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Multiplex Quantification of 12 European Union Authorized Genetically Modified Maize Lines with Droplet Digital Polymerase Chain Reaction

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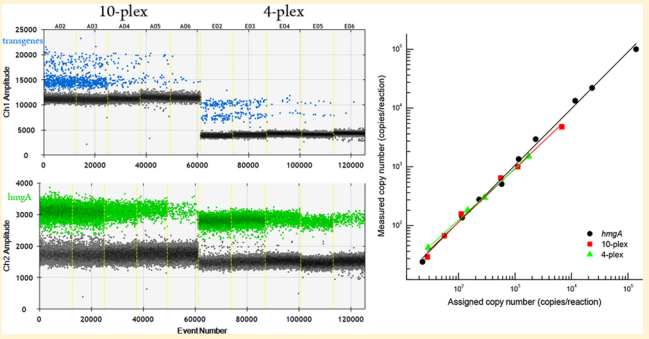
Supporting Information

ABSTRACT: Presence of genetically modified organisms (GMO) in food and feed products is regulated in many countries. The European Union (EU) has implemented a threshold for labeling of products containing more than 0.9% of authorized GMOs per ingredient. As the number of GMOs has increased over time, standard-curve based simplex quantitative polymerase chain reaction (qPCR) analyses are no longer sufficiently cost-effective, despite widespread use of initial PCR based screenings. Newly developed GMO detection methods, also multiplex methods, are mostly focused on screening and detection but not quantification. On the basis of droplet digital PCR (ddPCR) technology, multiplex assays for quantification of all 12 EU authorized GM maize lines (per April first 2015) were developed. Because of high sequence similarity of some of the 12 GM targets, two separate multiplex assays were needed. In both assays (4-plex and 10-plex), the transgenes were labeled with one fluorescence reporter and the endogene with another (GMO concentration = transgene/endogene ratio). It was shown that both multiplex assays produce specific results and that performance parameters such as limit of quantification, repeatability, and trueness comply with international recommendations for GMO quantification methods. Moreover, for samples containing GMOs, the throughput and cost-effectiveness is significantly improved compared to qPCR. Thus, it was concluded that the multiplex ddPCR assays could be applied for routine quantification of 12 EU authorized GM maize lines. In case of new authorizations, the events can easily be added to the existing multiplex assays. The presented principle of quantitative multiplexing can be applied to any other domain.



Many countries in the world regulate the cultivation and trade with genetically modified organisms (GMO).[1] Most commonly there is an approval system and mandatory labeling above a certain threshold. The enforcement of such regulations depends on the ability to detect and quantify the presence of GMOs in food, feed, and seed products. In the European Union (EU) this is enforced for all authorized GMOs through Regulation (EC) 1829/2003.[2] This regulation specifies that the developer of a GMO has to provide a method for quantifying the specific GMO event and funding for a collaborative trial to validate the method. Quantification of the GMO is done relative to a species specific reference gene. Thus, there exists a set of validated event specific GMO detection methods (available at http://gmo-crl.jrc.ec.europa.eu/gmomethods/). These are all standard curve based real-time PCR (qPCR) methods. In Europe, the labeling threshold for authorized GMO events is specified in Regulation 1829/2003.[2] This regulation states that labeling: "*shall not apply to foods containing material which contains, consists of or is produced from GMOs in a proportion no higher than 0.9% of the food ingredients considered individually or food consisting of a single ingredient...*" Thus, the labeling threshold is on a *per ingredient* basis, not on a *per GMO* basis. In analytical terms, ingredient in this context is interpreted as species.[3] So, it is the cumulative concentration, e.g., of all authorized maize GMOs relative to the total quantity of maize that determines if the threshold is exceeded or not. The technical guidance for sampling and detection of GMOs[4] states that the GMO content should be expressed as ratio of GM part in relation to the taxon specific part, meaning that GMO content can either be measured as additive concentration of individual authorized events or it can be measured as the total concentration of this group directly. The latter appears as a more cost-efficient approach, but hitherto it has not been possible to implement due to lack of suitable analytical methods.

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Standard curve based qPCR is sensitive and robust and is regarded as the gold standard for GMO analysis. As the number of authorized GMOs has increased over time, the required number of qPCR analyses has increased correspondingly. An analysis regime based only on separate event specific qPCRs is no longer cost efficient. As a more cost efficient approach, many laboratories now employ one of several alternative screening approaches, testing for presence of genetic elements commonly found in GMOs.[5] The observed presence/absence pattern for each sample can be compared with a reference table of presence/absence of the corresponding elements in known GMOs, e.g., using the GMOseek software.[6,7] At and above the limit of detection (LOD), the presence of GMOs containing elements that are not observed in the sample can effectively be excluded. The list of remaining candidate GMOs after screening serves as a starting point for quantification and confirmation of events present in the sample. Recently a multiplex version of one of these screening approaches[8] was developed as a single qualitative pentaplex PCR.[9]

Multiplex PCR analysis obviously has the potential to improve the cost efficiency, but a multiplex PCR is a complex experimental system with potential interference between oligonucleotides and amplification products (target and non-target). The development of quantitative methods to a high level of multiplexing has proven difficult. Although, several multiplex qPCRs exist, only two (duplex) are interlaboratory validated: one in Europe[10] and one in Japan.[11]

Digital PCR (dPCR) as a concept was invented in 1992.[12] In dPCR the reaction mixture is distributed into a large number of partitions, where each partition contains zero, one, or more copies of the target nucleic acid. Positive and negative signal in partitions is scored at end point of the PCR and the initial DNA concentration can be calculated using the Poisson distribution.[13] Digital PCR does not depend on PCR efficiency for DNA quantification to the same degree as standard curve based qPCR. Partial inhibition of a PCR will not affect quantification with dPCR as only discrete data on signal and not amplitude of the signal will be used in the calculations. Digital PCR can be performed in normal 96-well PCR plates using fluorescence probes,[14] and different approaches have been explored for the use in GMO analysis.[15,16] Although one of these was formally collaborative trial validated as a simplex dPCR approach for quantitation of GTS 40-3-2 (RoundupReady) soya (Marco Mazzara et al., personal communication), this experimental setup did not improve cost efficiency significantly due to large chemical consumption and low statistical power.

The dPCR is measuring the absolute number of copies in the sample; therefore, the final result for GM content is given as copy number ratio of GM event and taxon specific gene. Most widely used reporting unit for qPCR is nevertheless the mass ratio, as the majority of reference materials are certified for the GM% in mass. Thus, although the PCR reaction is amplifying the actual DNA copies, the final result is converted in mass ratio based on the standard curve of reference material. This fact made the topic unclear already from the beginning. The official unit of measurement has however never been exactly defined for the whole GMO field but rather for individual scopes (prepackaged food,[17] EU unauthorized GMOs[18] and recently for reporting of proficiency test results [EURL-GMFF Invitation letter, 2015]). As the EU Commission is apparently headed toward accepting only mass fraction as the accepted unit of measurement, an ENGL working group on the Unit of measurement has been recently established, who will try to explain the conversion of copy number ratio to mass fraction. For the purpose of this manuscript, the conversion factor set in EURL Technical Guidance 619/2011[19] was used.

Presently, two designated dPCR technologies are available: microfluidic/chip based dPCR and emulsion (droplet) based dPCR.[20] Several studies have tested the applicability of dPCR for GMO detection.[21−25] The practicability and applicability of using the droplet dPCR (ddPCR) in routine GMO diagnostics was recently shown for duplex reactions.[24] In the present study it was explored whether ddPCR could be multiplexed to a high degree and utilized to quantify a group of GMOs for compliance with the EU labeling regulation.[2] The analytical procedure of GMO testing include sample preparation, DNA extraction and purification, (q)PCR amplification, and data evaluation. These steps are nowadays often referred to as modules.[5] There are several advantages of using the modular approach in GMO testing, especially it is more favorable when the methods are being validated.[26] This study was focused only on the module of PCR amplification and does not include the evaluation of other modules. To be able to analyze GMO content per ingredient in a cost efficient manner and in line with the EU regulatory requirements,[2] a set of validated qPCR modules was used and combined for multiplex ddPCR. In this manuscript, the data on the performance of the developed assays are presented and their practicability, applicability, and potential impact is discussed.

EXPERIMENTAL SECTION

Test Material. A list of all CRM test materials is provided in Table S-15 the Supporting Information. They all have certified mass/mass (m/m) GM maize/wild-type maize material ratios. Routine diagnostic samples containing maize events (seeds or flour) and samples from proficiency programs (USDA and EURL) were also used in this study (Table S-12 in the Supporting Information). A specificity study was conducted on samples containing either GM maize events not authorized in the EU, DNA from non-GM maize/sweet corn, and GM and non-GM soybean, rape seed, rice, and linseed samples without maize (Table 2).

DNA Extraction. DNA was extracted and purified from 200 mg of starting material for all samples using a cetyltrimethylammonium bromide (CTAB) protocol with RNase-A solution and proteinase-K solution for removal of RNA and proteins from the sample (as described in annex A.3 of ISO 21570:2005[27]), with small adaptations. Dilutions of the extracted stock DNA solutions were made in nuclease- and protease-free water (Sigma-Aldrich Chemie GmbH, Munich, Germany). All samples were stored at −20 °C.

Primers and Probes Mix Preparation. The *hmgA* gene was used as the endogenous reference gene for maize, to which relative quantity of the GM events was estimated. For quantification of GM maize lines, the construct (for Bt11) or event (all others) specific qPCR modules were used. Nucleotide sequences of probes and primers used are presented in Tables S-4 and S-5 in the Supporting Information. The GM content in each sample was determined by simplex ddPCR. A total of 13 primer and probe sets were prepared for simplex reactions targeting individual events, constructs or the endogene, as well as 12 sets for duplex reactions where primers and probes for endogene (*hmgA*) and individual events or construct were mixed. The preparation of 10-plex and 4-plex primer and probe (PPP) mixes was done as follows. For 10-plex, primers and probes for the endogene (*hmgA*) and nine

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events, GA21, MON810, MON863, DAS1507, MIR604, MON88017, MON89034, MIR162, and T25, were mixed (for final concentrations see Table S-4 in the Supporting Information). For 4-plex, primers, and probes for the endogene (*hmgA*), two events, DAS59122 and NK603, and one construct, Bt11, were mixed (for final concentrations see Table S-5 in the Supporting Information). Primers and probes were purchased from Eurofins MWG Operon (Ebersberg, Germany) or from Integrated DNA Technologies (Leuven, Belgium). Primers and probes were shipped lyophilized and diluted in nuclease- and protease-free water (Sigma-Aldrich Chemie GmbH, Munich, Germany) upon reception.

Droplet Digital PCR Reactions and Data Analysis. The reaction mix for all reactions, simplex as well as multiplex, was composed of 10 μL of 2xddPCR Supermix for probe (No dUTP) (Bio-Rad, Pleasanton, CA), 6 μL of primer and probe mix, 4 μL of DNA template (or 4 μL of nuclease- and protease-free water for nontemplate control), mixed into a 20 μL reaction volume. Droplets were generated in 8-well cartridges, using the QX100 droplet generator (Bio-Rad, Pleasanton, CA). Water-in-oil emulsions were transferred to a 96-well plate and amplified in a T100 PCR cycler (Bio-Rad, Pleasanton, CA). Thermal cycling conditions were 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of a two-step thermal profile comprising of 15 s at 95 °C and 60 s at 60 °C at ramp rate 2.5 °C/s. After cycling, each sample was incubated at 98 °C for 10 min and then cooled to 4 °C. Plates were then transferred to the QX100 droplet reader (Bio-Rad, Pleasanton, CA). Data acquisition and analysis was performed using QuantaSoft (Bio-Rad, Pleasanton, CA) software. Positive droplets, containing amplification products, were discriminated from negative droplets without amplification products by applying a fluorescence amplitude threshold (Figure S-1 in the Supporting Information). The threshold was set manually, using both the fluorescence amplitude vs event number (1D amplitude) and the histogram of events vs amplitude data streams, on each of the FAM and VIC channels (2D amplitude). Data generated by the QX100 droplet reader were rejected from subsequent analysis if a clog was detected by the Quantasoft software or if a low number of droplets (<8000) was measured per 20 μL PCR. After being exported, the data were further analyzed in Microsoft Excel spreadsheets. The number of template copies per μL was calculated using the volume of 0.85 nL per droplet. A digital MIQE checklist is made available in Table S-16 in the Supporting Information.

Comparison of Simplex and Multiplex Reactions. The performance of ddPCR 4-plex and 10-plex assays was compared to simplex assays. Both *hmgA* simplex and event/construct simplex reactions were performed on individual DNA samples in two dilutions (containing from 3 000 to 40 000 target DNA copies per reaction) each in a duplicate. The 4- and 10-plex assays were also tested on individual DNAs but only in duplicate of one dilution.

Dynamic Range, Repeatability, Limits of Detection, and Quantification. A dilution series in terms of target copies was prepared with mixture of DNA from all 12 transgenic maize events extracted from the CRMs (each line was present approximately in equal number of target copies). The quantity of each GM maize event in the DNA mix solution was determined by duplex ddPCR (individual event and *hmgA* in the same reaction on 6× dilution). These values were used to calculate the assigned copy number of targets in the dilution series of DNA mix. The assigned values were from 6724 to 0.01 copies of transgenes covered by 10-plex and from 1734 to 0.002 copies of transgenes covered by 4-plex. The dilution series for both multiplexes contained from 139 302 to 0.2 copies of the endogene *hmgA*. Twelve replicates of the dilution series were measured by ddPCR (three separate runs in 3 days each containing four replicates). Another dilution series was prepared in terms of transgene content, where the dilutions of DNA mix 1 for 10-plex contained 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 0.9, and 2% of all transgenes found in 10-plex, and 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 0.9% of all transgenes found in 4-plex for DNA mix 2. Six replicates of these dilution series were measured by ddPCR. The absolute/relative (copy number/%) limit of quantification (a/rLOQ) and absolute/relative limit of detection (a/rLOD) for ddPCR were determined based on these experimental results. The LOQ was determined as the aLOQ or rLOQ in the sample, where the relative standard deviation (RSD) of all replicates was below 25%. The LOD was determined as the aLOD or rLOD in the sample, where all replicates still produced a positive signal.

Specificity. To test specificity the DNA extracts from 12 different samples (3 GM maize events not authorized in the EU, 2 GM soybean events, one GM rice event, one GM oilseed rape event and five different nontransgenic samples; Table 2) were tested with the 4- and 10-plex ddPCR assay. Reactions were performed in duplicates per sample per assay.

Fitness for Purpose. To assess whether the new assays are fit for purpose, 13 different samples from proficiency programs (USDA and EURL) and four routine diagnostic samples (Table 3) were tested with both multiplex assays. To obtain a robust mean value of GM content, the proficiency test reports were used to make an average of all reported values per event, then to calculate the sum of all present events, and finally to calculate standard deviation and *Z*-score values. For the results to be directly comparable, the conversion of ddPCR results (measured as GM% in copy number ratio) into the mass ratio (GM% in mass fraction) was done according to EURL Technical Guidance 619/2011.[19] For sample 189/09, containing GM maize in which the transgene originated from the male parent, another conversion using the factor 0.36 (based on information from Holst-Jensen et al.[3] and our previous ddPCR analyses of those GM lines [data not shown]) was also performed. For routine diagnostic samples, the additive concentration of GM maize events was calculated based on results of quantification with qPCR performed in the NIB laboratory for official control under ISO17025 accreditation.

Robustness. The robustness of the ddPCR multiplex assay was evaluated by making small modifications to the protocol. These included (1) use of a PCR cycler with slower ramp rate (GeneAmp 9700 PCR cycler [Applied BioSystems, Foster City, CA]), (2) different annealing temperatures (61 and 59 °C), (3) use of another Mastermix (2xddPCR Supermix for probe containing dUTP [Bio-Rad, Pleasanton, CA]), (4) pipetting all eight reaction mixes to the cartridge in one step with a multichannel pipet, and (5) transfer of generated droplets to PCR plate with an automatic electric multichannel pipette. Two samples from the repeatability experiments (DNA mix, 60× and 120× dilution) were used in the robustness experiments, and the results for normal conditions were taken from the repeatability experiments. The 60× sample was tested in duplicates with both multiplex assays, whereas only one reaction was tested for the 120× sample per multiplex assay.

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RESULTS AND DISCUSSION

Selection of PCR Modules. In the EU there is no consistent official definition of an event (see discussion in ref 3). Consequently, single transformations, multiple transformations, and crosses of transformations appear as events in the official EU registry (http://ec.europa.eu/food/dyna/g_register/index_en.cfm). There are currently (per first April 2015) 37 such events of maize authorized in the EU (Table S-1 in the Supporting Information). Twelve of the authorized events (1−12 in Table S-1 in the Supporting Information) are what can be denoted single events, while the remaining 25 (13−37 in Table S-1 in the Supporting Information) are stacked events (hybrid crosses between the 12 single events). Validated event specific qPCR modules are available for each of these 12 single events (Table S-2 in the Supporting Information and http://gmo-crl.jrc.ec.europa.eu/gmomethods/). As far as technically possible, the multiplexes were designed using methods validated by the European Network of GMO Laboratories (ENGL). For GA21 there are two alternative validated modules from different developers (QT-EVE-ZM-007, 112 base pair (bp) and QT-EVE-ZM-014, 101 bp). Both modules target the 5′ junction between the maize genome and the inset. The QT-EVE-ZM-007 fulfilled the validation criteria in a relative concentration range of 0.98−4.26% GMO while the QT-EVE-ZM-014 fulfilled the criteria in the concentration range 0.09−8.0% GMO. The latter module thus appeared to be more robust. In addition, there is an 8 base pair (bp) perfect match between the probe in the QT-EVE-ZM-007 module and the reverse primer of the ENGL validated NK603 specific module (QT-EVE-ZM-008) that can be avoided by choosing QT-EVE-ZM-014. In the ENGL validated Bt11 module (QT-EVE-ZM-006), the forward primer and probe targets the pUC19 derived sequence. Consequently, commonly found pUC19 contamination in molecular biological reagents could possibly interfere with primer and probe. On the basis of previous experience, the module works with some mastermixes but not with others. Therefore, it was decided to substitute it with the construct specific module designed and in-house validated by Brodman et al.[28] The latter module is in-house verified for use in routine analyses for detection and quantification of Bt11 in one of our laboratories (NIB) and has a record of successful use by at least one other laboratory in GeMMa proficiency testing schemes over nearly a decade (see reports from the proficiency testing rounds; http://fapas.com/proficiency-testing-schemes/gemma/). A qPCR targeting the maize *hmgA* reference gene was chosen as the taxon specific module (QT-TAX-ZM-002).[27] By focusing on PCR modules, different extraction procedures of different samples were not tested. On the basis of the experiences from qPCR, there might be some influences of extraction procedures of different complex or processed real-life samples on the performance of PCR. However, as dPCR is less sensitive to different inhibitors coming from such samples,[25,29] the effect should not be as pronounced as with qPCR.

Combining Modules into Multiplex Assays. As the selected qPCR modules were designed and validated for use in simplex qPCRs, it needed to be checked carefully that the modules can be multiplexed. As a first step, obvious interactions between primers and probes were analyzed *in silico* using the software Autodimer.[30] All primers and probes were aligned with a local alignment algorithm with a score of +1 for match and a penalty of −1 for mismatch. Three putative interactions with a score ≥7 were identified (Table S-3 in the Supporting Information). There was an 18 bp perfect match between the probes for DAS59122 and MON88017. This interaction will most likely not result in the generation of false positive signals, but it can reduce the effective free probe concentration in the PCR and potentially lead to generation of false negatives. A 9 bp match between the probe of MIR604 and the reverse primer of DAS59122 and an 8 bp match between the probes of MIR604 and MON88017 were carefully evaluated and it was concluded that the latter was unlikely to cause significant interference. Pooling of all the 12 event/construct-specific modules and the *hmgA* maize reference gene module into one multiplex was considered. However, on the basis of the putative interactions (Table S-3 in the Supporting Information) it was decided to divide the 12 event/construct specific PCRs onto two multiplex assays. Initially, multiplexing was tested under qPCR conditions with two hexaplex combinations; one with GA21, MON810, MON863, MON88017, MON89034, and NK603, and another hexaplex with Bt11 (QT-EVE-ZM-006), DAS1507, DAS59122, MIR162, MIR604, and T25. Both hexaplexes were found to be acceptable (data not shown). Then the hexaplexes were transferred to ddPCR and observed that Bt11 did not yield a signal, while the GMO concentration was underestimated for NK603 and DAS59122 (data not shown). The event specific Bt11 module was substituted with the module developed by Brodmann et al.[28] as discussed above and decided to combine the modules for GA21, MON88017, MON89034, MIR162, MIR604, T25, MON810, MON863, DAS1507, and *hmgA* in a decaplex (10-plex) and the modules for NK603, Bt11, DAS59122 and *hmgA* in a tetraplex (4-plex) assay, respectively. These combinations were successively adopted as the final assay designs and circumvented the putative interactions described above.

Characterization of Multiplex Assays on Individual GMOs. The ddPCR system used in the experiments enables the use of two different fluorophores (FAM and VIC/HEX channel, respectively). The idea was to label all probes for GMOs with FAM and the probe for the maize endogene with HEX (Tables S-4 and S-5 in the Supporting Information). Quantification of individual GMOs using the 4-plex and 10-plex assays was compared to quantification under simplex conditions. Both multiplex assays were comparable to the corresponding simplex conditions (Table 1), as the bias was consistently below 25%. Thus, it was concluded that both multiplex assays were suitable for use in subsequent performance assessment experiments.

Limit of Quantification and Dynamic Range. The absolute limit of quantification (aLOQ) is the lowest target copy number in a sample that can be reliably quantified with an acceptable level of precision and accuracy.[31] The aLOQ of each target group in the ddPCR assays was estimated as the lowest copy number within the dynamic range with a relative standard deviation (RSD) of the measured copy number ≤25%.[31] Four replicates of each reaction were performed on 3 separate days on serial dilutions of DNA mixture. The RSD of all 12 replicates was used to determine the aLOQ, which was 42 and 29 copies per reaction for transgenic lines in the 4-plex (Table S-6 in the Supporting Information) and the 10-plex (Table S-7 in the Supporting Information), respectively. For the endogene, *hmgA*, the aLOQ was 24 copies (Table S-8 in the Supporting Information). Acceptable values of aLOQ for qPCR, published[15,32] as well as implemented in our routine laboratories, range from 30 to 100 copies. Both developed

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Table 1. Performance Comparison of Multiplex Assay against Simplex Conditions on Individual DNA Material of GM Lines

	average GM %		
GM event	simplex	multiplex	bias multiplex to simplex (%)
DAS1507	5.71	6.09	6.65
DAS59122	3.48	3.59	3.16
GA21	34.09	27.11	−20.47
MIR162	58.99	59.12	0.22
MIR604	38.47	38.21	−0.68
MON810	3.83	3.63	−5.22
MON863	6.07	6.16	1.48
MON89034	60.27	55.77	−7.47
NK603	2.48	2.02	−18.47
T25	96.44	87.33	−9.45
Bt11	1.90	2.06	8.42
MON88017	48.90	54.78	12.02

multiplex assays fit within this range. As both multiplex assays are intended for quantification of total amount of 12 EU authorized GM maize lines, the sum of all transgene copies against the average of *hmgA* copies from both assays was also calculated. With this approach, the estimated aLOQ is 35 copies of transgenes (Table S-9 in the Supporting Information). The data is summarized in Figure 1. For a GMO detection method to be fit for purpose it must also be able to quantify low amounts of transgenic material in a high amount of nontransgenic material (e.g., ≥0.1%). The observed relative LOQ (rLOQ) for the 4-plex and 10-plex were 0.068% and 0.058%, respectively (Table S-10 in the Supporting Information). Quantitative performance at this level is compliant with the EU legal requirements (Regulation EC 1829/2003[2]) and the performance requirements for GMO detection methods as specified by the European Network of GMO Laboratories.[31]

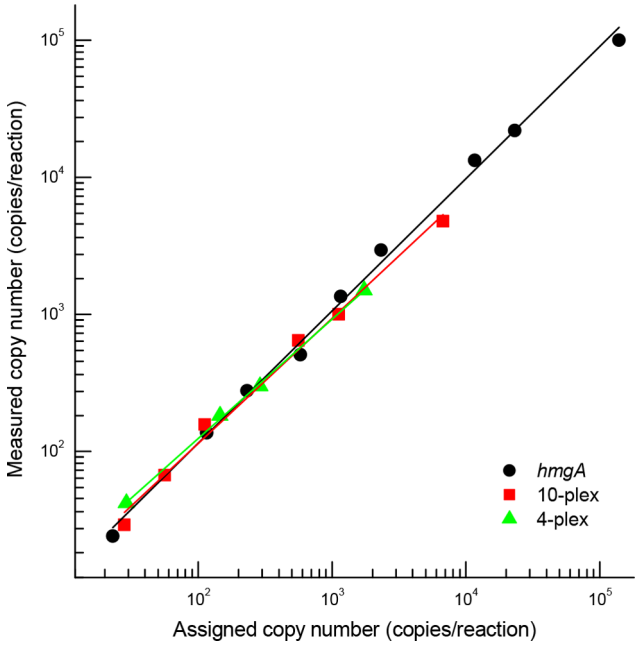

Figure 1. Dynamic range and correlation between measured copy number with the two multiplexes and assigned copy number on a mixture of equal amounts of each of the 12 maize events (as described in the Experimental Section). Each data point represents the average of three independent experiments with four replicates. Linear response for GM copy number mesured with the 4-plex (green triangles) is illustrated by the green line, linear response for GM copy number measured with the 10-plex (red squares) is illustrated by red line and linear response for maize reference gene copy numbers measured with *hmgA* in the 4-plex and in the 10-plex (black circles) is illustrated by black line. R^2 values originate from linear regression over the linear values and were 0.9898, 0.9943 for transgenes in 4-plex and 10-plex, respectively, and 0.9965 for *hmgA*.

Repeatability. To test whether both multiplex assays produce repeatable results, the performance was assessed on the level of target copies per reaction. Data from the experiments performed for aLOQ and dynamic range determination were used. All along the quantitative dynamic range, the RSD of the determined transgene copies in both multiplex sets remained below the threshold for acceptance for quantitative methods (RSD < 25%[31]) (Tables S-6−S-8 and S-10 in the Supporting Information).

Sensitivity. To assess the sensitivity, the absolute limit of detection (aLOD) was determined. The aLOD is the lowest target copy number in a sample that can be reliably detected but not necessarily quantified.[31] The aLOD was determined as the lowest copy number value for which all replicates were scored as positive. There does not yet exist a consensus on the number of positive droplets that are required to score a ddPCR replicate as positive, but 2, 3, and 5 droplets are commonly used thresholds. In this study, a threshold of 3 positive droplets was applied for scoring a replicate as positive. The aLOD was estimated to be 10 and 24 copies for the *hmgA* in the 4-plex and 10-plex, respectively, and 7 and 17 copies for the transgenes in the 4-plex and 10-plex, respectively. These results are compliant with the performance requirements for GMO testing methods in the EU[31] (i.e., aLOD < 25 copies).

Specificity. The specificity of the multiplex ddPCR assays was verified on the samples of unauthorized maize lines, nontransgenic maize, and other plant species (transgenic and nontransgenic) listed in Table 2. The false positive rate in dPCR could be presented in two ways: (a) as the whole

Table 2. False-Positive Rates Observed with ddPCR

sample	% of positive droplets for transgenes in 4-plex[a]	% of positive droplets for transgenes in 10-plex[a]
Bt176 nonauthorized GM maize	0	0.0038
DP98140 nonauthorized GM maize	0	0
MON87460 nonauthorized GM maize	0.0042	0
nontransgenic sweet corn	0	0.0034
nontransgenic maize seeds	0.0048	0
MON89788 soybean	0.0079	0.0042
MON40-3-2 soybean	0	0
GT73 rapeseed	0	0
nontransgenic rape seeds	0.0076	0
LL62 rice	0	0
nontransgenic rice grains	0	0
nontransgenic linseed	0	0
water	0.002	0

[a]Droplets from two replicates were considered for calculation.

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replicate or (b) as per partition (droplet). Acceptance values of false negative rates ≤5% are widely adopted[33] and a false positive rate below 5% of the replicates (wells) is a translation of this threshold. These are also the thresholds proposed in a recently published guidance document.[34] In present experiments, a false-positive rate of 0% was observed over all replicates (Table S-11 in the Supporting Information), showing a good specificity of the assays. When the false positive rate was assessed over droplets in each replicate, the observed false positive rate was below 0.015% (data not shown). The latter satisfies the acceptance criterion for a false positive rate per partition set to <0.2% for dPCR.[34] The percentage of positive droplets for transgenes in both replicates of a sample is presented in Table 2. Detailed results of false-positive rates are presented in Table S-11 in the Supporting Information. In addition, for all experiments performed in this study, the no-template-controls (NTC) were negative.

Fitness for Purpose. Both proficiency test samples and certified reference materials were used to assess the closeness of agreement between the average value obtained with the multiplex ddPCR method and the accepted reference values of these materials.[31] Thirteen proficiency test samples containing a variety of currently EU authorized maize events were analyzed and for all of these ddPCR results deviated less that 25% from the robust mean value (Table S-12 in the Supporting Information). In proficiency tests, each result gets assigned a *Z*-score value, indicating the statistical distance from the mean value of all results. All values where $|Z| < 2$ pass the proficiency test. A theoretical *Z*-score were calculated to assess whether the results from multiplex ddPCR would have passed the proficiency test. In all 13 cases $|Z| < 2$, therefore the results would have passed the test. Four samples from our routine diagnostic activity were included to test different matrixes. These samples have a high concentration of GM maize. The measured values of ddPCR were compared to results obtained earlier with standard curve based qPCR. In all four cases were the ddPCR values within ±25% of the qPCR values. To compare measured ddPCR values to accepted reference value or certified values of CRMs, the mass/mass concentration were converted to copy number ratio. The conversion to GM% in mass fraction was done according to EURL Technical Guidance on implementation of Regulation 619/2011.[19] A conversion factor "GM % in DNA copy number ratio = 50 % [GM% in mass fraction]" is used for crops hemizygous for an event-specific insert (e.g., hemizygous GM maize), while the conversion factor "GM % in DNA copy number ratio = 100 % [GM % in mass fraction]" is used for crops homozygous for an event-specific insert (e.g., homozygous GM soybean). This factor is not a precise value applicable to each sample, but rather a pragmatic approach for harmonization among laboratories in the EU. Because of specific seed composition in maize, the actual conversion factors are higher or lower, based on the parental origin of GM plant and the tissue in question.[3] Nevertheless, over the entire dynamic range the values measured with ddPCR deviated <25% from the certified values (Table S-13 in the Supporting Information).

Robustness. Robustness is defined as a measure for the capacity of a method to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.[31] Within the EU, a GMO detection method is deemed robust, if despite varied parameters the determined analyte copy number does not change more than 30%.[31] Six different modifications to the original procedure were tested experimentally, including different annealing temperatures, different PCR cyclers, different Mastermix, and different pipetting techniques. When comparing the overall GM % determined by both multiplex assays together, none of the modifications caused a change in result >30% for both samples, indicating that the difference may be the result of increased variability between replicates. A significant effect of PCR cycler and multichannel pipetting was observed in only one of two samples (Table 3). A detailed look at the results, comparing the measured copy numbers for individual assays can confirm that the changes of the procedure affected the results randomly (Table S-14 in the Supporting Information). When the results of the experiments obtained with the original protocol were compared to results obtained with modified protocols as one group, the RSD of the determined copy number did not exceed the threshold of 25% at concentrations ≥ LOQ (data not shown). Thus, the overall robustness of both assays was shown to be acceptable.

Table 3. Robustness of the Multiplex Assays Tested by Introducing Small Changes to Original Protocol Presented As Bias % of Determined GM Content Compared to Original Protocol

	bias % of GM content		
changes introduced	DNA mix 60×	DNA mix 120×	average
annealing at 61 °C	−5.5	6.4	1.4
annealing at 59 °C	−20.0	−21.5	−19.9
different Mastermix	−8.7	22.4	7.8
different PCR cycler	38.6	−1.4	20
multichannel pipetting to cartridge	−5.1	39.5	18.2
automatic pipetting to plate	−9.6	10.9	1.6

Practicability, Applicability, and Impact. Prior to introduction of new technology in a laboratory, practicability for its daily use has to be verified.[35] To benchmark the multiplex ddPCR for quantification of 12 EU authorized GM maize lines against existing qPCR methods, calculations were made, using information on overall costs of performed analysis (personnel, consumables, indirect costs, etc.; Table 4). Routine sample analysis with qPCR consists of several different steps, depending on the customer's order. Usually the testing includes an initial screening analysis, identification of GM lines on the basis of positive screening results, and final quantification of specific GM lines identified in the sample. Genetically modified botanical impurities, such as presence of some GM soybean (the impurity) in a maize gluten (the ingredient) are commonly detected.[5] These will typically interfere with screening approaches and complicate the interpretation, e.g., giving the impression that the sample is likely to contain GM maize. Direct application of the presented multiplex ddPCR assays will emend the interpretation problem with respect to EU authorized GM maize events. However, screening could aid to uncover that a sample contains a botanical impurity or a GM maize event not authorized in the EU.[5] Thus, the choice to apply screening or not is also a matter of the scope of analysis. For the reasons given above, different combinations of methods were used in calculations for qPCR in combination with different numbers of samples analyzed simultaneously (1, 5 and 11). Multiplex ddPCR assays for quantification of 12 EU authorized GM maize lines were taken as a reference, to which

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Table 4. Practicability and Cost-Effectiveness of the Developed Multiplex Assays in Comparison to Existing qPCR Approaches[a]

Testing pipeline	Steps prior quantification	Number of tested samples	Estimated hands-on time (hours)	Working days until final result (days)	Relative final price per sample (%)
Direct quantification of twelve approved GM maize lines with 4-plex and 10-plex multiplex assays with ddPCR	/	1	3	0.8	100
		5	4	0.9	100
		11	5	1.0	100
Direct quantification of twelve approved GM maize lines in simplex reactions with qPCR	/	1	8	2.0	272[b]
		5	16	4.3	292[b]
		11	25	7.5	300[b]
Initial screening with qPCR, all samples negative	5x Splex SE	1	2	0.5	83
		5	6	1.3	105[b]
		11	8	1.5	105[b]
Initial screening with qPCR, all samples negative	Pplex SE	1	1	0.4	31
		5	3	0.6	39
		11	5	1.0	39[b]
Initial screening, identification of six GM maize lines, quantification of one GM maize line with qPCR	Pplex SE, 6 specific lines	1	8	1.5	175[b]
		5	14	2.8	167[b]
		11	20	4.4	159[b]
Direct identification of twelve approved GM maize lines, quantification of two GM maize lines with qPCR	12 specific lines	1	10	1.8	226[b]
		5	14	3.1	195[b]
		11	18	4.8	185[b]
Initial screening with qPCR and quantification of twelve approved GM maize lines with 4-plex and 10-plex multiplex assays with ddPCR	Pplex SE	1	4	1.1	131[b]
		5	7	1.5	139[b]
		11	10	2.0	139[b]

[a]Testing pipeline, different scenarios of possible procedures for testing a diagnostic sample are taken into account; ddPCR, droplet digital PCR; qPCR, quantitative real-time PCR with standard curve; 5x Splex SE, screening phase using five simplex qPCRs to determine the presence of P-35S, T-nos, ctp2-cp4-epsps, bar, and pat elements; Pplex SE, screening phase using pentaplex assay to determine the presence of P-35S, T-nos, ctp2-cp4-epsps, bar, and pat elements.[9] Working days until final result, hands-on time and time needed for PCR cycling was considered. An 8-h working day was used for calculation. Relative final price per sample, ddPCR testing for 1, 5, or 11 samples with 4- and 10-plex assays was taken as a reference. For purpose of comparison the relative value of 100 was set to these setups. Values shaded with the same color should be compared, as they correspond to the equal number of tested samples. [b]More than one 96-well reaction plate is needed to perform all the reactions.

all other approaches were compared (Table 4). As a rule of thumb, the cost per sample is reduced by approximately 50% in the case of 5 samples and by approximately 60% in the case of 11 samples, independently of analysis strategy (data not shown). Only relative values are presented, as the costs for specific consumables, personnel, and other costs vary between laboratories and countries. Additionally, the working days until final result were calculated to visualize the influence of number of runs needed in some testing pipelines. The calculations demonstrate that qPCR testing is more cost efficient than multiplex ddPCR only when all samples are negative and analyzed with pentaplex qualitative qPCR screening[9] or when a single sample analyzed with simplex qualitative qPCR screening[8] yielded a negative result. Our experience is that GMOs are detected in approximately 50% of the analyzed food/feed samples, but the ratio of positive samples can of course vary from laboratory to laboratory. A common scenario is that an initial screening is followed by qualitative identification of 6 events and finally quantification of one of these. This scenario is 60−70% more costly than direct quantification with the multiplex ddPCR approach. As pentaplex screening of negative samples is highly cost efficient, an interesting approach could be to combine an initial pentaplex qPCR screening and use multiplex ddPCR to quantify the positive samples. As expected, the use of standard curve based singleplex qPCR directly is the most costly approach in the comparison. Unlike for qPCR, the cost of Mastermix is a large contributor to the cost of ddPCR, as can be read indirectly out of Table 4. Digital droplet PCR is a relatively new technology. The costs of Mastermix can be expected to decrease when the technology will gain volume and if new suppliers enter the market. This could further improve the cost efficiency of ddPCR. It has previously been demonstrated that ddPCR performs comparably to qPCR in duplex reactions for quantification of single events.[24] Although not reflected in the costs, the total analysis time is worth considering. The multiplex ddPCR analysis of as much as 11 samples (one full 96 well plate) can be finished in 1 day, whereas in cases other than pentaplex screening of negative samples, from 2 to almost 8 working days are needed (Table 4). The present study demonstrates that increasing the multiplexing using ddPCR is highly cost-effective. Therefore, it can be concluded that quantification of currently EU authorized GM maize lines in routine samples using multiplex ddPCR is

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practical for GMO laboratories, with better throughput and cost-effectiveness than qPCR.

Extension of Multiplex Assays. There is a high possibility of authorization of new events in the future; therefore, it is important that it will be possible to add additional events to the multiplexes. The 10-plex system is already close to the limit, as the overall concentration of the probes in the reaction is much higher than recommended for the simplex reaction. This results in a high fluorescence value of negative droplets and makes the difference between negative and positive droplets smaller. With potential rain, the threshold setting could be rather challenging. Thus, the 10-plex could probably not be extended a lot, but the 4-plex assay still has some room left for extensions and is more appropriate for addition of future authorized events. In the process of manuscript revision, another GM maize event (MON87460) was authorized in the EU. Preliminary tests were performed, adding the event to the 4-plex resulting in a 5-plex reaction. All of the testing combinations were comparable to the equivalent assays (data not shown) and were within the limits of acceptance criteria.[31] Shortly, the 5-plex assay produced a comparable result on MON87460 DNA as simplex assay (bias of 7.8%). 5-plex and 4-plex assays on DNA which were used in the rLOQ 4-plex also produced comparable results (bias of 7.8%). The quantification of the DNA mixture of all four events targeted by the 5-plex assay produced a result comparable to the sum of quantifications of individual lines (bias of 6.1%). Although the full in-house validation was not performed with this 5-plex assay, it was shown that addition of new events to the multiplex system is possible in the case of new authorizations.

Summary. The purpose of this study was to take the ddPCR to a higher level by introducing a multiplex approach and evaluating its suitability and conformity to the strict minimum performance parameters in the field of GMO diagnostics. The applicability of ddPCR for GMO diagnostics has previously been investigated and demonstrated to be comparable to qPCR (reviewed in[36]). However, the multiplexing capabilities of ddPCR for GMO detection were previously only demonstrated as duplex amplification of transgene and endogene target.[24] The presently reported, newly developed multiplex assays, combining interlaboratory validated simplex qPCR assays, were implemented with only slight optimization of primer and probe concentrations. The multiplex assays achieved a wide dynamic range, with lower limit of quantification below 50 target copies per reaction and upper limit around 100 000 target copies. Both multiplex assays showed good sensitivity, suitable for GMO testing. All of the tested parameters comply with international recommendations[31,35] and are comparable to those reported for interlaboratory validated qPCR simplex assays. Quantitation using multiplex ddPCR appears to be highly robust. At least three factors contribute to this. First, each technical replicate consists of more than 10 000 individual PCRs. Second, the calculations are based on the distribution of discrete signals, which is a qualitative measure, and not signal intensity and Cq-values, which are quantitative measures. Third, the reference gene and the transgene target are analyzed in the same well and thus the pipetting errors associated with sample volumes will cancel in the calculation of GMO content. From the perspective of a diagnostic lab, one of the most important parameters of a method is its practicability. The characteristics of the ddPCR system enable quantification of GMO content in samples performed in much lower number of reactions, thus reducing the cost of analysis, and decreasing the uncertainty linked to dilution pipetting errors.[22] Comparison of cost-effectiveness in the case of routine GMO diagnostics of both the multiplex ddPCR assays and qPCR demonstrate an overall better cost efficiency of the first, whenever samples are positive for GM events and better cost efficiency of the latter, when the screening phase gives negative results. To be employed in routine testing, methods need to be properly validated through interlaboratory validations and verified during their introduction in laboratories to demonstrate their fitness for the purpose. The ddPCR performance demonstrated in the present study already verified the suitability of the method for testing routine samples. Collaborative trial validation, planned within the DECATHLON project (www.decathlon-project.eu), is expected to reinforce this conclusion. Nonetheless, it was not the purpose of this study to achieve better performance than qPCR, which is still a golden standard in GMO detection, but rather to show that new techniques based on PCR amplification are comparable in terms of performance parameters, but can outperform qPCR in terms of cost-effectiveness. The most cost efficient approach can be qPCR screening of all samples prior to multiplex ddPCR quantification of the screening positive samples or ddPCR alone of all samples. The best basis to decide is probably the expected GMO negative rate as experienced by the laboratory. To conclude, the presented ddPCR multiplex assays could enable more cost-effective GMO quantification, especially when the system is extended to include additional relevant plant species. Moreover, the principle of multiplexing presented here could be applied to any other domain where several targets can be quantified as a group.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01208.

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Notes

The authors declare no competing financial interest.

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