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SPECIAL ISSUE PAPER

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Cloned plasmid DNA fragments as calibrators for controlling GMOs: different real-time duplex quantitative PCR methods

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Abstract Analytical real-time PCR technology is a powerful tool for implementation of the GMO labeling regulations enforced in the EU. The quality of analytical measurement data obtained by quantitative real-time PCR depends on the correct use of calibrator and reference materials (RMs). For GMO methods of analysis, the choice of appropriate RMs is currently under debate. So far, genomic DNA solutions from certified reference materials (CRMs) are most often used as calibrators for GMO quantification by means of real-time PCR. However, due to some intrinsic features of these CRMs, errors may be expected in the estimations of DNA sequence quantities. In this paper, two new real-time PCR methods are presented for Roundup Ready soybean, in which two types of plasmid DNA fragments are used as calibrators. Single-target plasmids (STPs) diluted in a background of genomic DNA were used in the first method. Multiple-target plasmids (MTPs) containing both sequences in one molecule were used as calibrators for the second method. Both methods simultaneously detect a promoter 35S sequence as GMO-specific target and a *lectin* gene sequence as endogenous reference target in a duplex PCR. For the estimation of relative GMO percentages both "delta C_T" and "standard curve" approaches are tested. Delta C_T methods are based on direct comparison of measured C_T values of both the GMO-specific target and the endogenous target. Standard curve methods measure absolute amounts of target copies or haploid genome equivalents. A duplex delta C_T method with STP calibrators performed at least as well as a similar method with genomic DNA calibrators from commercial CRMs. Besides this, high quality results were obtained with a standard curve method using MTP calibrators. This paper demonstrates that plasmid DNA molecules containing either one or multiple target sequences form perfect alternative calibrators for GMO quantification and are especially suitable for duplex PCR reactions.
Electronic Supplementary Material Supplementary material is available for this article if you access the article at http://dx.doi.org/10.1007/s00216-003-2372-5. A link in the frame on the left on that page takes you directly to the supplementary material.

Keywords Reference materials · Cloned plasmid DNA standard · Real-time PCR · Duplex quantification · GMO

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Introduction

Analytical methods for GMOs test for the presence of the newly introduced DNA or expressed novel proteins encoded by the DNA. Numerous DNA- and protein-based methods have been developed in the last decade [1, 2, 3, 4]. For determining the level of GMO contamination, the DNA-based real-time polymerase chain reaction (rt-PCR) technique is most often used. PCR methods must be able to reliably quantify the GMO content in different matrices at various low levels down to 0.9% for authorized GMOs and 0.5% for nonapproved GMOs which received a favorable scientific risk assessment [5, 6, 7]. These thresholds represent the lowest levels of adventitious contamination considered by the EU. Threshold values are fixed on the level of single ingredients or plant species. This requires both a GMO-specific target and a species-specific target to be quantified absolutely. For this double quantification appropriate external calibration standards with well-known amounts of target are needed.

Two things are important for DNA-based quantification of GMOs: (1) the way in which the relative GMO percentage is determined and (2) the type of calibrators used. Two methods are available for deriving a relative percentage of GMO: the "delta C_T method" and the "standard curve method". In the delta C_T method both C_T val-

ues are directly compared to each other. The difference between the C_T values is used for directly calculating the GMO content. In the standard curve method a standard curve is set up for each target, expressed in absolute numbers of haploid genome copies. Comparison of copy numbers results in a percentage [4].

It is generally recognized that the use of reliable reference materials determines the quality of analytical measurement data, which in their turn form the basis for decision making. Ideally, a reference material should be internationally recognized and certified. Generally, a distinction is made between single-substance or pure RMs and matrix RMs [8].

Most GMO methods of analysis use genomic DNA (gDNA) solutions, derived from either powdery certified reference materials produced by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), or 100% pure GM material such as seeds, leaves or grains. The powder CRMs as such are matrix RMs. Due to the lack of pure RMs, solutions of DNA isolated from powder CRMs are used as calibrators. For genomic DNA, however, the measured analyte is not pure but is present in a background of plant cell material. For a number of real-time GMO quantification methods, calibration curves are set up with serial dilutions of one single DNA standard (e.g., 5% CRM) in water [9, 10, 11, 12] or in wild-type DNA [13, 14, 15, 16]. Block and Schwarz [17] call these "diluted genomic standards" and "mixed genomic standards", respectively. The first are expressed in absolute copy numbers or absolute amounts of DNA, the second in relative percentages. On the basis of these approaches a number of methods have been developed which detect both targets simultaneously in a duplex PCR [11, 18].

All methods mentioned so far are screening or construct-specific methods; transformation event-specific methods with gDNA as calibrators have been developed for Roundup Ready soybean [19], Mon810 maize [20], and Bt11 maize [21]. An alternative gDNA approach is to use CRMs with different, precisely known GMO percentages and to plot delta C_T values as a function of the concentration (in %). All delta C_T methods developed for GMOs so far are duplex [22, 23], one being in addition event-specific for Roundup Ready soybean [24]. Analytical methods for GMO detection are validated through international ring trials using CRMs, due to the lack of other material in Europe. However, a drawback of these RMs is their limited availability. When using these CRMs, one is limited not only in working range (0–5% GMO only) but also in the number of GM events. Their suitability for quantification of GMOs may also be doubted on the basis of biological and molecular composition. A lot-to-lot variation of the exact GMO content cannot be excluded since the GM and non-GM seeds or grains used to produce these CRMs can have different ploidy levels [25].

Because powder CRMs exhibit some major limitations, a high demand has arisen for alternative types of reference materials. Holck et al. [26] developed an event-specific method for Mon810 maize using single PCR amplicons as calibrators. Pardigol et al. [25] published a method using "hybrid amplicons", containing both transgene and reference gene targets in a tandem orientation on the same molecule. A few years ago our research group introduced cloned plasmid DNA standards [27]. Both an endogeneous and an event-specific target were separately cloned in a plasmid vector (single-target plasmid, STP). Dilution series were then made in water and relative quantification methods were developed based on two separate absolute quantifications. More recently Kuribara et al. [28] described a sort of "multiple-target plasmid (MTP) standard", containing different target sequences in one single vector. Next to those single- and multiple-target "pure" plasmids, plasmids mixed with genomic DNA have been tested by Block and Schwarz [17]. Because of the advantages of plasmid standards in terms of easy and cost-efficient production and distribution and long-term stability, they presented plasmid standards as "gold standards". They stated, however, that genomic DNA should be added to plasmid DNA as background in order to be usable as calibrator for the quantification of unknown genomic DNA samples.

In this paper, we present two new duplex real-time PCR methods using two types of plasmid DNA calibrators: (1) STPs containing only one target and (2) MTPs containing several sequences next to one another in the same vector. In a first duplex delta C_T method, we used in-house-developed STP standards diluted in genomic DNA and resulting in GMO percentages from 0.75 up to 50% (mixed STPs: STP-%). This delta C_T method will be compared to the conventional duplex delta C_T method with Fluka CRMs. A second method uses the pure MTP standards developed by the Japanese research group, with equal amounts of both the GMO and reference gene targets, each expressed in copy numbers (MTP-cp). The second method uses absolute numbers of copies and quantification is thus based on two separate calibration curves for both targets.

Methods and materials

Genomic DNA calibrators

The DNA calibrators used for each method and their concentrations are summarized in Table 1. Genomic DNA calibrators (G) are CRMs produced by the Institute of Reference Materials and Measurements (IRMM, Geel, Belgium) and purchased from Fluka (Sigma, Belgium). DNA was extracted from 20-mg amounts of CRMs containing 0.1, 0.5, 1, 2, and 5% Roundup Ready soybean (IRMM-410S), with the DNeasy Plant Mini Kit from Qiagen (Westburg, The Netherlands). DNA concentrations were measured with GeneQuant and 20 ng/μl solutions were made (50 ng in final PCR). Verification of the RR contents was done during the certification process, as described by Trapmann et al. [29].

Multiple-target plasmid DNA calibrators

As a second type of DNA calibrator we used plasmid reference molecules constructed by Kuribara et al. [28] and validated in an interlaboratory trial by Shindo et al. [30]. The plasmid contains a DNA sequence of a region specific for event 40-3-2 (Roundup Ready soybean), sequences of the *Cauliflower Mosaic Virus* promoter p35S and *Agrobacterium tumefaciens* nopaline synthase terminator T*nos* as well as a *lectin* gene sequence specific for soybean in one and the same vector. The product is linearized DNA,

Table 1 Types of calibrators used in this study, together with their concentrations. G=genomic DNA, STP=(mixtures of) single-target plasmid DNA, MTP=multiple-target plasmid DNA, C_T=threshold cycle number, cp=copies or number of haploid genome equivalents (final absolute amounts in the PCR tube)

Method	Calibration curve (s)	Type of calibrator	Concentration of calibrator	Calibration curve(s) in	Quantitative results in ....
G-%	1 (delta C_T)	G	0-0.1-0.5-1-2-5%	Table 2 Fig. 1a	Table 3
STP-%	1 (delta C_T)	STP	0.75-1.75-2.75-3.75-4.75-7.75-10-25-50% *lectin* 2E4 p35S: 150-350-550-750-950-1,550-2,050-5,050-1E4	Table 2 Fig. 1b	Table 3
MTP-cp	2 (cp)	MTP	*lectin* 20-125-1500-2E4-2.5E5 p35S: 20-125-1500-2E4-2.5E5	Table 2 Fig. 1c	Table 3

digested with a restriction enzyme and diluted with salmon sperm DNA [28]. Plasmid solutions containing 20, 125, 1,500, 20,000, and 250,000 copies (absolute amounts in 2.5 µl) were provided as one set (GM Soybean (RRS) Detection Plasmid Set) by Diagenode (Luik, Belgium). Calibration of the MTP standards was done by Kuribara et al. [28].

Single-target plasmid DNA calibrators

STP molecules diluted in genomic DNA were used as a third type of DNA calibrator. Two different STPs were constructed: one containing a 422-base-pair (bp) fragment of the p35S-plant border specific for the RRS transformation event [EMBL J308514, 31, 32] and one containing a 795-bp fragment of the soybean *lectin* endogeneous gene [GenBank K00821]. Generated PCR amplicons were separately cloned in pCR2.1 vectors using the TOPO TA Cloning kit (Invitrogen, Belgium). Clone analysis, plasmid DNA preparation, and concentration measurement were performed as described previously [27]. Taking into account the size of the plasmid and the molecular weight of ds-DNA (965 Mb weigh 1 pg, [20]), a mixture was made of the two plasmids in the ratio 2:1, containing 1E5 copies of the p35S junction fragment and 5E4 copies of the *lectin* fragment per 2.5 µl of DNA. This mixture was diluted in pure wild-type soybean genomic DNA extracted from CRM 410S-0, in such a way that for each calibrator approximately 50 ng is present in the final PCR. Dilution resulted in a series of STP standards containing 50, 25, 10, 7.75, 4.75, 3.75, 2.75, 1.75, and 0.75% of the event-specific junction fragment over the reference gene fragment. The total *lectin* level of 2E4 copies is thus based on plasmid plus gDNA diluent levels (Table 1).

Before using the STP standards as calibrators, the copy number levels were assessed in-house. The nine STP samples were analyzed as unknowns in real-time PCR experiments using the MTP DNA standards as calibrators. Absolute copy numbers were estimated for the nine STP-% samples in duplo and in two independent runs. Measurement of four replicates for each of the calibrators resulted in precise and true copy number determinations, with a mean relative standard deviation (RSD) of 11.6% and a mean error of 19% (data not shown).

Unknown samples

As unknown samples, in all three types of real-time PCR methods, the CRM series from IRMM was used (0, 0.1, 0.5, 1, 2, and 5% RRS). Different DNA solutions were extracted and measured as described above.

Duplex real-time PCR reactions

All products and reagents were purchased from Applied Biosystems (The Netherlands). Duplex reactions were carried out with the TaqMan GMO Soy 35S detection kit on the ABI Prism 7000 sequence detection system. The kit is specially designed for duplex amplification of a p35S target and a soybean *lectin* gene target in the same tube. Besides a negative control and a positive control (100% RRS), the kit contains Ampli*Taq* Gold polymerase and a master mix including $MgCl_2$, dNTPs, uracyl *N*-glycosylase (UNG), the passive reference dye ROX, and primers and probes for both target sequences. The p35S system's probe is 5′-FAM-labeled while the probe for *lectin* is 5′-VIC-labeled. The PCR master mix has been developed and optimized for duplex quantification of low concentrations of GMO such as the CRMs from Fluka. Nevertheless, the kit does not contain calibrators, leaving the choice of appropriate calibrators to the user. Details about primer and probe sequences, amplicon sizes, and reaction component concentrations are not provided within the kit.

Reactions were carried out in 96-well microtiter plates in a total volume of 25 µl, containing 22 µl master mix, 0.5 µl Ampli*Taq* Gold, and 2.5 µl DNA. All reactions in all runs were performed in duplicate. For each plate, a negative control and a positive control, both from the duplexing kit, together with a no-template control (NTC) were analyzed in duplo. Plates were closed with optical adhesive covers and spin off for 15 s at 12,000 rpm. After initial steps at 50 °C for 2 min and at 95 °C for 10 min, for activation of the UNG enzyme and the Ampli*Taq* Gold polymerase respectively, a two-step protocol of 95 °C for 15 s and 60 °C for 1 min was followed for 45 cycles.

Real-time PCR data analysis

After completion of the PCR a threshold of 0.2 was chosen and the baseline was fixed between cycles 3 and 15. Data were generated and analyzed with the ABI Prism 7000 SDS software. For estimation of raw copy numbers (method MTP-cp), calibration curves were set up separately for the *lectin* gene fragment and the p35S target by plotting C_T values versus the logarithm of the concentration in copy numbers. For the genomic DNA calibrators and the mixed STP calibrators, the differences between the C_T values of the p35S and the *lectin* sequence (delta C_T values) were calculated and plotted against the logarithm of the concentration in percentages (methods G-% and STP-%). For all methods three independent runs were performed. The precision was estimated by calculating standard deviations (SDV) and relative standard deviations (% RSD) or coefficients of variation (% CV) on the C_T values (MTP-cp method) or on the delta C_T values (G-% and STP-% methods). For the copy number method, relative GMO percentages of unknown samples were calculated by dividing the number of copies of the p35S by that of the *lectin* gene and multiplying by 100. For the delta C_T methods, a percentage was directly derived from the calculated delta C_T value. The accuracy of the quantitative results (%) was estimated through the SDV and % RSD values (precision) and the percentage of error or bias (trueness).

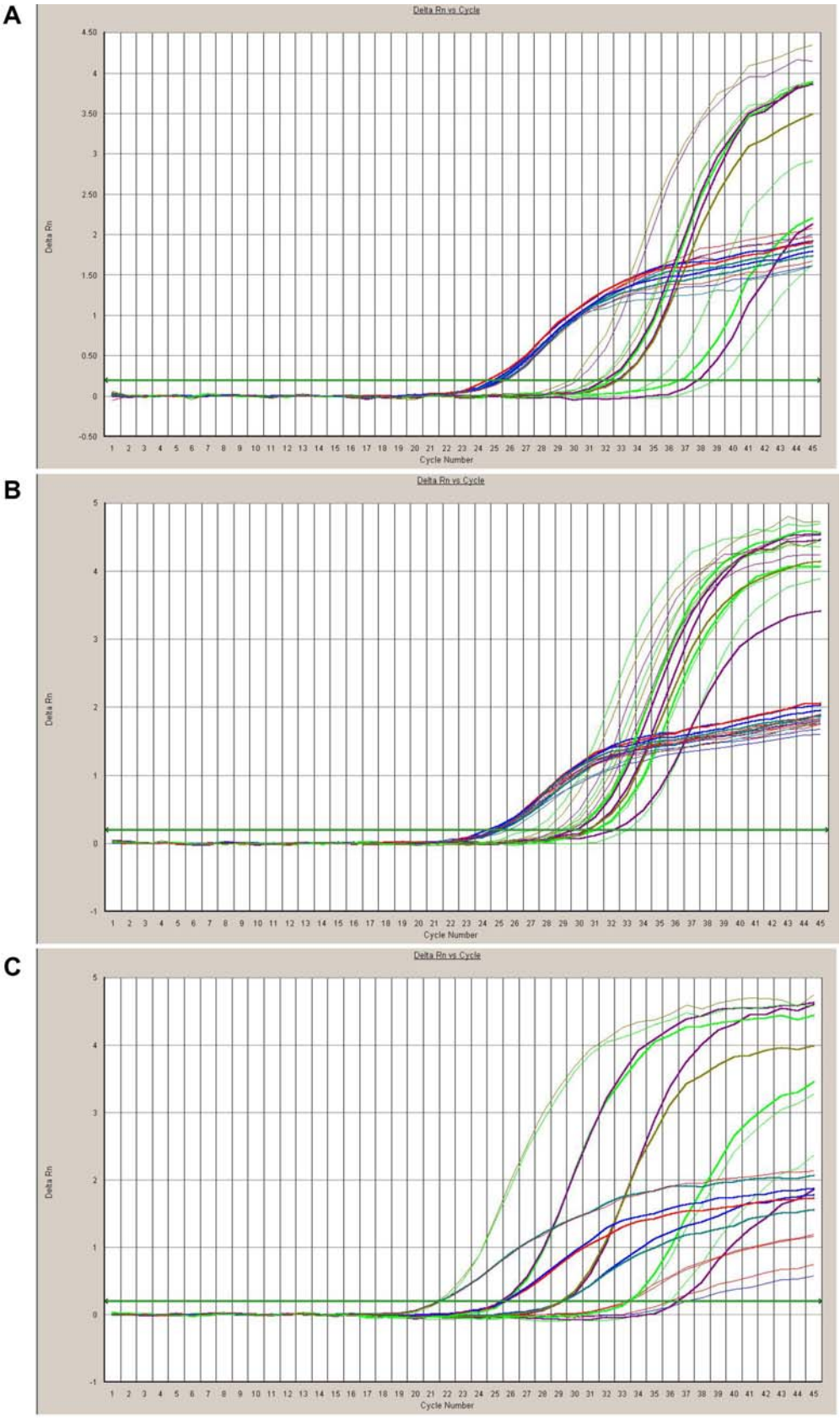

Fig. 1a–c Amplification plots of the p35S and the *lectin* targets for the different G-% standards shown in S1 (**a**), for the different STP-% standards shown in S2 (**b**), and for the different MTP-cp calibrators shown in S3 (**c**)

Results and discussion

Setup of calibration curves

In order to be able to compare genomic to plasmid DNA calibrators, delta C_T calibration curves were first set up with genomic DNA standards containing 0.1, 0.5, 1, 2, and 5% (w/w) of RRS. In three independent runs, C_T values for both the p35S and the *lectin* target were measured and delta C_T values calculated (see S1). These were plotted as a function of the GMO content (%). An example of the *lectin* and p35S amplification plots of the different G-% standards can be seen in Fig. 1a. The characteristics of the delta C_T curves are summarized in Table 2. The regression correlation coefficient R^2 demonstrates the degree of correlation between the concentrations of GMO (%) and the delta C_T values obtained after amplification of both targets. The commercial kit perfectly allows duplex amplification of low percentages of GMOs. However, the correlation coefficients of the resulting delta C_T curves are below the minimum acceptable value of 0.98 (0.96) in two of the three runs. With a mean intermediate precision RSD value of 4.76%, the measurements are highly repeatable (Table 2).

A similar delta C_T method was developed by diluting a mixture of two STPs containing a fragment of the *lectin* gene and the p35S/plant junction in genomic DNA from a wild-type soybean sample. Measured C_T and delta C_T values for the two replicates of the different STP calibrators in each of the three runs can be seen in S2. Typical amplification plots for this type of calibrator are shown in Fig. 1b. Table 2 reports the mean measured delta C_T values and characteristics of the resulting calibration curves. Within-run repeatability is slightly lower than for the G-% method, with a mean RSD value of 11.3%. However, in contrast to the G-% method, with mean delta C_T values from 4.69 to 10.03 and a small working range of 5–0.1% GMO, the delta C_T method with STP-% calibrators allows a wider working range. Reported delta C_T values range from 1.46 (50% GMO) to 7.59 (0.75%). The minimum acceptable regression correlation coefficient of 0.98 is obtained in all runs (Table 2).

Three independent runs were performed with the MTP standards containing 20, 125, 1,500, 2E4, and 2.5E5 copies of both the *lectin* gene and the p35S target. Measured signals (C_T values) for six replicates of each calibrator can be found in S3. For this method, C_T values for both targets are plotted against the absolute numbers of copies. Mean C_T values for both targets range between 21.52 (250,000 cp) and 35.85 (20 cp). Intermediate precision is very high, with mean RSD values of only 1.27% for the p35S system and 0.87% for the *lectin* system (Table 2). Figure 1c shows that for each MTP calibrator, the amplification plots of both the p35S and the *lectin* gene targets cross the threshold fluorescence of 0.2 at exactly the same point. This is as expected because the numbers of copies are exactly the same for both targets. Not only the C_T values but also the standard curves of the two PCR systems are perfectly matching in runs 1 and 2 and almost matching in run 3. This can be seen in the equivalence between the slopes, Y-intercepts, and R^2 values of the p35S and the *lectin* system not only within one run, but also between the three runs for the MTP-cp method (Table 2).

Quantification of GMO samples

In order to investigate the suitability of the different types of calibrators for relative quantification, a series of six samples with well-known GMO contents (in %) were analyzed as unknowns: 0, 0.1, 0.5, 1, 2, and 5% RRS. Table 3 shows the quantitative data obtained after all runs, for the three methods. For the delta C_T methods, unknown per-

Table 2 Summary of the main characteristics of the calibration curves obtained in the different runs for the three methods. Measured results and statistical data of the distinguished calibrators can be found in S1, S2, and S3 in the Electronic Supplementary Material for the three methods, respectively. *Optimal Y-intercept values are 6.644 for delta C_T methods (G-% and STP-%) and 40 for standard curve methods (MTP-cp), see also text. **Mean calculated values of delta C_T (G-% and STP-%) or C_T(MTP-cp) are given for the highest (first value) and the lowest (second value) calibrator points

Method	Run number	C_T	R2	Intercept	Deviation to optimal intercept (%)*	Slope	Efficiency	Devation to $E = 1$ (%)	Mean calculated values**	*Mean % RSD*
G-% (0.1–5)	1	Delta Ct	0.96	7.08	6.56	–3.35	0.99	1	4.87 – 10.20	*4.76*
	2	Delta Ct	0.99	6.98	5.06	–3.57	0.91	9	4.66 – 10.76	
	3	Delta Ct	0.96	6.66	0.24	–2.68	1.36	36	4.54 – 9.12	
STP-% (0.75–50)	1	Delta Ct	0.99	6.59	0.81	–3.26	1.03	3	1.21 – 7.03	*11.3*
	2	Delta Ct	0.98	7.31	10.02	–3.29	1.01	1	1.62 – 8.20	
	3	Delta Ct	0.98	6.95	4.61	–3.25	1.03	3	1.56 – 7.55	
MTP-cp (20–2.5E5)	1	p35S	1	40.86	2.15	–3.59	0.9	10	21.50 – 36.23	*1.27 (p35S)*
		lectin	1	40.99	2.48	–3.60	0.9	10	21.59 – 36.51	*0.87 (lectin)*
	2	p35S	0.99	40.31	0.78	–3.57	0.91	9	21.47 – 35.95	
		lectin	0.99	40.41	1.03	–3.57	0.91	9	21.68 – 36.26	
	3	p35S	0.99	39.12	2.2	–3.36	0.98	2	21.27 – 34.76	
		lectin	0.99	39.73	0.68	–3.42	0.96	4	21.59 – 35.39	

Table 3 Quantitative estimates of six samples analyzed as unknowns with the three types of calibrators. Given are the mean % RSD and % error values of six replicates over three runs, together with the overall mean imprecision and bias of all samples. Measured results and statistical data for the different samples can be seen in S4, S5, and S6 in the Electronic Supplementary Material for the three methods, respectively

Method	Calculated values	Run	0		0.1		0.5		1		2		5		Mean % RSD & % error (0-5%)	Mean % RSD & % error (0.5-5%)
	Mean % RRS	1	0.03	0.04	0.09	0.02	0.55	0.38	1.06	0.97	1.43	1.44	4.76	5.66		
		2	0.01	0	0.17	0.47	0.57	0.62	1.47	1.05	2.52	2.06	–	–		
		3	0.01	0.01	0.06	0.14	0.53	0.59	0.96	0.85	1.72	1.1	1.27	5.67		
G-%	Overall mean RRS %		0.02		0.19		0.54		1.06		1.71		4.34			
(0.1-5)	Mean % RSD		57.37		60.92		13.11		12.86		15.44		50.96		35.11	23.09
	Mean % error		1.59		86.67		12.67		12.67		24.17		17.4		25.86	16.73
	Mean % RRS	1	0.02	0.02	0.12	0.11	0.42	0.4	0.61	0.75	1.41	1.08	–	–		
		2	0.01	0.03	0.16	0.05	0.76	0.8	0.79	1.65	2.79	3.06	6.18	6.64		
		3	0.02	0.02	0.17	0.27	0.57	0.82	0.79	0.85	2.27	2.52	4.59	6.64		
	Overall mean RRS %		0.02		0.15		0.63		0.91		2.18		6.02			
STP-% (0.75-50)	Mean % RSD		16.67		37.63		10.79		23.2		10.86		15.48		19.11	15.08
	Mean % error		2		50		37.33		24		34.5		20.3		28.02	29.03
	Mean % RRS	1	0.02	0	0.17	0.18	0.72	–	1.45	0.86	2.2	1.29	1.88	3.15		
		2	0.01	0.03	0.15	0.05	0.64	0.67	0.67	1.32	2.13	2.32	4.43	4.73		
		3	0.01	0.02	0.12	0.19	0.39	0.54	0.53	0.57	1.47	1.61	2.91	4.14		
	Overall mean RRS %		0.02		0.15		0.62		0.9		1.84		3.54			
MTP-cp (20-2.5E5)	Mean % RSD		66.67		35.6		13.22		29.22		16.36		21.64		30.45	20.11
	Mean % error		1.67		46.67		27.33		20.33		15.67		29.13		2347	23.12

centages of GMO are directly derived from measured delta C_T values. Mean relative values of 0.02, 0.19, 0.54, 1.06, 1.71, and 4.34 were obtained with G-% calibrators (Table 3). Although relatively high mean values for RSD (35.11%) and error (25.86%) were obtained, the trueness and precision are still acceptable. These high values are mainly attributed to the 0.1% sample, the lowest GMO-positive sample analyzed as unknown (mean RSD of 60.92% and mean error of 86.67%). If the 0% and the 0.1% samples are excluded, the mean RSD and error values are much lower (23.09 and 16.73%, respectively, Table 3).

With STP-% calibrators, the mean quantitative results for 0, 0.1, 0.5, 1, 2, and 5% RRS are 0.02, 0.15, 0.63, 0.91, 2.18, and 6.02%, respectively (Table 3). These results are very precise (mean RSD of 19.11%) while again the mean bias is relatively high (28.02%). Also for this method, 0.1% RRS is the sample delivering the highest RSD (37.63%) and the highest error (50%). Errors were also higher than 30% for the 0.5% and 2% RRS samples (Table 3).

With the MTP calibrators (MTP-cp method), genomic DNA samples were quantified with a mean RSD of 30.45% and with errors varying largely from 2% to about 47% (mean error of 23.47%) (Table 3). The mean calculated concentrations are 0.02, 0.15, 0.62, 0.9, 1.84, and 3.54% RRS. As with the delta $C_T$methods (see above), the lowest unknown sample (0.1%) delivered unacceptable high imprecision (35.6% RSD) and bias (46.67% error).

For all methods, the measured C_T values and the calculated delta C_T values (G-% and STP-% methods) copy numbers of the p35S and *lectin* gene target (MTP-cp method), together with statistical data on the percentage results per run are reported in S4, S5, and S6, respectively.

Evaluation of different calibration standards

The three different types of calibrator standards tested in this work are compared and evaluated in terms of correlation coefficient, slope, and PCR amplification efficiency of the calibrator curves, precision (% RSD) of the C_T or delta C_T values, and precision (% RSD) and trueness (% error) of the quantitative results. Table 2 is a compilation of the calibration curve characteristics per method, while Table 3 summarizes the quantitative results. For GMO methods of analysis, the precision and trueness of the quantitative estimations are acceptable if values for RSD and error are not higher than 20% [33]. Correlation coefficients should be at least 0.98.

With regard to the calibration curves, the MTP method working with equal numbers of copies (MTP-cp) scores the best, followed by the two delta C_T methods. The MTP-cp calibrators have an excellent performance in real-time duplex PCR. For the calibration curves, correlation coefficients of at least 0.99 are always obtained as well as PCR amplification efficiencies of at least 90% (mean deviation of 7.3% from the ideal efficiency of 1 or 100%, Table 2). If working with absolute standard curves, plotting C_T values against absolute copy numbers, the Y-intercept is the number of cycles needed to amplify one single copy. Theoretically, in a PCR with 45 cycles, it is assumed that one copy has a C_T value of 40. In the case of delta C_T methods, where delta C_T values are plotted as a function of the relative GMO %, the Y-intercept reflects the difference in C_T values between the amplified endogenous target and the GMO target, for a 1% GMO-containing sample. Ideally, a 1:10 ratio should correspond to a difference in measured C_T of –3.322. This means that for a 1% GMO sample the ideal delta C_T is 6.644. In our MTP method, the mean deviation of the Y-intercept from the ideal value of 40 is only 1.5%. For the G-% and STP-% methods, the mean deviations are 3.95 and 5.15%, respectively (Table 2). Finally, the MTP-cp method has the highest precision for the different calibrator points (mean RSD of 1.07%, Table 2). Both delta C_T methods resulted in lower correlation coefficients and lower precision of the measurements. Both methods can however be considered as performing well.

A good calibration curve forms the basis for real-time PCR quantification but does not guarantee precise and accurate quantification. We tested the suitability of the different calibrator types by analyzing real GMO samples containing precisely known concentrations expressed as percentage RRS. It must be remarked that these unknown samples are equal to one of the calibrator sets used and as a consequence part of the measurements were carried out in a closed system. Generally, high values for % RSD and % error are reported. The highest precision was obtained with the STP-% method (RSD of 19.11%), while trueness was the highest for the MTP-cp calibrators (bias of 23.47%). Values varying from 20% up to 35% for single-lab imprecision and bias in quantitative real-time PCR data have also been reported in other studies, especially if concentrations at the 0.1% level are taken into account [13, 25]. As demonstrated in Table 3, precision and trueness are much higher (RSD and error generally below 25%) if the 0% and 0.1% samples are excluded. In summary, all three types of calibrators are suitable for the relative quantification of RRS.

Discussion

From Table 3 it can be concluded that both MTP calibrators and mixtures of STP calibrators with genomic DNA (STP-%) are fit for relative quantification of GMOs in a real-time duplex PCR. Duplex amplification of both transgenic DNA and endogenous reference DNA in the same tube has several advantages over simplex detection. Duplex assays are not subject to random differences in the reaction conditions from tube to tube, such as pipetting errors. They are less labor-intensive and less expensive than real-time simplex PCR reactions, since fewer reagents are needed for one run [11, 18, 24]. Finally, the chance of cross contamination is reduced since both targets are amplified within the same reaction [34]. A disadvantage of multiplex PCR methods is the need for more optimization work. The PCR kit used in this study contains reagents in concentrations optimized for duplex amplification. Primer

and probe concentrations for the *lectin* gene sequence, the target superior to the GMO-specific target, are limited compared to those specific for the much weaker p35S system. This is demonstrated by the fact that amplification plots reach the plateau phase of the PCR much earlier for the *lectin* system than for the p35S system (Fig. 1).

Duplex quantification methods for GMOs based on real-time PCR have been described in the literature but only with use of genomic DNA CRMs as calibrators [11, 18, 22, 23, 24]. Due to some intrinsic features of genomic DNA standards, errors may be introduced when using this type of calibrator for the estimation of DNA copy number values [20]. First, CRMs are produced based on weight equivalents whereas PCR quantification of a GMO content can only be based on genome equivalents, being relative ratios of DNA molecules. Because there is no exact relationship between a weight or number of grains and a number of DNA molecules, the suitability of such CRMs for DNA copy number determinations may be doubted. Since the number of DNA molecules in one unit of GM material may be different from the DNA amount in a unit of non-GM material, mutual differences may be expected between a weight percentage of GMO and a percentage based on genomes. Second, the production of CRMs includes intensive homogenization and grinding for minimization of the particle size variation. These steps, however, can lead to severe DNA degradation. Third, a variation in exact GMO content from lot to lot cannot be excluded since plants with different zygosities and ploidy levels can be mixed. A fourth drawback is the limited availability of these CRMs, not only in concentration range but also in GMO events [4, 25, 35, 36].

In contrast to the CRMs, plasmid calibrators can be produced on a large scale without the need of agricultural products. Because of the smaller product size, they have a higher stability and can be stored for a long time without loss of quality. They can be made available for a wide range of GMO events and DNA target concentrations. Their production process is simple and less costly than that of CRMs based on mixing agricultural products such as seeds or grains [17, 20, 25, 27, 28]. It must be remarked, however, that working with plasmid DNA requires careful laboratory setup and practice in order not to cause contamination problems.

We have used mixtures of STPs in a background of genomic DNA, in relative GMO concentrations from 50 to 0.75%, to set up a single delta C_T calibration curve. Although this method showed good results regarding both the calibration curve and the quantity estimates (acceptable precision and trueness), delta C_T methods have disadvantages too. A requirement for delta C_T methods is that the two targets are amplified with the same efficiency in the PCR. This is because final quantity estimates of percentage GMO are based on a ratio of one PCR cycle to another PCR cycle. This is not the case for quantity estimates from standard curve methods where two absolute quantifications are done with separate calibration curves. A C_T value is compared here only with C_T values of the same amplicon, being amplified with the same efficiency.

Normally, one could assume that the PCR efficiencies for two targets which are very similar to each other in structure and length would be the same. At least this should be the case if the two fragments are amplified in separate tubes. However, if a GMO-target sequence and an endogenous gene sequence are amplified in the same tube, slight mutual differences in PCR efficiency may be expected. This difference in amplification efficiency between a transgenic DNA sequence and a *lectin* gene sequence is likely to have an influence also in the delta C_T method based on mixtures of plasmid with genomic DNA. When using plasmid calibrators in a background of genomic DNA for setting up delta C_T standard curves, the PCR efficiencies of both targets should first be tested and compared. To the contrary, different target sequences present in one MTP DNA molecule are very likely to be amplified with the same efficiency (no significant differences in slopes between p35S and *lectin* systems in MTP-cp method, Table 2).

The comparison between STP and MTP calibrators can be summarized as follows. First, as the amplification efficiencies between both targets are exactly the same, MTPs would be more suitable than STPs for delta C_T methods. Only an additional amount of *lectin* copies, for example present in genomic wild-type DNA, would need to be added to MTPs in order to obtain percentages. Second, MTP RMs could be spiked in different backgrounds of genomic DNA, thereby delivering more "matrix matched" calibrators. Third, as both target sequences to be amplified are present, less handling is required for the preparation of the dilution series to be used as calibrators in the PCR than if two STPs are used in mixtures.

A disadvantage of MTP calibrators is that they are more complicated and time-consuming to construct because several subsequent cloning steps are involved. Besides this, the MTPs used in this study only contain sequences of regulatory elements (promoter 35S and terminator *nos*) and a construct-specific sequence. Since we used a commercial kit optimized for duplexing a p35S sequence and an endogenous target, the presented methods are only screening methods. The limitations of screening tests are well known: detection of a general element such as promoter sequences does not necessarily indicate the presence of GMOs and screening does not allow for identification or quantification of GMOs. Since different GM events may contain different copies of this promoter, this sequence is not suitable for accurate quantification [1, 4]. The only way to unequivocally identify a specific GM event is to target the junction between the T-DNA construct and the adjacent plant genome at the integration site. This junction and, more specifically, a sequence of the integration-border region of transgenic Roundup Ready soybean at the p35S site, is present in the STP molecule used in this work as a GMO-specific target. This plasmid can thus be used for event-specific quantification, as previously reported [27].

Conclusions

In this paper we have described two new real-time duplex assays for GMO quantification. Plasmid DNA calibrators were compared with the classic genomic DNA standards from the commercially available CRMs. Since these CRMs are made by mixing GM seeds with non-GM seeds in certain concentrations, a relative percentage of GMO here represents a weight/weight percentage. Because percentages based on weights are not exactly the same as those based on genome copies, the suitability of these CRMs for the estimation of DNA copy numbers may be doubted. In answer to the growing need for alternative types of calibrators for GMOs, several authors have already reported on the use of plasmid DNA calibrators [17, 20, 22, 27, 28, 30]. With regard to the ease of production, storage, and distribution, the high stability, and the universal applicability, plasmid DNA calibrators are preferred above genomic DNA calibrators originating from CRMs. We have proven that, for duplex quantification based on a delta C_T curve, STPs mixed with genomic pure soybean DNA with concentration levels from 50 to 0.75% perform at least as well as genomic DNA samples from commercially available CRMs (5–0.1%). The STP calibrators allow quantification in a wider range and the different concentrations show a higher correlation compared to genomic DNA standards, which are independent DNA extractions.

One drawback of plasmid DNA calibrators is that they, as such, only contain the pure analyte and are not similar to real samples of interest. However, the plasmid DNA RMs used in this study were made "matrix matching" by spiking them in a background of genomic DNA. Another prerequisite for delta C_T methods is that equal PCR amplification efficiencies for both targets are obtained. Because of this drawback of delta C_T methods, we also developed a duplex quantitative method with plasmid DNA calibrators expressed in copy numbers. Optimal calibration curves were set up with MTP DNA standards containing different DNA target sequences. In addition, quantitative results were delivered with high precision and accuracy. In summary, plasmid DNA molecules containing multiple fragments next to each other show excellent performance in a real-time duplex PCR with a commercial GMO quantification kit.

Throughout this study, a commercial kit was used with optimized reagent concentrations for duplex PCR but targeting (next to endogenous *lectin* gene) only the promoter 35S. The primers and probe specific for the p35S element could be used in combination with the MTP fragments developed by the Japanese researchers as well as with our STPs containing the p35S T-DNA/plant junction fragment of Roundup Ready soybean. However, because we stuck to the use of this commercial kit, reactions were only screening and not event-specific.

Future developments of real-time duplex PCR methods could aim at transformation event-specific sequences, such as the p35S/plant border of Roundup Ready soybean present in the STP used. This junction, also called "cross-border region" or "edge fragment", is the only unique signature of a transformation event [1, 3, 4, 37]. As junctions of different commercialized GMOs have been characterized and cloned already [38], event-specific duplex quantification assays could be developed with these plasmid DNA calibrators.

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