Semi-Quantification of GM Maize Using Ready-To-Use RTi-PCR Plates

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Abstract European Union (EU) legislation foresees that food and feed are labelled for their genetically modified organism (GMO) content when the ingredients contain authorised GMOs in a proportion higher than 0.9 %. Non-authorised GMOs are not allowed on the EU market. Exception is made for feed materials containing traces of GM events lawfully placed on the market in non-EU countries and for which an authorisation is pending in the EU or expired: in these cases, a minimum required performance limit (MRPL) of 0.1 % is applied. Considering that the number of GMOs placed on the EU market is constantly growing, laboratories need to expand their capacity accordingly to be able to identify and, if necessary, quantify any GM material. To this purpose, our laboratory had previously developed a multi-target ready-to-use system, also known as a pre-spotted plate (PSP), which allows the qualitative detection of up to 44 GM events in one single real-time (RTi) PCR experiment. Should any event be detected, the laboratory would proceed with its quantification. This study evaluates the possibility to use the data generated by the PSP system in a semi-quantitative manner, allowing a categorisation of the GM quantity in the sample. ΔCq values were calculated and modelled via linear regression to estimate limits indicating whether the GM content is (1) above, (2) below or (3) near a defined quantity and thus requires further quantification. Identifying the GM events present in a sample and simultaneously discriminating whether it needs or not further quantification would offer to testing laboratories a valuable gain in time and resources. Six maize GM events frequently found in food and feed were selected for this evaluation (Bt11, MON810, MON88017, MON89034, NK603 and TC1507). The feasibility of the approach was successfully verified in-house using spiked and proficiency test samples.

Keywords GMO · Labelling · Semi-quantification · Ready-to-use · Real-time PCR · Pre-spotted plates

Introduction

The control of food and feed for GMO contamination represents an ever-growing task as more and more GM events are authorised for the European market. Testing laboratories must constantly expand their capacity to detect, identify and quantify GMOs to verify whether products comply with the current legislations. Notably, these include traceability requirements that foresee that food and feed are labelled when an ingredient contains authorised GMOs in a proportion higher than 0.9 %. Below this threshold, labelling is not required, provided that GMO traces are due to adventitious contaminations (European Parliament and Council of the European Union 2003). Non-authorised GMOs are not allowed. The sole exception being feed matrices in which contamination by GMOs that are authorised in non-EU countries and for which the authorisation in EU is pending or expired is tolerated at very low level (European Commission 2011). Considering the challenge in detection and identification of GMO at such a low concentration level, harmonised analytical procedures were established in order to guarantee the reproducibility of the analysis. In particular, reference procedures are necessary

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to assess whether the GM content exceeds the minimum required performance limit (MRPL) of the methods (i.e. 0.1 %) and therefore does not comply with EU provisions (European Commission 2011).

The pre-spotted plate (PSP) consists of a real-time (RTi) PCR-based multi-target and ready-to-use analytical system for GMO detection and identification that was developed by our laboratory (Querci et al. 2009) to simplify the analytical workflow of the EU GMO testing community. The system can be custom-ordered from primer and probe providers and allows the identification of up to 44 GM events in a single RTi-PCR experiment, thus providing testing laboratories with a rapid and efficient testing workflow. As of today, PSPs are used exclusively to screen for the qualitative presence of GM events in food and feed samples. If a GM event is detected, a quantitative assay follows to assess the product compliance with labelling provisions. Quantification of GM content by RTi-PCR is laborious: two quantitative assays must be run in parallel, one taxon- and one GM event-specific assay, to quantify the ingredient and the GM content, respectively, and a series of standard samples for assay calibration must be prepared.

Semi-quantification represents an intermediate analytical approach, between qualitative and quantitative methods, yielding an approximation of the quantity of a substance (Miller-Keane and O'Toole 2003). Signals generated during the analysis of samples can be confronted with defined limits to then establish whether the sample contains an amount of analyte above or below a defined threshold.

In RTi-PCR assays, the detection of the target yields a signal characterised by a quantitation cycle (Cq) that is directly related to the initial DNA input so that the higher the Cq value, the lower the initial amount of target sequence and vice versa. The use of ΔCq values i.e. the difference between the Cq values observed from a target and a reference, for the quantification of a genetic target with respect to a reference has been used for many years and also in the GMO field (Vaïtilingom et al. 1999). However, although semi-quantification by using RTi-PCR is already described for medical applications (Filipits et al. 2011; Weber and Feder 2013), it is not yet applied for monitoring the GM content in food and feed.

In 2012, Kluga et al. made a first attempt at using results generated by a PSP to get an approximation of the GM content. More specifically, Kluga's study correlated the ΔCq values, obtained by calculating the difference between the Cq of the event-specific assay (i.e. Cq_{GM}) and the Cq of the taxon-specific assay (i.e. Cq_{REF}), to the relative amount of GM material in food and feed. Ultimately, these authors established a cut-off ΔCq value above which the GM content could be considered negligible.

Although this approach does not provide the exact quantification of GM content, it would allow laboratories to discriminate between samples that do not need full quantitative assessment and samples that do need further investigations, therefore greatly reducing the number of quantitative tests to perform. However, only the theoretical aspects of this approach had been outlined so far, without the experimental verification of its reliability.

This work describes the development and the evaluation of the semi-quantification strategy using the already existing PSP system. In this study, the semi-quantification is done with respect to the labelling threshold of 0.9 % foreseen for authorised GMOs (European Parliament and Council of the European Union 2003) and for 0.1 % applicable to those GMOs falling under the MRPL regime (European Commission 2011).

To demonstrate the feasibility of the approach, six maize GM events were chosen based on their frequency of occurrence in EU official samples (Rosa et al. 2016). The associated certified reference materials (CRMs) were tested at different GM concentration levels by RTi-PCR for the taxon- and the event-specific assays and Cq signals were then combined to calculate ΔCq values (Cq_{GM}–Cq_{REF}). These were in turn modelled to obtain the prediction intervals (i.e. intervals in which future observations will fall within a defined probability) of ΔCq values expected for a range of GM content between 0.1 and 5 %. The predictions of ΔCq values for the desired GM contents are then calculated from the model.

The applicability of the estimated limits to results generated on PSPs was then verified in-house on spiked and proficiency test samples, thereby confirming the reliability of the approach at predicting whether the GM content of the samples is (1) above, (2) below or (3) close to the threshold. Only the latter cases would then need further quantitative assessment.

Material and Methods

Sample Preparation

Three types of samples were used in this study: (1) CRMs from six GM maize events were used for the generation of datasets for the calculation of ΔCq limits; (2) spiked samples and (3) proficiency tests (PT) samples of known GM content were used for the performance evaluation of the defined ΔCq semi-quantification limits.

CRMs of the GM events Bt11, MON810, MON88017, MON89034, NK603 and TC1507 were obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) or the American Oil Chemists' Society (AOCS, Urbana, IL, USA). CRMs were also used for spiked samples preparation (see below for details). Samples from PT rounds were from the European Union Reference Laboratory for GM Food and Feed (EURL GMFF, Joint Research Centre, Ispra, Italy), from the US Grain Inspection, Packers &

Stockyards Administration (GIPSA, US Department for Agriculture, WA, USA) and from FAPAS (Fera Science Ltd., York, UK).

DNA was extracted using the Nucleospin® kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's instructions, DNA integrity was then assessed by agarose gel electrophoresis and its concentration was determined using the Picogreen dsDNA quantification kit (Invitrogen, Molecular Probes, Eugene, OR, USA) with the Bio-Rad VersaFluor™ Fluorometer. DNA extracts were furthermore tested for the absence of PCR inhibitors as described in Annex 2 of ENGL (2011) using a maize-specific reference system (Mazzara et al. 2009).

Five concentration levels (0.1, 0.5, 1, 2 and 5 %) for each GM event were directly obtained from the corresponding CRMs. Whenever the CRM at the concentration of interest was not available, reference samples were prepared by mixing the DNA from CRM of a higher concentration with its wild-type counterpart as described in the Annex 3 of ENGL (2011).

Spiked samples were prepared by mixing maize DNA containing 0.1, 0.5, 1, 2 and 5 % (w/w) of GM events with non-target DNA (Lambda DNA, Thermo Fisher Scientific, Waltham, MA, USA) in a proportion 1:4, 1:16 and 1:32 of the total DNA per reaction (e.g. 100 ng of the sample 1:4 spike level 1 % GM contains the following: 25 ng of maize DNA 1 % GM and 75 ng of non-target DNA). Fifteen spiked samples were prepared for each of the six GM maize event, for a total of 90 spiked samples.

PCR Setup

RTi-PCRs on CRMs and spiked samples were performed in 50 μL of amplification mix containing 100 ng DNA, 1× TaqMan® Universal PCR Master Mix no UNG (Life Technologies, Carlsbad, CA, USA), 900 nM of each primer and 250 nM of the FAM/TAMRA-labelled probe and loaded onto a 96-well plate. The thermal profile used was as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. Data acquisition was set on the amplification step. RTi-PCR runs were performed using the 7900HT Fast Real-Time PCR System and the 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) and the data analysed using the SDS 2.4 and the 7500 software v2.0.6, respectively, by setting the baseline between 3 and 15 cycles and threshold at 0.2 ΔRn.

Methods used in this study are those described in Querci et al. (2009) and they are the taxon-specific assay hmg of *Zea mays*, and the GM event-specific assays for Bt11, MON810, MON88017, MON89034, NK603 and TC1507. The analyses of proficiency tests samples were performed using custom PSP manufactured by Life Technologies (Carlsbad, CA, USA) containing 45 and 12.5 pmol of dried primers and probes. In each well 50 μL of amplification mix containing 100 ng DNA, 1× TaqMan® Universal PCR Master Mix no UNG (Life Technologies, Carlsbad, CA, USA) was added and analysed as previously described.

Statistical Model and Calculation of ΔCq Semi-Quantification Limits

For each of the 6 GM events, 5 concentration levels (0.1, 0.5, 1, 2 and 5 %) were prepared independently by 2 operators. Each operator ran 2 PCR experiments on 2 different instruments, resulting in 4 ΔCq values (Cq_{GM}–Cq_{REF}) per combination of operator/instrument/sample. A total of 80 ΔCq values per GM event were then obtained (5 concentrations × 2 operators × 2 instruments × 4 replicates). Details of the experimental design are displayed in Fig. 1.

Models identifying the probability of distribution of ΔCq values at a defined GM content were built for each GM event independently by pooling the 80 ΔCq values.

The algorithm for ΔCq limits estimation was as follows: the relation between the (log) percentage GM (pGM) and the ΔCq values was modelled as a heteroskedastic linear model with an exponential increase in σ. For the systematic (mean) linear model, ordinary least squares were used:

$$\log_{10} p_{GM} = a + b\,\Delta Cq \tag{1}$$

where a and b are the respective intercept and slope of the model. For the construction of prediction intervals, bootstrap estimates ($n = 100$) of the standard deviation of the ΔCq values were regressed against the systematic ΔCq values (double log scale):

$$\log_{10} \hat{\sigma} = c + d \log_{10} \overline{\Delta Cq} \tag{2}$$

where c and d are the respective intercept and slope of the model. Rather than the mean estimate for $\hat{\sigma}$, the 95 % upper confidence bound is used in order to minimise the risk of underestimating the ΔCq variability. For a pGM, the range of observable ΔCq values (98 % prediction interval) is then given by:

$$\Delta Cq_{L,\ U} = \overline{\Delta Cq} + z_{\alpha/2}\,\hat{\sigma}_{95\%} \tag{3}$$

Where $z_{\alpha/2}$ is the corresponding lower or upper Z value (in this case either $z_{0.01}$ or $z_{0.99}$), $\hat{\sigma}_{95\%}$ is the 95 % upper confidence of the ΔCq standard deviation (as extracted from the linear model in R using *predict()* and $\overline{\Delta Cq}$ as new data point), with $\overline{\Delta Cq}$ found via Eq. 1 as:

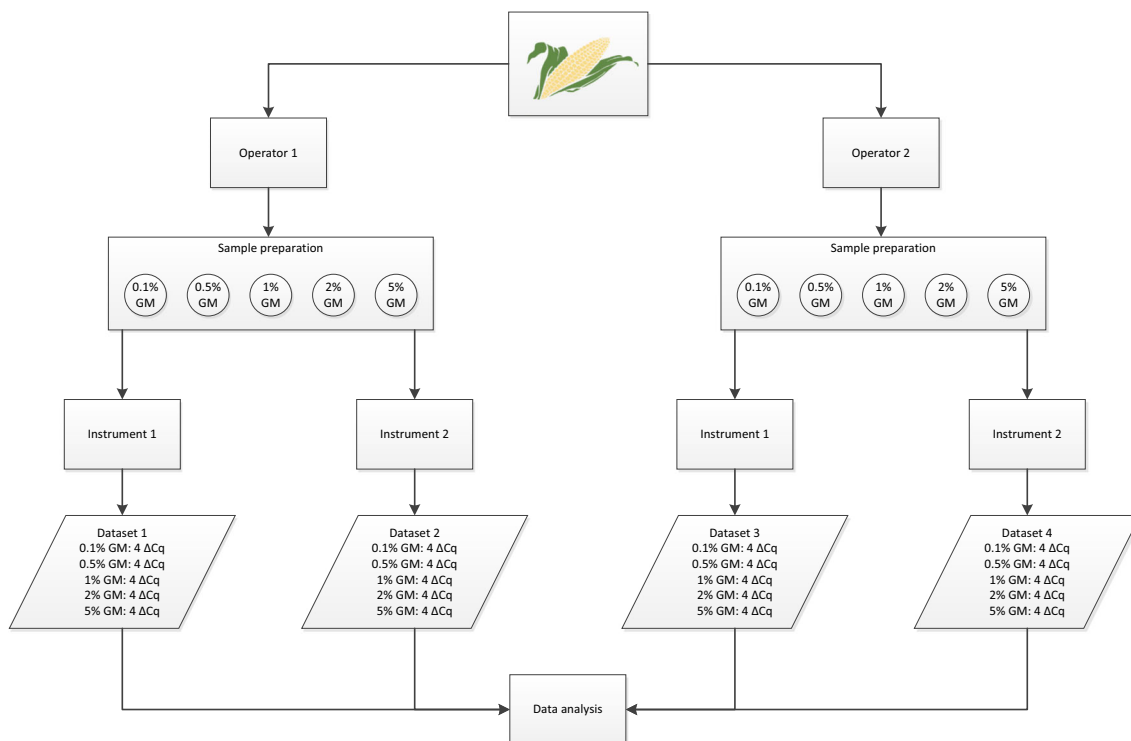$$\overline{\Delta Cq} = \frac{\log_{10} p_{GM} - a}{b} \tag{4}$$

Fig. 1 Experimental design followed to obtain the dataset for the estimation of ΔCq semi-quantification limits. For each of the six GM maize events, a total of 80 ΔCq values were generated

All calculations and curve fitting were done using R version 3.2.3 (R Core Team 2012). Parameter modelling was accomplished using the standard linear modelling function (*lm*).

Background data and scripts are given in Online Resource.

In-House Verification of ΔCq Semi-Quantification Limits

Spiked samples were analysed in eight replicates at PSP reaction conditions to assess the reliability of the inferred ΔCq semi-quantification limits. Four tests per combination of GM event/concentration/spike level/instrument were loaded for a total of 120 tests per GM event.

In addition, 18 samples from proficiency test programmes were analysed in duplicate on PSP. The GM content was predicted by the observed ΔCq, and the categorisation was then assessed against the value calculated from participants' data.

Results and Discussion

This study shows that semi-quantification aiming at identifying samples with GM content below or above a defined limit (i.e. 0.9 and 0.1 %) is feasible, reliable and achievable using standardised ready-to-use RTi-PCR plates, aka PSP.

Z. mays was adopted as a model since it can be considered the most challenging among the GM crops for the number of GM events on the market (European Commission 2003) and for the genome composition that may influence the ratio between the GM inserts and endogenous sequences targeted for quantification (Holst-Jensen et al. 2006).

ΔCq Limit Calculation

ΔCq limits are statistically inferred from the analysis of reference samples with the aim to predict the GM content. The case study described here identifies ΔCq limits defining an interval allowing to categorise samples as follows: (1) the GM maize content exceeds the target GM content (i.e. greater than 0.9 or 0.1 %), (2) the GM maize content is below the target GM content (i.e. lesser than 0.9 or 0.1 %) or (3) an additional standard quantitative assay is necessary (i.e. GM content close to 0.9 or 0.1 %). Actual ΔCq obtained from the samples analysed on PSP can then be compared to the ΔCq semi-quantification limits and a judgement call regarding the target concentration can be taken.

Figure 2 displays the statistical model developed for MON810 with the inferred values for the evaluation of the GM content with respect to the thresholds of 0.9 % (Fig. 2a) and to 0.1 % (Fig. 2b), respectively. Only
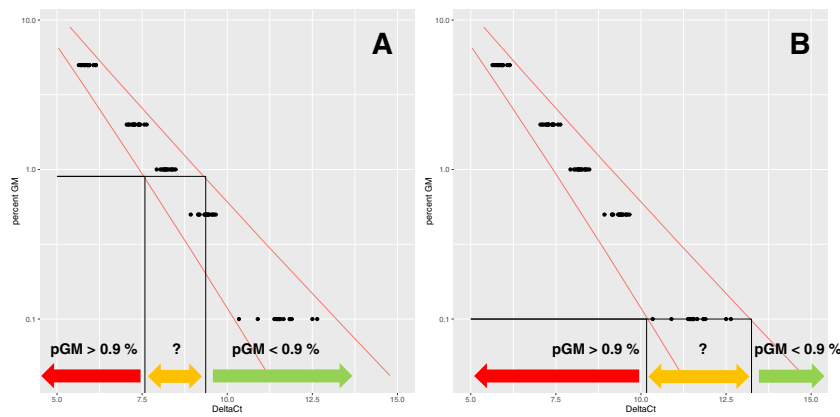
Fig. 2 Visual representation of ΔCq semi-quantification limits for MON810 for a GM content of 0.9 % (**a**) and 0.1 % (**b**). The area included within the *red lines* represents the bilateral 98 % prediction interval (unilateral prediction at 99 %). pGM: GM percentage

samples for which the ΔCq values fall within the range of the semi-quantification limits will need a full quantitative assessment. Table 1 displays ΔCq semi-quantification limits for the six GM events for both scenarios: 0.9 %$_{1st}$ and 0.1 %$_{1st}$ represent the limits below which the actual ΔCq values from the samples can be correlated to a GM content above the threshold, while values above the 0.9 %$_{99th}$ and 0.1 %$_{99th}$ limits indicate a GM amount below the threshold.

The limits for the categorisation with respect to the 0.9 % threshold allow the classification of samples containing one GM event: considering a sample in which the GM event MON810 is detected, if ΔCq$_{MON810}$ < 7.57 (i.e. 0.9 %$_{1st}$ limit), the GM content is above the labelling threshold; on the other hand, when ΔCq$_{MON810}$ > 9.38 (i.e. 0.9 %$_{99th}$ limit), the sample is in line with labelling provisions.

The application of this approach for the categorisation of samples containing more than one GM event is feasible; however, it requires tuning semi-quantification limits for the different scenarios. For example, if two GM maize events are detected, the half of the threshold (i.e. 0.45 %) is used for the calculation of ΔCq limits. In this case, if both GM events display an expected GM content below the 0.45 %$_{99th}$ limit the sample complies with labelling provisions, because the sum of the two GM quantities is expected to be below 0.9 %; otherwise, if both GM events display ΔCq values indicating a

concentration above 0.45 %$_{1st}$, the GM maize content exceeds the EU labelling threshold.

However, a decision on the sample with respect to the labelling provisions can nevertheless be taken when the semi-quantification of one of the GM events indicates that the 0.9 % threshold is passed.

Similarly, the semi-quantification can be used for the evaluation of samples with respect to lower concentration levels. Although mandatory analytical procedures are necessary for the control of samples at the MRPL (European Commission 2011), ΔCq semi-quantification limits can be calculated for the evaluation of a 0.1 % GM content. The semi-quantification limits shows that the GM content is negligible when the ΔCq from the sample is above 11.88–14.08, depending by the GM event, and that it is above the MRPL when the ΔCq is below 9.43–11.00.

Using the methodology described in Kluga et al. (2012), the theoretical ΔCq corresponding to GM contents of 0.9 % and 0.1 % are of 6.8 and 10.0, respectively. However, these values are not always included in the range identified by the corresponding ΔCq semi-quantification limits. Rather, each combination of GM event/assay is characterised by specific values confirming that the genetic background (i.e. zygosity) of the reference material (RM) and the efficiency of the assays have an impact on the ΔCq value.

Table 1 ΔCq semi-quantification limits for the six GM maize events

GM level	Percentile	Bt11	MON810	MON88017	MON89034	NK603	TC1507
0.9 %	1st	7.73	7.57	6.53	7.74	8.23	6.97
	99th	10.35	9.38	8.32	9.37	10.18	8.20
0.1 %	1st	11.00	10.15	9.43	10.92	10.69	9.61
	99th	14.03	13.27	11.88	13.14	14.18	12.03

Assessment of ΔCq Semi-Quantification Limits

In order to evaluate whether the ΔCq limits can be applied to real-life samples, in which the maize ingredient represents only a portion of the constituents, the DNA solutions from CRMs/RMs were spiked with non-target DNA (Table 2). In this assessment, also dilution levels in which the concentration of the GM target is very low (<20 copies) were included, thus simulating a laboratory routine scenario.

Results showed that the ΔCq semi-quantification limits provide the expected results when applied to the data obtained by simulating PSP reaction conditions on spiked samples. When ΔCq limits for 0.9 % are applied for the evaluation of samples containing 0.1 or 5 % GM, only a small subset (between 2.1 and 6.3 %) of the samples needs a full quantification assessment. On the other hand, samples with a GM content of 0.5 and 2 % more often require additional quantification (between 14.6 and 52.1 %). Almost all samples at 1 % of GM content are categorised as requiring quantification, as expected. Samples with a low target DNA concentration (i.e. 1:32 spike level) display 44.7 % rate of no amplification for the GM event-specific assay; this can be explained by the fact that the theoretical copy number of the target at this level are below the limit of detection of the methods (between 20 and 5 copies).

At the 1:32 spike level, a small fraction (17 %) of samples at 1 % are wrongly classified as containing less than 0.9 % GM (Table 2). However, considering that analyses are normally performed in duplicate, it would thus take two simultaneous mis-categorisations to result in a false classification of the sample. Therefore, the probability to wrongly categorise a sample with very low target content is expected to be below 5 % (i.e. $0.17 \times 0.17 = 0.03$ or 3 %).

Similar results are obtained when ΔCq limits for 0.1 % are applied to the spiked samples. Again, a reduction in the accuracy of the classification of samples is observed when the target DNA is at a limited concentration (spiked levels 1:16 and 1:32).

In order to simulate a routine testing scenario, PT samples were analysed in duplicate on PSPs (Table 3). Results show that the categorisation of samples as below the 0.9 % threshold is achievable for a GM content below 0.4 % (i.e. when the 0.9 %$_{99th}$ semi-quantification limits are applied). For samples with GM content higher than 0.36 %, the limit of 0.1 %$_{1st}$ provides an appropriate discrimination power, indicating a GM amount above the MRPL. ΔCq limits of 0.9 %$_{1st}$ and 0.1 %$_{99th}$ were not assessed for lack of availability of PT samples at higher/lower concentrations.

This preliminary evaluation on PT samples thus confirms the reliability of the approach: samples below the labelling threshold were directly identified and those near to 0.9 % were

Table 2 Assessment of semi-quantification limits on spiked samples. Pooled data from spiked samples from the six GM events are displayed and expressed in percentage per each category

pGM	Spike level	Tot.	n. ΔCq	% not detected	0.9 %			0.1 %		
					<0.9 %	Quant.	>0.9 %	<0.1 %	Quant.	>0.1 %
0.1	1:4	48	48	2.1	97.9	0.0	0.0	0.0	97.9	0.0
0.1	1:16	48	40	16.7	77.1	6.3	0.0	0.0	72.9	10.4
0.1	1:32	47	26	44.7	53.2	2.1	0.0	0.0	48.9	6.4
0.5	1:4	48	48	0.0	47.9	52.1	0.0	0.0	33.3	66.7
0.5	1:16	48	48	0.0	52.1	47.9	0.0	0.0	41.7	58.3
0.5	1:32	47	45	4.3	46.8	48.9	0.0	0.0	42.6	53.2
1	1:4	48	48	0.0	0.0	97.9	2.1	0.0	0.0	100.0
1	1:16	48	48	0.0	6.3	89.6	4.2	0.0	6.3	93.8
1	1:32	47	47	0.0	17.0	70.2	12.8	0.0	10.6	89.4
2	1:4	48	48	0.0	0.0	14.6	85.4	0.0	0.0	100.0
2	1:16	48	48	0.0	0.0	35.4	64.6	0.0	0.0	100.0
2	1:32	47	47	0.0	0.0	31.9	68.1	0.0	0.0	100.0
5	1:4	48	48	0.0	0.0	2.1	97.9	0.0	0.0	100.0
5	1:16	48	48	0.0	0.0	0.0	100.0	0.0	0.0	100.0
5	1:32	47	47	0.0	0.0	0.0	100.0	0.0	0.0	100.0

pGM: GM content in percentage; spike level: fraction of DNA from RM at known GM concentration in non-target DNA; Tot.: number of test per pGM/spiked level combination; n. ΔCq: number of ΔCq values; % not detected: no amplification rate. 0.9 % and 0.1 %: rate of ΔCq values per pGM/spiked level combination categorised according ΔCq semi-quantification limits, Quant.: full quantitative assay is necessary

Table 3 Results of the semi-quantification on sample from proficiency tests rounds

Reported mean (%) from participants (provider)	GM event	ΔCq	0.90 %	0.10 %	ID sample
0.07 (0.1)	TC1507	12.83	<0.9 %	<0.1 %	USDA PT04/11 C04.6
		11.55	<0.9 %	Quant	
0.07 (0.1)	MON810	12.45	<0.9 %	Quant	USDA PT04/15 C4.1
		12.84	<0.9 %	Quant	
0.08 (0.1)	MON810	12.55	<0.9 %	Quant	USDA PT04/14 C4.4
		13.22	<0.9 %	Quant	
0.1 (0.1)	MON88017	10.46	<0.9 %	Quant	USDA PT04/14 C4.4
		10.22	<0.9 %	Quant	
0.12 (0.1)	BT11	12.50	<0.9 %	Quant	USDA PT04/11 C04.2
		12.37	<0.9 %	Quant	
0.13 (0.1)	MON88017	10.61	<0.9 %	Quant	USDA PT04/15 C4.1
		11.01	<0.9 %	Quant	
0.13 (0.2)	MON810	11.31	<0.9 %	Quant	USDA PT04/15 C4.3
		12.03	<0.9 %	Quant	
0.14 (0.2)	MON810	11.65	<0.9 %	Quant	USDA PT04/14 C4.5
		11.71	<0.9 %	Quant	
0.26 (0.5)	MON810	11.24	<0.9 %	Quant	USDA PT04/14 C4.2
		10.97	<0.9 %	Quant	
0.29 (0.5)	MON810	10.81	<0.9 %	Quant	USDA PT04/14 C4.3
		10.76	<0.9 %	Quant	
0.31 (0.5)	TC1507	9.58	<0.9 %	>0.1 %	USDA PT04/15 C4.2
		9.18	<0.9 %	>0.1 %	
0.34 (0.5)	NK603	11.10	<0.9 %	Quant	USDA PT04/14 C4.3
		11.16	<0.9 %	Quant	
0.35 (0.5)	MON89034	8.73	Quant	>0.1 %	USDA PT04/15 C4.3
		9.30	Quant	>0.1 %	
0.36 (0.20)	MON810	10.56	<0.9 %	Quant	EURL CT1/13 Level 2
		10.05	<0.9 %	>0.1 %	
0.38 (0.30)	TC1507	8.54	<0.9 %	>0.1 %	EURL CT2/11 Level 1
		8.56	<0.9 %	>0.1 %	
0.38 (0.8)	TC1507	8.45	<0.9 %	>0.1 %	USDA PT04/14 C4.4
		8.45	<0.9 %	>0.1 %	
0.39 (0.8)	TC1507	8.21	<0.9 %	>0.1 %	USDA PT04/15 C4.1
		8.51	<0.9 %	>0.1 %	
0.39 (0.5)	MON89034	9.71	<0.9 %	>0.1 %	USDA PT04/14 C4.5
		9.54	<0.9 %	>0.1 %	
0.42 (0.5)	MON88017	8.46	<0.9 %	>0.1 %	USDA PT04/14 C4.3
		8.76	<0.9 %	>0.1 %	
0.50 (0.5)	BT11	9.36	Quant	>0.1 %	USDA PT04/14 C4.5
		9.30	Quant	>0.1 %	
0.52 (1)	MON89034	7.92	Quant	>0.1 %	USDA PT04/14 C4.3
		8.61	Quant	>0.1 %	
0.59 (0.65)	NK603	10.48	<0.9 %	>0.1 %	USDA PT04/11 C04.2
		10.63	<0.9 %	>0.1 %	
0.59 (0.68)	MON88017	7.62	Quant	>0.1 %	EURL CT2/12 Level 1
		8.52	<0.9 %	>0.1 %	
0.63 (0.5)	BT11	9.39	Quant	>0.1 %	USDA PT04/15 C4.3
		9.59	Quant	>0.1 %	
0.68 (1)	BT11	9.29	Quant	>0.1 %	USDA PT04/14 C4.2
		8.51	Quant	>0.1 %	
0.7 (0.6)	MON810	9.54	<0.9 %	>0.1 %	USDA PT04/11 C04.6
		9.26	Quant	>0.1 %	
0.70 (0.8)	MON88017	7.60	Quant	>0.1 %	USDA PT04/15 C4.2
		7.67	Quant	>0.1 %	

Table 3 (continued)

Reported mean (%) from participants (provider)	GM event	ΔCq	0.90 %	0.10 %	ID sample
0.73 (n.a.)	MON89034	8.06	Quant	>0.1 %	FAPAS GeM MU39
		8.05	Quant	>0.1 %	
0.86 (1.42)	MON88017	7.90	Quant	>0.1 %	EURL CT2/12 Level 2
		6.77	Quant	>0.1 %	
0.90 (1.0)	MON88017	6.88	Quant	>0.1 %	USDA PT04/15 C4.3
		6.87	Quant	>0.1 %	
0.90 (n.a.)	NK603	9.82	Quant	>0.1 %	FAPAS GeM MP22
		8.83	Quant	>0.1 %	
0.91 (0.66)	MON810	8.65	Quant	>0.1 %	EURL CT1/13 Level 1
		8.49	Quant	>0.1 %	
0.91 (n.a.)	TC1507	7.14	Quant	>0.1 %	FAPAS GeM MU33
		6.94	>0.9 %	>0.1 %	
0.92 (1.0)	NK603	9.00	Quant	>0.1 %	USDA PT04/15 C4.2
		8.73	Quant	>0.1 %	
0.93 (1.0)	MON88017	7.34	Quant	>0.1 %	USDA PT04/14 C4.5
		7.17	Quant	>0.1 %	
1.07 (0.89)	TC1507	7.22	Quant	>0.1 %	EURL CT2/11 Level 2
		6.95	>0.9 %	>0.1 %	
1.18 (1.3)	NK603	8.62	Quant	>0.1 %	USDA PT04/11 C04.6
		8.26	Quant	>0.1 %	
1.21 (2)	MON89034	7.37	>0.9 %	>0.1 %	USDA PT04/14 C4.4
		7.35	>0.9 %	>0.1 %	
1.38 (2.0)	MON89034	7.89	Quant	>0.1 %	USDA PT04/15 C4.1
		7.36	>0.9 %	>0.1 %	
1.44 (1.2)	MON810	8.47	Quant	>0.1 %	USDA PT04/11 C04.2
		8.51	Quant	>0.1 %	

Quant full quantitative assay is necessary, *n.a.* not available

classified as samples for which a subsequent quantitative assay is necessary.

Although this study demonstrates that the approach provides the expected results on both spiked and PT samples, further investigations are still necessary in order to demonstrate the transferability of this approach. A collaborative validation study according to international standards (International Organization for Standardization 1994; Horwitz 1995; Gustavo González and Ángeles Herrador 2007; ENGL 2015) would demonstrate its reproducibility.

Practicability and Impact

The implementation of the semi-quantification for the analysis of real-life samples is expected to have a significant impact on the reduction of analytical costs. While the cost is influenced by several factors, such as sample type, composition and analytical technology used (Milavec et al. 2014), decreasing the number of full quantitative assays has a direct effect on the overall cost. This can be illustrated using the data generated in a recent study (Rosa et al. 2016), for which the composition of

135 real-life samples containing GM events was communicated to our laboratory. Results of quantifications performed by laboratories showed that ~40 % of the GM events were at 0.1 % or not quantifiable (i.e. below the limit of quantification) and ~30 % were at 5 % or above. At these concentrations, the semi-quantification displays a high capacity of correct categorisation and therefore could have spared ~70 % of quantification analyses if it had been used.

Conclusions

The approach described in this work successfully built on and verified experimentally the theoretical assumptions that were made by Kluga et al. (2012). By including the main sources of uncertainty to the calculation of ΔCq limits (such as the impact of the CRM zygosity and the uncertainty associated to the assays, operators and instruments), it was shown that the difference between the Cq of the event-specific and the taxon-specific assays (ΔCq = Cq_{GM}–Cq_{REF}) could be correlated to the initial GM concentration in PSP-based experiments.

Indeed, results indicated that our approach is able to predict the maximum and the minimum ΔCq values expected for a defined GM content, therefore allowing a classification of the samples in one of the three following categories: below, above or close to the chosen GM content (in this case, 0.9 and 0.1 %). The six commonly found authorised GM maize events adopted as a case study could be readily assigned to one of the three categories, thus proving that a full quantitative assay represents an unnecessary additional step in many instances.

Categorisation of samples with respect to a defined threshold can also be achieved by a control by attributes (Montgomery 2009). Notably, the sample is divided in subsamples and each of them is then analysed for the presence or the absence of the analyte. The number of positive/negative results determines if the threshold is passed or not. This approach is commonly used for the categorisation of seed lots (Remund et al. 2001; Kobilinsky and Bertheau 2005); however, its application is not straightforward because it requires the analyses of many replicates for each sample. Therefore, it is not indicated when multiple targets have to be investigated.

New technologies have been recently applied to GMO testing. For instance, the droplet digital PCR is promising in terms of cost effectiveness and reliability (Morisset et al. 2013; Köppel et al. 2015; Dobnik et al. 2015; Lievens et al. 2016). However, this technology is not yet available in most of testing laboratories, while PSP and the semi-quantification can be easily implemented with the standard GMO laboratory equipment.

The challenge of discriminating between samples needing or not subsequent quantification experiments was also raised by the GMO testing community; in the last few years, the European Network of GMO Laboratories discussed the possibility to issue guidelines for the assessment of cut-off values for the interpretation of qualitative RTi-PCR experiments. However, the adoption of such approaches for official control is still debated.

While this study demonstrates the feasibility of the semi-quantification strategy, further work is necessary to extend the approach to all GM events authorised or in the pipeline of getting authorisation in the EU. Moreover, further studies are necessary to demonstrate its reproducibility and transferability to other laboratories equipped with different RTi-PCR platforms.

In conclusion, the PSP-based semi-quantification is promising and its application can have a significant impact on the GMO testing workflow, helping control laboratories reducing cost and time for their analyses.

References

Dobnik D, Spilsberg B, Bogožalec Košir A, et al. (2015) Multiplex quantification of 12 European Union authorized genetically modified maize lines with droplet digital polymerase chain reaction. Anal Chem 87:8218–8226. doi:10.1021/acs.analchem.5b01208

European Commission (2003) EU Register of authorised GMOs. http://ec.europa.eu/food/dyna/gm_register/index_en.cfm. Accessed 17 Sep 2015

European Commission (2011) Commission regulation (EU) no 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired Text with EEA relevance. Official Journal of the European Union, Brussels

European Network of GMO Laboratories (ENGL) (2011) Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm

European Network of GMO Laboratories (ENGL) (2015) Definition of minimum performance requirements for analytical methods of GMO testing. In: http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm. http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm. Accessed 22 Oct 2015

European Parliament, Council of the European Union (2003) Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. 7

Filipits M, Rudas M, Jakesz R, et al. (2011) A new molecular predictor of distant recurrence in ER-positive, HER2-negative breast cancer adds independent information to conventional clinical risk factors. Clin Cancer Res 17:6012–6020. doi:10.1158/1078-0432.CCR-11-0926

Gustavo González A, Ángeles Herrador M (2007) A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. TrAC Trends Anal Chem 26:227–238. doi:10.1016/j.trac.2007.01.009

Holst-Jensen A, De Loose M, Van den Eede G (2006) Coherence between legal requirements and approaches for detection of genetically modified organisms (GMOs) and their derived products. J Agric Food Chem 54:2799–2809. doi:10.1021/jf052849a

Horwitz W (1995) Protocol for the design, conduct and interpretation of method-performance studies. Pure Appl Chem 67:331–343

International Organization for Standardization (1994) ISO 5725:1994 Accuracy (trueness and precision) of measurement methods and results

Kluga L, Folloni S, Van den Bulcke M, et al. (2012) Applicability of the "real-time PCR-based ready-to-use multi-target analytical system for GMO detection" in processed maize matrices. Eur Food Res Technol 234:109–118. doi:10.1007/s00217-011-1615-5

Kobilinsky A, Bertheau Y (2005) Minimum cost acceptance sampling plans for grain control, with application to GMO detection. Chemom Intell Lab Syst 75:189–200. doi:10.1016/j.chemolab.2004.07.005

Köppel R, Bucher T, Frei A, Ulrich H (2015) Droplet digital PCR versus multiplex real-time PCR method for the detection and quantification of DNA from the four transgenic soy traits MON87769, MON87708, MON87705 and FG72, and lectin. Eur Food Res Technol 241:521–527. doi:10.1007/s00217-015-2481-3

Lievens A, Jacchia S, Kagkli D, et al. (2016) Measuring digital PCR quality: performance parameters and their optimization. PLoS One 11:e0153317. doi:10.1371/journal.pone.0153317

Mazzara M, Grazioli E, Savini C, Van den Eede G (2009) Report on the verification of the performance of a MON810 event-specific method on maize line MON810 using real-time PCR—validation report and protocol

Milavec M, Dobnik D, Yang L, et al. (2014) GMO quantification: valuable experience and insights for the future. Anal Bioanal Chem 406:6485–6497. doi:10.1007/s00216-014-8077-0

Miller-Keane, MT O'Toole (2003) semiquantitative. In: Miller-Keane Encycl. Dict. Med. Nursiring, Allied Heal. http://medical-dictionary.thefreedictionary.com/semiquantitative. Accessed 15 Jun 2016

Montgomery D (2009) Control charts for attributes. In: Introduction to statistical quality control, 6th edn. John Wiley & sons

Morisset D, Štebih D, Milavec M, et al (2013) Quantitative analysis of food and feed samples with droplet digital PCR. PLoS One doi:10.1371/journal.pone.0062583

Querci M, Foti N, Bogni A, et al. (2009) Real-time PCR-based ready-to-use multi-target analytical system for GMO detection. Food Anal Methods 2:325–336. doi:10.1007/s12161-009-9093-0

R Core Team (2012) R: A language and environment for statistical computing. http://www.r-project.org/. Accessed 17 Sep 2015

Remund KM, Dixon DA, Wright DL, Holden LR (2001) Statistical considerations in seed purity testing for transgenic traits. Seed Sci Res 11:101–119. doi:10.1079/SSR200166

Rosa SF, Gatto F, Angers-Loustau A, et al. (2016) Development and applicability of a ready-to-use PCR system for GMO screening. Food Chem 201:110–119. doi:10.1016/j.foodchem.2016.01.007

Vaïtilingom M, Pijnenburg H, Gendre F, Brignon P (1999) Real-time quantitative PCR detection of genetically modified maximizer maize and roundup ready soybean in some representative foods. J Agric Food Chem 47:5261–5266

Weber KE, Feder, IS (2013) Estimation of delta-cq values with confidence from qpcr data. http://www.google.com/patents/EP2583209A1?cl=en. Accessed 18 Jan 2016