent on annealing temperature and primer concentrations.

Finally, the validated ddPCRTM-systems for the most prevalent GMO traits we present here are fit for routine analytic. The assessed MU and performance, analysing proficiency test samples revealed in general similar performance as real time PCR. In contrast to earlier findings ddPCRTM was not superior to real time PCR. A remaining

Table 3 Compilation of proficiency test results (Fapas and GIPSA) using real time PCR and ddPCR™

GMO trait	True value %	ddPCR value %	Real time PCR value %
T25	0.8	0.4	0.28
	1.86	0.69	0.88
	0.39	0.32	0.11
GA21	1.0	1.27	1.09
	1.0	0.34	0.60
Bt176	0.5	0.13	Neg
	0.36	0.26	0.29
	1.45	1.22	1.33
Mon863	0.08	0.08	0.07
	0.2	0.1	0.23
	0.2	0.07	0.16
	1.5	0.69	1.71
	0.2	0.35	0.23
	0.2	0.44	0.33
	1.5	1.22	1.14
	0.2	0.33	0.21
	0.07	0.07	0.02
	0.2	0.13	0.04
Bt11	0.5	0.22	0.22
	0.5	0.27	1.1
59122	1.33	1.5	0.58
	1.5	1.2	3.28
	1.0	0.67	2.07
	0.5	0.36	0.7
	0.2	0.62	0.12
TC1507	1.0	2.5	2.6
	1.5	3.6	4.6
	0.5	0.1	0.3
NK603	0.2	0.05	0.09
	0.8	0.96	0.4
	0.5	0.21	0.3
	1.0	0.61	0.62
MIR604	1.5	0.98	1.54
	1.5	1.5	1.4
	1.0	0.88	0.8
	1.0	1.17	1.1
	0.5	0.17	0.38
MON810	0.1	0.04	0.1
	0.2	0.15	0.22
	0.5	0.29	0.39
RR	0.2	0.06	0.08
	0.2	0.3	0.1
	0.5	1.22	2.64
RR	1.5	3.83	2.64
	0.5	0.64	0.7
	0.5	0.55	0.5
	0.45	0.69	0.73

Table 3 (continued)

GMO trait	True value %	ddPCR value %	Real time PCR value %
	0.65	0.34	0.65
2704	0.49	0.42	0.0
	0.12	0.1	0.0
	0.34	0.08	0.0
	0.11	0.16	0.0

The true value corresponds to the value according to the recipe weight to weight. In 23 cases real time results were more close to the true value, in 25 cases ddPCR™ was superior

Bold values show better performance of ddPCR or real time PCR

advantage is still that results can be generated without the use of reference material once the conversion factors were determined.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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