

# Optimization and Verification of Droplet Digital PCR Even-Specific Methods for the Quantification of GM Maize DAS1507 and NK603

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**Abstract** In recent years, digital polymerase chain reaction (dPCR), a new molecular biology technique, has been gaining in popularity. Among many other applications, this technique can also be used for the detection and quantification of genetically modified organisms (GMOs) in food and feed. It might replace the currently widely used real-time PCR method (qPCR), by overcoming problems related to the PCR inhibition and the requirement of certified reference materials to be used as a calibrant. In theory, validated qPCR methods can be easily transferred to the dPCR platform. However, optimization of the PCR conditions might be necessary. In this study, we report the transfer of two validated qPCR methods for quantification of maize DAS1507 and NK603 events to the droplet dPCR (ddPCR) platform. After some optimization, both methods have been verified according to the guidance of the European Network of GMO Laboratories (ENGL) on analytical method verification (ENGL working group on “Method Verification.” (2011) Verification of Analytical Methods for GMO Testing When Implementing Interlaboratory Validated Methods). Digital PCR methods performed equally or better than the qPCR methods. Optimized ddPCR methods confirm their suitability for GMO determination in food and feed.

**Keywords** Droplet digital PCR · GMO quantification · Food and feed · Method verification

## Introduction

Methods for detection and quantification of genetically modified organisms (GMOs) are routinely used by various laboratories (national reference laboratories, control laboratories,

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and research institutes). In the European Union (EU), the company applying for authorization of GMOs as food and feed must prove its safety for human and animal health and provide proper GM event-specific detection and quantification method. This method is validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) [1] as an integral part of the authorization process according to the Regulation (EC) No 1829/2003 [2].

Currently, all GMO detection and quantification methods submitted for validation to the EURL-GMFF are developed and optimized for the qPCR platform (<http://gmo-crl.jrc.ec.europa.eu/gmomethods/>) [3]. Therefore, validated qPCR methods are widely used in European control laboratories, which have to act according to the requirements of the ISO 17025 standard [4–7]. Nevertheless, qPCR also has certain drawbacks, for example, the significant impact of inhibitors present in processed or composite samples might hamper amplification efficiency and in consequence affect the analytical result. Another obstacle that may limit the use of qPCR for a wide range of unauthorized in EU GM events is the necessity for certified reference materials (CRMs) used as a calibrant.

Digital PCR as qPCR is based on the polymerase chain reaction principle and utilize fluorescently labelled probes or DNA intercalating dye. The significant difference and the novelty is that dPCR works by partitioning PCR mix into many individual small-volume PCR reactions. In chamber digital PCR, the reaction mix is divided on a dedicated array [8] of up to 20,000 partitions. In droplet digital PCR (ddPCR), up to 10 million droplets are generated out of the reaction mix with the use of a special kind of oil [9]. The PCR reaction takes place independently in each partition. There can be none, one, or a few copies of the target sequence present in each reaction unit. The positive and negative signals from each partition are counted by the software. The name of the technique comes from the digital signal of the obtained result—0 and 1 stand for absence and presence of the target sequence, respectively. Contrarily to qPCR, it is an end point analysis, but the large number of individual results obtained for each sample enables precise quantification with the use of statistical tools, namely, the binomial Poisson distribution [10]. However, it was shown that Poisson statistics measures a limited source of variability and the solution to this would be splitting samples into subsamples to get more results for analysis from one sample [11].

Digital PCR has many applications, e.g., copy number determination, gene expression analysis, and low copy detection. One of its promising applications is GMO quantification. This technology has several features that can make it more suitable for GMO detection than the currently most popular qPCR. Firstly, the analysis is absolute, which means that the GMO content is counted purely as a ratio of the number of target sequence copies to the number of the reference gene copies in the sample and there is no need to use the calibration curve for quantification. The obligation to use CRMs in qPCR can be a serious limitation. The availability of reference materials is limited and can be problematic in the case of less popular transformation events or unauthorized GMOs. CRMs for all authorized events must be available. Nevertheless, some of them are produced in a limited number of concentrations (e.g., 0 and 100% GMO) (<https://ec.europa.eu/jrc/en/reference-materials>, <https://www.aocs.org>).

There is an ongoing discussion on the units of measurement to express the quantitative result of GMO analysis. Most of the CRMs available on the market are certified for the mass fraction of a given transformation event and only a few are also certified for GM copy number per haploid genome equivalent (cp/HGE). According to the Regulation (EU) No 619/2011 [12], the certified value of the GMO content shall be given in mass fraction and, where available, in copy number per haploid genome equivalent. When quantifying GM food/feed using the dPCR, results are expressed in cp/HGE. Comparing this result to the results

expressed in mass fraction obtained by qPCR requires establishing a conversion factor which takes into account the genetic constitution of the CRMs used in qPCR.

Another common problem associated with the PCR analysis is the inhibition of the reaction. Inhibitors may originate from the original sample and be added as a result of the sample processing or the procedure used for DNA extraction [13]. In all kinds of PCRs, the presence of inhibitors contribute to the reduction of PCR efficiency. In qPCR, inhibition results in poorer amplification, improper amplification plot, and late threshold cycles, which can significantly impact the results of quantification. In dPCR, the analysis of each chamber/droplet is qualitative, so the impact of inhibitors in terms of PCR efficiency is not that significant. Moreover, the concentration of inhibitors in positive partitions is decreased. The reaction may be delayed but the final result is unchanged (0 and 1 results) and the quantification is based on the statistical assumptions [10].

Digital PCR being less prone to inhibitions is also expected to detect low copy number of the target sequence. The limit of detection (LOD) and the limit of quantification (LOQ) of ddPCR methods for GMO detection are lower than those in the case of qPCR [14]. That is why it can be useful for analysis of samples with low GMO admixtures.

Several methods of GMO detection have been recently assessed to be used on the dPCR platform [10, 15–17]. Many of them were based on the methods previously developed for qPCR. Digital PCR is a novel technique in the approach to quantification, but the basics of PCR remain unchanged. That is why transferring qPCR methods to the dPCR platform can be usually done with minor modifications resulting from the recommendations of the equipment producers. Sequences of primers and probes as well as the reaction temperature profile can be maintained unchanged. However, some reaction conditions may require optimization, the reason of which can be e.g. a dedicated mastermix (different from that in the original method) used in digital PCR. Duplexing and multiplexing of several events in one reaction are also possible with digital PCR [18].

The ISO 17025 standard [4] requires verification of the officially validated method in each laboratory to test the performance of the method in particular conditions. If fundamental changes have been introduced in the method, the laboratory should perform a full validation. In this paper, we describe the transfer of two qPCR methods for GM event quantification (DAS1507 and NK603) onto the ddPCR platform. Both methods are officially validated by EURL-GMFF and are routinely used for official control in national reference laboratories. Optimization of reaction conditions and method verification were performed according to the recommendation, “Verification of Analytical Methods For GMO Testing When Implementing Interlaboratory Validated Methods” [19].

## Materials and Methods

### DNA Extraction

The following CRMs were purchased from IRMM (Geel, Belgium): DAS1507 maize—ERM-BF418A, ERM-BF418B, ERM-BF418C, and ERM-BF418D; NK603 maize—ERM-BF415A, ERM-BF415B, ERM-BF415C, ERM-BF415D, and ERM-BF415E. For the specificity test, CRMs of different GM events were used: ERM-BF410dn (Roundup Ready Soy), ERM-BF413ek (MON810), ERM-BF416c (MON863), ERM-BF419a, and ERM-BF419b (H7-1) from IRMM, Belgium. Standards of leaf DNA from T25 event (100 and 0%),

MON531 cotton DNA (100 and 0%), and GT73 canola (100 and 0%) were purchased from AOCS (IL, USA). The DNA extraction from CRMs was performed with the use of NucleoSpin Food kit (Macherey Nagel). The DNA concentration at a wavelength of 260 nm and the 260/280 ratio were measured using a NanoPhotometer Pearl (Implen).

### Droplet Digital PCR Analysis

PCR analyses were performed with the QX200 ddPCR apparatus (Bio-Rad). The PCR methods used were based on the officially validated qPCR protocols for DAS1507 and NK603 maize quantification [20, 21] with some modifications. The simplex format of the PCR reaction was used. In the case of the NK603 method, the high-mobility group gene (*Hmg*) instead of the alcohol dehydrogenase gene (*Adh1*) gene was used as a reference gene assay. In the original DAS1507 method, the reference gene (*Hmg*) remained unchanged. The unification of the reference genes used in various methods can be useful for routine work in the laboratory. The remaining sequences of primers and probes were not changed (Table 1). Instead of that routinely used in qPCR polymerase, the ddPCR Supermix for probes (Bio-Rad) was used. According to the ddPCR manufacturer, the TAMRA quencher in probes was replaced with the BHQ quencher. Five microliters of the DNA solution was added to 20  $\mu$ l of the reaction mix. Twenty microliters of this mixture was transferred to the droplet generation cartridge; subsequently, 70  $\mu$ l of the droplet generation oil was added. Forty microliters of the emulsion of generated droplets was transferred to the PCR plate and reaction was performed in the T100 Thermal Cycler (Bio-Rad). The reaction temperature profiles were maintained as in the qPCR method. End point PCR reaction readout was completed using the QX200 Droplet Reader (Bio-Rad). The GMO content in the sample was calculated as the number of target copies versus the number of the reference gene copies in the sample with the QuantaSoft software (Bio-Rad) manually setting the threshold line.

### Optimization of the ddPCR Conditions

To achieve the required method performance parameters, some modifications in the reaction conditions were applied. As recommended by the equipment manufacturer, the concentration of primers was increased from 300 nM (DAS1507 target sequence and *Hmg* gene) or 150 nM (NK603 target sequence) to 400 nM. The concentration of probes was also increased from 180 to 250 nM [17, 22]. Various amounts of DNA in the reaction were also tested: 80 ng as an equivalent of standard concentration used in qPCR analyses performed in our laboratory and

**Table 1** Primer and probe sequences

Primer/probe	Sequence
NK603-F	5'-ATGAATGACCTCGAGTAAGCTTGTTAA-3'
NK603-R	5'-AAGAGATAACAGGATCCACTCAAACACT-3'
NK603-P	5'-[6FAM]TGGTACCACGCGACACACTTCCACTC[BHQ]-3'
MaiY-F (DAS1507)	5'-TAGTCTTCGGCCAGAATGG-3'
MaiY-R (DAS1507)	5'-CTTTGCCAAGATCAAGCG-3'
MaiY-P (DAS1507)	5'-[6FAM]TAACTCAAGGCCCTCACTCCG[BHQ]-3'
ZM1-F ( <i>Hmg</i> )	5'-TTGGACTAGAAATCTCGTGCTGA-3'
ZM1-R ( <i>Hmg</i> )	5'-GCTACATAGGGAGCCTTGTCT-3'
ZM1-P ( <i>Hmg</i> )	5'-[6FAM]CAATCCACACAAACGCACGCGTA[BHQ]-3'

160 ng. In the case of NK603 quantification, the amount of 320 ng DNA was tested additionally. The impact of the enzymatic digestion of DNA on the PCR was also examined. The chosen restriction enzymes do not cut within the amplicon sequences. The DNA solution was digested with HindIII (DAS1507) or EcoRI (NK603) in 37 °C for 1 h before the PCR run. HindIII was inactivated in 80 °C for 20 min, and EcoRI in 65 °C for 20 min. Finally, the number of the PCR cycles was increased from 45 to 50.

## Method Verification

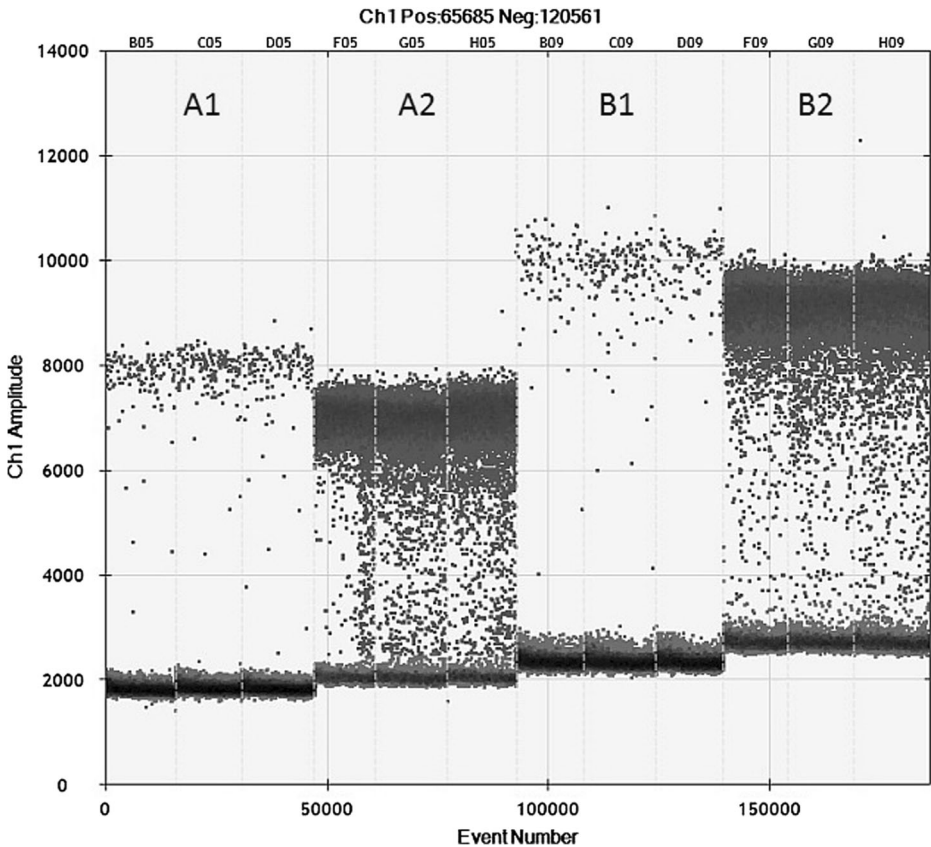
Methods were verified according to the requirements defined in the document “Verification of Analytical Methods for GMO Testing When Implementing Interlaboratory Validated Method” [19]. Critical parameters were assessed: dynamic range, trueness, precision, LOD, and LOQ. Additionally, the specificity of both methods has been investigated. The method specificity was checked on several DNA samples extracted from GM maize events (T25, MON863, MON810) and various species: soybean, cotton, sugar beet, and canola. Moreover, equal amounts of DNA samples of the three GM maize samples were mixed with positive control (NK603 or TC1507) to check if the method is sensitive enough to detect the target sequence among other DNA samples.

## Results and Discussion

### Optimization of the ddPCR Conditions

The aim of the work was to transfer officially validated methods of DAS1507 and NK603 GM maize quantification from the qPCR to the ddPCR platform. It was decided to change the reference gene for NK603 quantification from *Adh1* that was in the original method into the *Hmg* gene. It is the way to harmonize methods of GMO detection which is a consequence of the modularity approach that was proposed previously for GMO analyses [1, 23]. Firstly, the optimization of the reaction conditions was performed. As the outcome of the ddPCR analysis, the software shows a plot of the amplitude of the droplet fluorescence for each analyzed sample. The cluster of negative droplets showing basic fluorescence should be clearly separated from the cluster of positive droplets with a higher fluorescence. A high number of droplets with an intermediate fluorescence (the “rain effect”) can be problematic for quantification, because it is hard to assess if they should be counted as positive or negative signals. It makes the threshold setup difficult. Some amount of “rain” is acceptable and does not have a serious impact on the result. Nevertheless, this effect should be avoided and optimization of reaction conditions is a good way to reduce the rain effect.

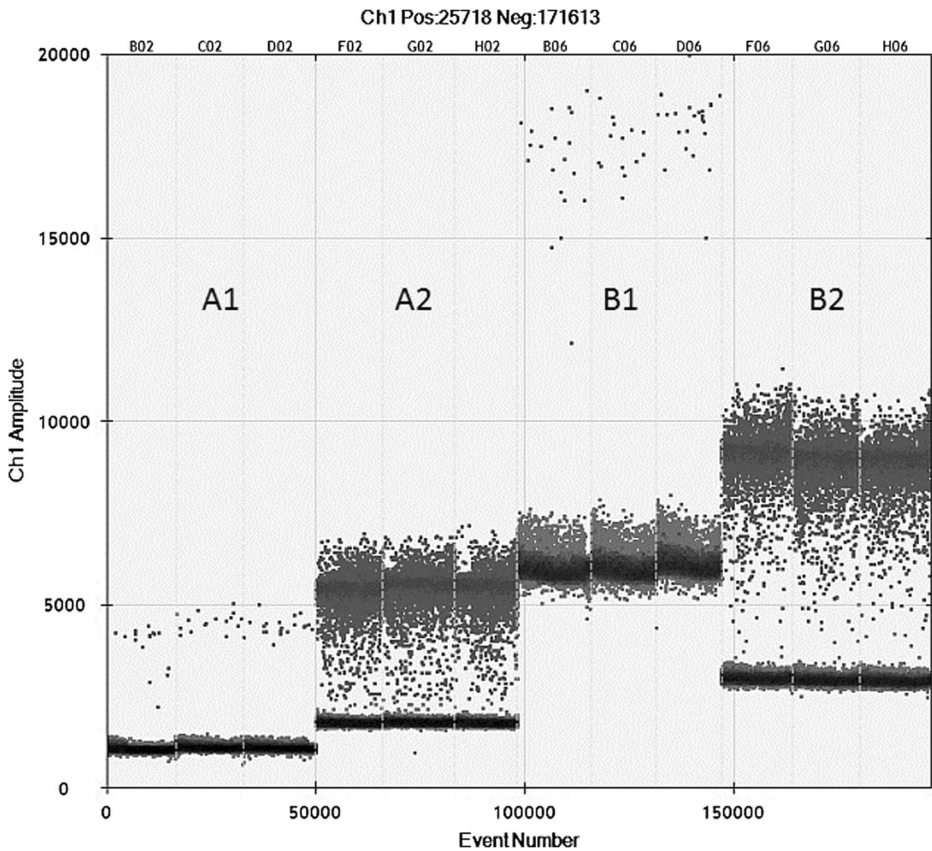
The manufacturer of the ddPCR equipment recommends the use of higher primer and probe concentrations than are routinely used in qPCR analyses. The increased concentration of primers and probes did not have any significant effect on the rain reduction and quantification results. Nevertheless, the higher (400 nM for primers and 250 nM for probes) concentrations of oligonucleotides in the reaction caused a higher fluorescence of positive droplets which means better separation of the clusters. This effect was observed in both methods, but in the case of the NK603 method the shift of fluorescence was higher, probably because the change of oligonucleotide concentration was larger (Figs. 1 and 2). In subsequent analyses for method verification, we decided to keep the original concentrations of oligonucleotides (300 nM for



**Fig. 1** The impact of primer and probe concentrations on DAS1507 method performance. A, 300 nM primers and 180 nM probes; B, 400 nM primers and 250 nM probes. 1, GM target sequence; 2, *Hmg* reference gene sequence (1% w/w CRM)

primers and 180 nM for probes) in the DAS1507 quantification method and switch to higher concentrations (400 nM for primers and 250 nM for probes) for NK603 quantification. An optimization of primer concentrations for DAS1507 and the *Hmg* gene have previously been verified and our findings confirm these results. The concentration of primers at the level of 300 nM was sufficient to achieve the acceptable resolution between positive and negative droplets [11].

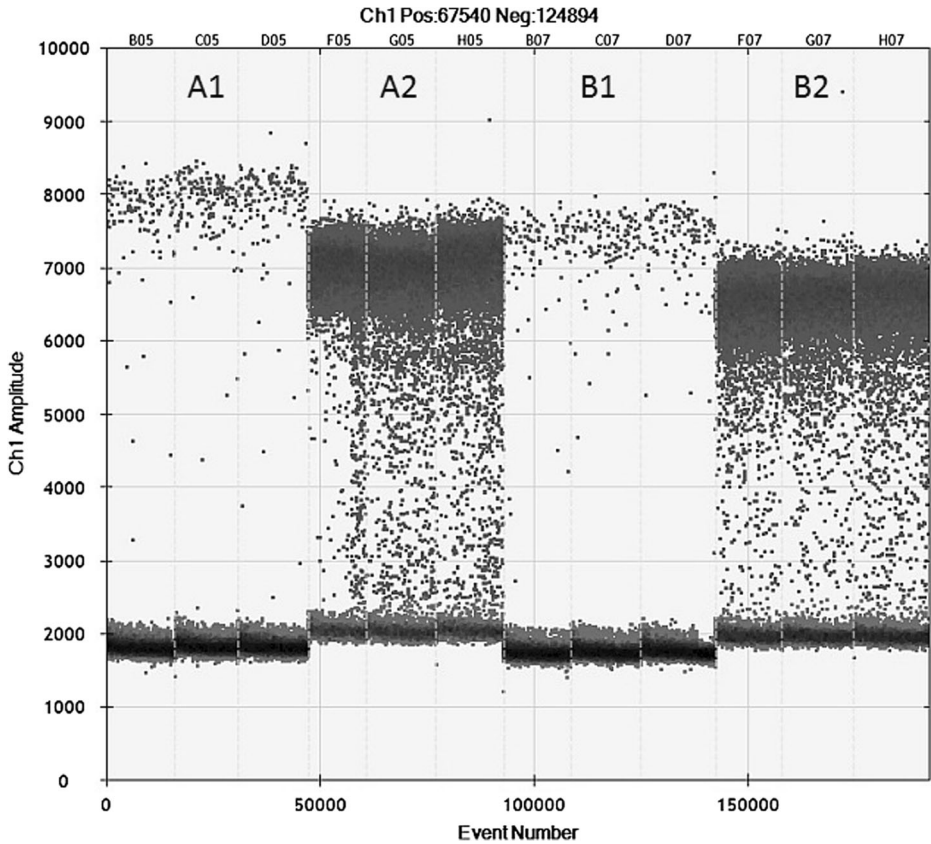
As the second part of the method optimization, the impact of increased DNA concentration on the method performance was evaluated. In qPCR analyses, we routinely use 100 ng DNA in 25  $\mu$ l reaction. In droplet digital PCR, we used 80 ng of DNA in 20  $\mu$ l reaction to obtain the same DNA concentration as that in qPCR analysis. We also tested the impact of increased DNA concentration (160 ng per reaction) for both methods. For the DAS1507 method, the rain effect was slightly bigger in the case of higher DNA input (Fig. 3) but this could be expected, because twice as much of the target sequence copies was present in the reaction. The concentration of DNA extracted from CRMs was assessed on the basis of UV absorbance and it can be biased by sample contaminations. What is more, the UV absorbance measurement does not discriminate between fragmented and not degraded DNA. That is why the spectrophotometric measurement should be treated as an approximation of DNA



**Fig. 2** The impact of primer and probe concentrations on NK603 method performance. A, 150 nM primers/50 nM probe (NK603) and 300 nM primers/180 nM probe (Hmg); B, 400 primers/250 nM probe. 1, GM target sequence; 2, *Hmg* reference gene sequence (1% w/w CRM)

concentration. The maximal number of target DNA copies in the ddPCR reaction should be 100,000–120,000 as recommended by the manufacturer [24]. A higher concentration could be problematic, because too many positive signals would negatively affect Poisson statistics calculation. Taking into account that the maize haploid genome mass is 2725 pg [25], 80 ng of maize DNA contains 29,358 genome copies and 160 ng, twice that much (58,715 copies). The measured number of copies per 20  $\mu$ l reaction in DAS1507 analysis is about 25,000 of *Hmg* gene (about 86% of the theoretical number of copies) when 80 ng of DNA is used and about 45,000 *Hmg* copies (77% of the theoretical number of copies) when 160 ng of DNA is used. This indicates that, in the case of higher DNA concentrations, analysis is less accurate which may be caused by the too high number of the positive signals to be accurately counted by the Poisson statistics. Another issue is that the highest recommended input of not digested DNA in PCR is 66 ng. In our analyses, 80 ng still performed well, but 160 ng seems to be an overload. Nevertheless, higher DNA concentrations do not have a significant impact on the analysis results. In subsequent analyses, we used 80 ng of DNA per reaction.

The quantification of the DNA concentration based on the UV absorbance can be seriously biased. In many cases, the presence of the different compounds absorbing UV light in the DNA solution results in the overestimation of the DNA concentration. NK603 analysis



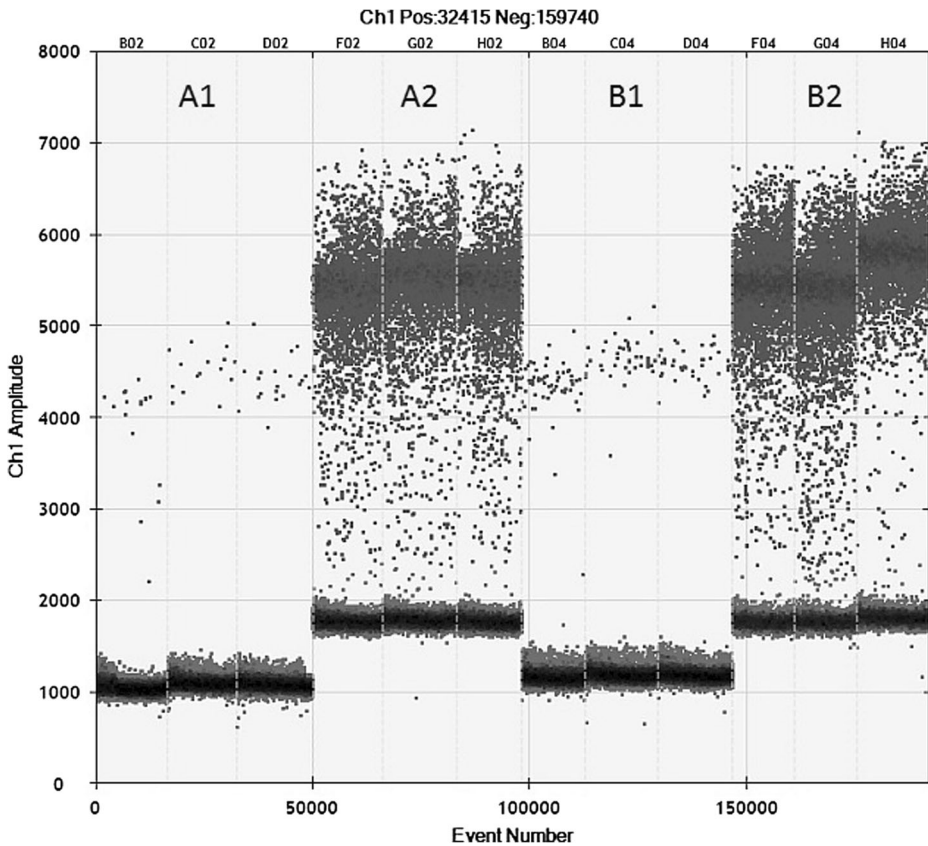
**Fig. 3** The impact of the amount of DNA on DAS1507 method performance. A, 80 ng; B, 160 ng. 1, GM target sequence; 2, *Hmg* reference gene sequence (1% w/w CRM)

resulted in five times less positive droplets for the reference gene system (about 6000) that was expected from the calculation of the number of target DNA copies added to the reaction (29,358). It was observed for all samples within the dynamic range. The explanation to this observation can be the inaccurate estimation of the DNA concentration by spectrophotometric measurement. The 260/280 nm ratio of the NK603 samples was about 2.12 which indicates the presence of some compounds that can absorb UV light and cause the overestimation of DNA concentration. Because in ddPCR the absolute number of target DNA copies in the sample which is close to the real value is indicated, it can be a valuable tool for DNA concentration determination. It was shown lately that ddPCR as a method for DNA concentration analysis performs equally or better than commonly used methods including qPCR and fluorimetry [26].

The low concentration of positive signals was observed in reduced number of results, especially in lower GMO concentrations as 0.1% NK603 w/w (data not shown). We decided to use 320 ng of DNA per well (on the basis of UV absorbance). The obtained result was about 24,000 measured copies of *Hmg* gene per well, as expected. Increasing the amount of DNA to 320 ng did not have any negative impact on the rain effect (Fig. 4). That is why we used this concentration for subsequent analyses.

The manufacturer of the equipment recommends digestion of genomic DNA outside the amplified sequence before the ddPCR. It should help to obtain accurate results of the analysis,





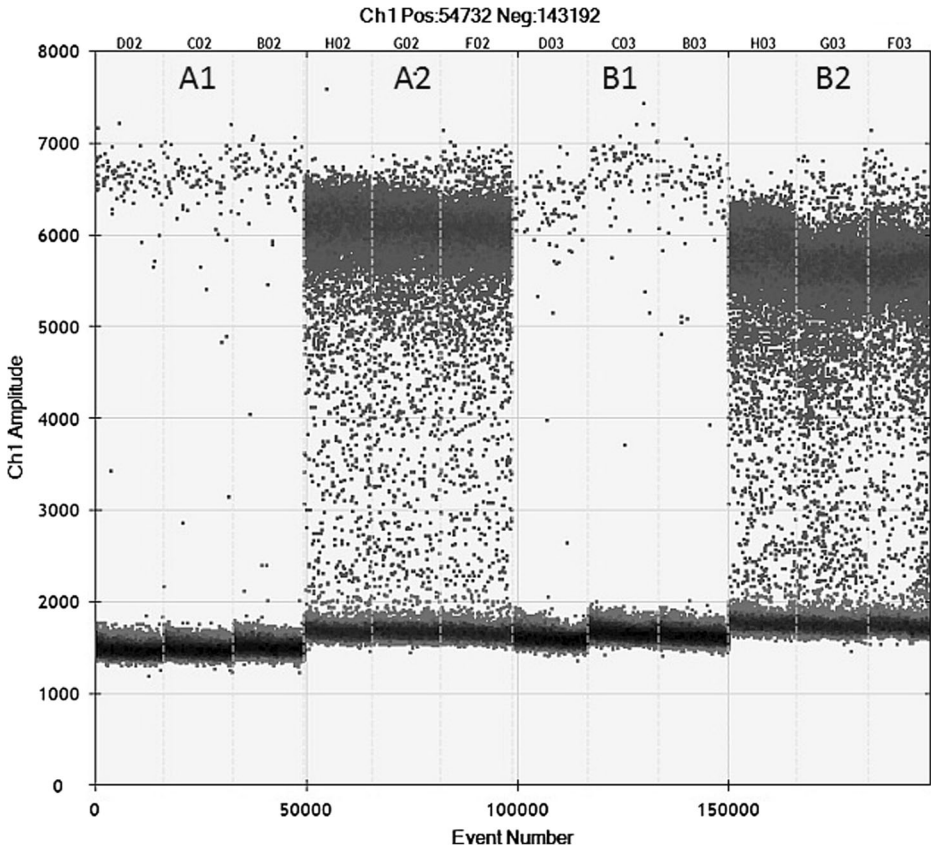
**Fig. 4** The impact of the amount of DNA on NK603 method performance. A, 80 ng; B, 320 ng. 1, GM target sequence; 2, *Hmg* reference gene sequence (1% w/w CRM)

because target sequences are evenly distributed between the small volumes of individual droplets. However, it was proved that in GMO quantification with ddPCR, the digestion of genomic DNA was not needed [14, 16]. Our findings are that digestion of DNA reduces slightly the rain effect (Fig. 5), but it does not have a significant impact on the final result of quantification. Therefore, in further optimization and verification experiments of both methods, undigested genomic DNA was used.

The next part of method optimization was testing the impact of the PCR cycle number. Increasing the number of PCR cycles from 45 to 50 was expected to reduce the rain effect which has been suggested by Lievens et al. [11], but actually it did not affect the analytical result (data not shown). Nevertheless, in subsequent analyses, we performed 50 cycles as this might be beneficial in routine GMO analysis of processed food and feed samples which might contain PCR inhibitors.

## Method Verification

The EURL-GMFF is responsible for validation of methods for GMO detection that are submitted with the dossier for GMO authorization in the EU. There is no legal requirements for the specific PCR platform to be used by the applicant, so some could expect that the dPCR



**Fig. 5** The impact of DNA digestion on DAS1507 method performance. A, digested; B, not digested. 1, GM target sequence; 2, *Hmg* reference gene sequence (1% w/w CRM)

method could be developed and submitted for validation. EURL-GMFF recommends verification of methods in the laboratories to check how laboratory conditions influence the performance of the method. When the officially validated method is not significantly changed in the laboratory, the full validation is not necessary [4]. The document published by ENGL is a guidance for proper GMO method verification in the laboratory [19]. We propose verification of GMO detection methods as a sufficient test for transferring of the qPCR method to the ddPCR platform.

## DAS1507

The dynamic range was tested on the basis of standard curves. Because in the ddPCR analysis the threshold cycle is not measured, classical standard curves cannot be created. The plot of the analysis result in copy numbers (cp/HGE) versus the certified GMO content in weight unit (w/w) served as a standard curve. Therefore, the slope measurement that is an indicator of the amplification efficiency was not applicable. Nevertheless, this parameter is not as significant in dPCR as in qPCR amplification, due to the fact that ddPCR results rely on the end point analysis. The reference materials of DAS1507 maize are certified for

the mass fraction unit only. Two standard curves were made for each method. Each level of GMO content (0.075, 0.1, 0.5, 1, 2, 5, and 10% *w/w*) was analyzed in five replicates. The linearity over the dynamic range 0.075–10% *w/w* was confirmed; the  $R^2$  coefficient of the curves was 0.99.

Trueness is defined as the closeness of agreement between the average value of a number of results and a certified value. Because CRMs certified for the copy number are not available, trueness for the DAS1507 method could not be assessed.

Precision defined as the relative repeatability standard deviation (RSDr) was calculated as the ratio of standard deviation to the mean value obtained from 16 results (PCR replications) from different PCR runs. RSDr was calculated for three GMO levels: at the expected LOQ, the highest from the dynamic range, and an intermediate value. RSDr should be below 25% as recommended by the EURL [1]. The obtained results fulfill this requirement (13.7% for the 10% GMO level and 16% for 1% GMO level). Precision at the 0.1% level is close to the acceptance limit—24.6% (Table 2).

The limit of detection is the lowest amount of GMO that can be reliably detected in the sample but not necessarily quantified. It was assessed as the lowest level of GMO content that gave positive result in 10 PCR replicates. The limit of quantification is the amount of GMO that can be reliably quantified in the sample. We determined it as the GMO level which gave results with RSD lower than 25% in 10 PCR replicates. The LOD for the DAS1507 quantification method was determined at the level of 0.05% *w/w* and LOQ at the level of 0.075% *w/w*. These levels are lower than those achieved during verification of the DAS1507 method for qPCR in our laboratory, which confirms the suitability of the ddPCR technology for quantification of genetically modified organisms. Additionally, the ddPCR format enables a direct quantification of DNA copies amplified in the sample and determination of absolute LOD and LOQ (number of DNA copies that can be reliably detected or quantified in a sample). The absolute LOD (aLOD) for the DAS1507 method was around 8 copies and for absolute LOQ (aLOQ), around 13 copies. These results are in line with previous findings obtained for other GMO quantification methods transferred to the ddPCR platform. Morriset et al. [14] reported aLOD at the level of 6 copies and aLOQ at the level of 18 copies for MON810.

Additionally, the specificity of both methods was tested. Recommendations for method verification indicate that for qPCR methods it is not necessary to check specificity if it was previously tested during method validation. However, specificity should be experimentally assessed if single elements like the brand of ready-to-use reaction mix, Taq polymerase, or the primer and probe concentrations are modified. We decided that the transfer of the qPCR method to the digital PCR platform and the use of different chemistry require assessment of the specificity. Different  $Mg^{2+}$  concentrations in the reaction buffer can affect the annealing temperature of primers and probes and result in unspecific amplification. For the specificity test, DNA from three GM maize events (T25, MON863, and MON810) and from Roundup Ready Soybean, MON531 cotton, H7-1 sugar beet, and GT73 canola was used. The

**Table 2** Result precision

GMO content ( <i>w/w</i> )	DAS1507 RSDr (%)	NK603 RSDr (%)
0.1%	24.6	23.2
1%	16	11.1
10%	13.7	–

reference materials for specificity tests were chosen based on the popularity of the transformation events representing different plant species. None of these matrices showed any amplification of the target sequence. Additionally, the mix of three maize events' DNA was spiked with DAS1507 DNA to check if the method enables detecting the target DNA in the mix of other sequences. A number of positive signals was detected in the DNA mixture. Taking these results into consideration, the specificity of the method had been confirmed.

### NK603

The standard curves for the NK603 quantification method verification were made from four GMO levels: 0.1, 0.5, 1, and 2% w/w NK603, which was determined by the availability of the CRMs. Two standard curves were created and each level was analyzed in five replicates. The dynamic range of the NK603 quantification method was determined between 0.1 and 2% w/w NK603. The  $R^2$  coefficient of standard curves was  $> 0.99$ .

Trueness was measured for the 2% w/w NK603 CRM, which is certified also for the copy number unit (cp/HGE). The measured value obtained from 16 replicates of the sample is 0.96% cp/HGE NK603, which is very close to the certified value 0.95% cp/HGE NK603. The RSDr values fall within the acceptable range (11.1% for 1% GMO level and 23.2% for the 0.1% GMO level—Table 2), but at the 0.1% level precision is close to the acceptance limit. The LOD of the method was determined at the level of 0.075% w/w NK603 and the limit of quantification was determined at the level of 0.1% w/w NK603. These results are in line with the validation data obtained in our laboratory for the qPCR method. It confirms that the ddPCR NK603 quantification can be successfully applied in routine analyses of plant material. Absolute LOD was obtained at the level of 9 DNA copies and aLOQ at the level of 12 copies.

The test for method specificity was performed in a similar way as that for the DAS1507 quantification method. The same matrices were used and the DNA mixture was spiked with DNA from 2% NK603 CRM. The obtained results confirmed the specificity of the method. The target sequence was detected in the mixture of other DNA sequences and negative samples did not show any amplification.

### Conclusions

In this work, we report the successful transfer of two officially validated qPCR methods (DAS1507 and NK603) for GMO quantification to the ddPCR platform. Some essential changes were introduced in the protocols but the primer and probe sequences and temperature profiles of the PCR reaction remained unchanged. The optimization of reaction conditions showed that the concentration of DNA and oligonucleotides in the PCR might need some adjustment. Higher primer and probe concentrations than those in the qPCR analysis are recommended to obtain better separation of positive and negative signals in end point PCR. Our experience show that measuring the DNA concentration with the UV spectrophotometer can underestimate the real DNA content. We recommend the initial measurement of the plant DNA copy number in a sample by digital PCR to assess the actual DNA input in the reaction. The verification of optimized ddPCR methods confirms their suitability for GMO detection and quantification in food and feed.

## Compliance with Ethical Standards

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

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