Eur Food Res Technol (2008) 227:1149–1157
DOI 10.1007/s00217-008-0830-1

ORIGINAL PAPER

A statistical approach for evaluation of PCR results to improve the practical limit of quantification (LOQ) of GMO analyses (SIMQUANT)

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Received: 7 August 2007 / Revised: 10 January 2008 / Accepted: 19 January 2008 / Published online: 6 February 2008
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Abstract The predominant approach for quantification of genetically modified organisms (GMO) is the application of quantitative real-time PCR. However, for a large number of processed food and feed products, this approach is unsuitable, because they contain low amounts (mass) of amplifiable DNA. Here we present a novel approach, "Single molecule quantification" (SIMQUANT) for GMO quantification of samples with extremely low amounts of DNA. The approach is based on statistics and application of multiple qualitative parallel PCRs. Here the qualitative PCRs were done using real-time PCR setup, but this is not a requirement. The difference is that the quantitative real-time PCR requires that the target copy number exceeds the absolute limit of quantification (LOQ_{abs}) and provides quantity estimates by extrapolation from a linear regressional relationship between an observed cycle threshold (Ct) value and copy numbers, while with SIMQUANT the template DNA typically contains very few, e.g., one target copy per PCR volume and the quantity is estimated on the basis of observed ratio between positive and negative individual PCRs. The components of this analysis are the numbers of test samples, the size of each sample and the outcome in number and relative ratio of positive and negative test results. The approach results in a statistical estimate of the relative GM concentration based on the probability that one or more amplifiable GM template copies are present in discrete volumes. Thus, the approach is based on the ratio of discrete volumes without or with one or more PCR-amplifiable GM target copies. The approach described here can be used reliably with more than a 100-fold improvement of the practical LOQ (LOQ_{pract}) compared to real-time quantitative PCR based on standard curves.

Keywords MPN · Most probable number quantification · GMO · Limit of quantification (LOQ) · Event · Specific PCR · Limiting dilution PCR

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Introduction

Thresholds for labeling of GMOs or products derived from GMOs are in place in many countries, e.g., 0.9% in the EU, 1% in Australia and New Zealand, 3% in Korea and 5% in Japan and Indonesia. GMO quantification can be done by application of protein or DNA based methods, but is prevalently done by application of quantitative real-time PCR on processed materials. Unfortunately, due to presence of only minute amounts (mass) of intact, amplifiable DNA, it is often not possible to quantify the relative GMO content of several different kinds of processed or composite products even if the GMO content exceeds a relevant threshold. Such low amounts of DNA may be the result of removal, dilution or degradation of DNA during processing or storage. In the present study we have developed and tested a statistical approach to quantify GMO samples with extremely low amounts of amplifiable DNA. This method significantly expands the range of products that can be subjected to quantitative GMO testing and certification according to the relevant regulations and thresholds.

Real-time PCR is considered to be the most sensitive technology available for routine GMO detection. Despite this, we have found that the low amount of amplifiable DNA in many processed and composite samples is too low to permit normal real-time PCR based quantitation

(hereafter referred to as quantitative real-time PCR). This, of course, is a major problem since labeling is meant to ensure the stakeholders a freedom to chose between GM and non-GM products, and correct labeling is difficult to verify if analytical control cannot be performed in the context of GMO quantification.

We have previously distinguished between three types of quantification limits [1, 2]: (1) the absolute limit, i.e., the lowest number of initial template copies that can be quantified; (2) the relative limit, i.e., the lowest percentage of GM that can be quantified under exceptionally optimal conditions; and (3) the practical limit, i.e., the functional quantification limit of the sample under analysis (relative limit of a specific DNA extract). The absolute limit of quantification (LOQ_{abs}) for quantitative real-time PCR is normally in the range of 30–100 amplifiable copies of the target DNA per PCR reaction [1, 3, 4]. This is based on theoretical evaluation of stochastic variability in copy numbers at low concentrations in discrete volumes.

Conventionally, in quantitative real-time PCR a linear regressional relationship (a standard curve) is ultimately established for the copy number and responding fluorescence in calibrant reference materials, where the copy number is determined on the basis of mass of DNA (of the standard) and the size of the genome. The quantity of the test sample is determined by extrapolation from observed fluorescence in PCRs performed on the DNA from the test sample and the corresponding copy number of the standard curve (see [5] for details). Recently, the term PCR forming unit (PFU) was proposed to establish a clear distinction between target copies that amplify in PCR and crude mass based copy number estimates [5]. Because some target DNA copies may be damaged, fragmented or for other reasons fail to function as templates for a PCR, the number of copies serving as PCR templates may be lower than the copy number estimated from mass and genome size calculations. In such cases, the relative LOQ (LOQ_{rel}) calculated from the equivalent of 30–100 target copies (mass based estimates) may be underestimated (too optimistic).

The following two examples illustrate scenarios where quantitative real-time PCR cannot be applied to quantify the relative GM content, because the templates contain too few target PFU to comply with the LOQ_{abs}. (1) A 100% GM sample may contain only ten PFUs of each target, e.g., due to processing as discussed above. However, the GM-content is usually much lower than 100%, and the number of GM specific PFUs is therefore usually much lower than the number of corresponding reference PFUs. Thus, the ability to quantify the number of PFUs of the reference target sequence ($PFU_{ref} > LOQ_{abs}$) does not necessarily imply the ability to quantify the number of PFUs of the GM-specific target sequence (if $PFU_{GM} < LOQ_{abs}$). (2) In a PCR, where the template DNA contains approximately 200 references target PFUs and a GM concentration of 1% there will be only two GM PFUs on average.

Experience from GMO analysis of processed food and feed has shown that often the level of amplifiable DNA is too low, i.e., below the practical limit of quantification. This does not necessarily imply that the GMO percentage of an ingredient is low. As a matter of fact it may be as high as 100%. However, the DNA may be highly degraded or the ingredient derived from a GMO may constitute only a minor part of the product that is under analysis. In some proficiency tests (GeMMA proficiency testing scheme, arranged by FAPAS/CSL, UK) on such low copy number samples, more than 50% of the answers reported by the more than 50 participants were very imprecise (below 50% or above 200% of the assigned values). We suspect that this is because the laboratories might ignore the fact that the LOQ_{pract} is likely to be inferior to the method specific LOQ_{rel}. A LOQ_{pract} inferior to the method specific LOQ_{rel} will limit the range of samples from which quantitative results can be reliably obtained. Consequently, in the proficiency test, results may have been reported even when the copy number is less than the LOQ_{pract}, with a consequential high risk of reporting incorrect values.

Theoretically, the smallest number of PFU that can be detected in a PCR is 1. Experimentally, we have confirmed this using an event-specific real-time assay targeting GTS-40-3-2 (RoundupReady® soybean; RRS) [1] and diluted mass determined template DNA. Similar observations have been reported by others (e.g., [6, 7]) for other GMO related targets. In a set of reactions where template DNA has been diluted to approximately one PFU per reaction vessel, each PCR test performed will be either positive or negative depending on the presence or absence of target PFUs. Theoretically, if the number of PFUs of a specific target in each PCR is close to 1, then the ratio of positive to negative PCR tests can be used to estimate the number of PFUs in the sample by application of simple frequency distribution probability statistics. The GMO content can then be estimated by comparing the PFU estimates of the GM and reference targets, if necessary taking into consideration the differences in dilution factors. Here we explore this approach experimentally, using certified reference materials (CRMs), and other raw and processed reference materials to compare the SIMQUANT method (SIngle Molecule QUANTification PCR) with quantitative real-time PCR.

Materials and methods

GM and non-GM materials

Materials used in analysis were represented by 100% RRS leaf material, provided by Matforsk (Ås, Norway), and by

CRMs produced and obtained from the European Commissions Joint Research Centre, Institute for Reference Materials and Measurements (JRC, IRMM, Geel, Belgium) series IRMM-410 (5% RRS), −411 (5% Bt-176), −412 (5% Bt-11), −413 (5% MON810), −414 (4,4% GA21) and −415 (5% NK603). Processed reference materials were obtained from the Central Science Laboratory (CSL), Proficiency Testing Group, Sand Hutton, UK, through the GeMMA proficiency programme.

Sample preparation and DNA extraction

The CRMs and processed reference materials were received homogenized while the leaf material was freeze-dried and crushed prior to DNA extraction. DNA from soybean was extracted using a commercial kit (DNeasy plant mini/maxi kit, Qiagen, Vienna, Austria) and DNA from maize was extracted with a CTAB DNA extraction protocol [8].

DNA measurements and dilution of test samples

Total mass based DNA concentrations (ng/µl) were estimated by comparison to DNA standards of known mass based concentration either using SYBR™ Green I (S9430 Sigma, St Louis, MO, USA) on the LightCycler (Roche, Paolo Alto, CA, USA) as described in [1] or by photospectrometric measurements on a NanoDrop ND-1000 V3.1.0 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Test samples were diluted to an approximate concentration of one PFU per PCR vessel/volume based on DNA mass measurements. In some cases, crude results from quantitative real-time PCR analyses were also used to estimate the original PFU concentration prior to dilution, e.g., if real-time PCR indicated that the $LOQ_{abs} > PFU > 1$. Specific dilutions are described for each material and experiment in the following.

Most probable number (MPN) calculations

The following equations are used to estimate the number of target molecules per µl DNA solution (E) and 95% confidence interval (Y):

$$E = \frac{2.303}{V} \times \log_{10}\left(\frac{n}{q}\right)$$

$$Y = \frac{2.303}{V} \times \log_{10}\left(\frac{n}{q \pm 2\sqrt{\frac{q \times (n-q)}{n}}}\right)$$

where V is the volume (µl) of template DNA solution added to the PCR in each test, n is the number of tests, and q is the number of negative results [9]. An Excel spread sheet for calculation of MPN and confidence interval is made available by the Nordic Committee on Food Analysis (NMKL) (http://www.nmkl.org/Kurs/NMKLrepeng%20201201.xls). The relative error is calculated as the difference between result and assigned value, divided by the magnitude of the assigned value.

Primers and probes

We used the primers and TaqMan® probes described by Berdal and Holst-Jensen [1], targeting the RRS event-specific 3′-junction and the soybean lectin gene, respectively. In addition, we used primer/probe sets targeting the maize housekeeping (endogenous) genes adh, hmga, zein and inv1 [7], SSIIb [10], the cauliflower mosaic virus 35S promoter (P35S) [11–13], and the following GM maize events Bt11 [12–14], GA21 [15], NK603 [16], Bt176 [16, 17] and Mon810 [10]. The probes were labeled with 5′-FAM and 3′-TAMRA. All primers and probes were purchased from DNA technology (Århus, Denmark).

Quantitative real-time and SIMQUANT PCR analyses

All the PCR reactions were performed on the ABI Prism® 7900HT (Applied Biosystems, Foster City, CA, USA) except for lectin and RRS analyses which were run on LightCycler (due to a change of instrument in the laboratory in the course of the study). For developmental purposes and expediency, and to minimize the risk of carry over contamination with amplicons corresponding to the amplicons used in quantitative real-time PCR, we performed all PCR reactions as real-time PCR amplifications (closed system with no need for post-PCR handling), although SIMQUANT is based on qualitative tests. The reaction conditions were as described for each method (see references in section "Primers and probes"), both for quantitative real-time PCR and SIMQUANT analysis. Fluorescence was monitored on channel 1 of the LightCycler using software version 3.5. For the ABI results, the software ABI SDS (Sequence Detection System) version 2.2.1 was used. All the SIMQUANT PCRs were performed with DNA template diluted to an approximate concentration of one PFU per PCR.

Results

Effect of competing DNA

We wanted to examine initially if the positive:negative ratio is influenced by adding exogenous DNA, i.e., such

Table 1 PCR on samples with an average of one DNA target molecule with and without low purity exogenous DNA

	No exogenous DNA		Exogenous DNA present	
	RRS	Lectin	RRS	Lectin
Ratio negative PCR	11/16	10/16	10/16	11/16
Average Ct	38.3	38.4	40.6	39.2
SD (Ct)	0.74	0.81	2.10	2.07

that DNA might result in more negative PCRs. Therefore, we performed four series of reactions, each with 16 parallels. Two series were set up with the RRS GM target specific primers and probe, two series were set up with the lectin reference target specific primers and probe, and from each target one series was set up with 10 ng of standard purity maize DNA (CTAB extraction) as exogenous source and one was set up without exogenous DNA (Table 1). Even though both the Ct-values and their variability was heavily influenced by the exogenous low purity DNA, no effect was seen on the qualitative results (positive:negative ratio).

Quantification of samples with low target copy numbers

Six samples of processed RRS reference materials with low absolute GM target concentration were taken from recent proficiency tests. We previously reported the GM concentration to be less than the LOQ_{pract} for most of the samples with quantitative real-time PCR. With SIMQUANT we were able to obtain quantitative GM estimates for all six samples (Table 2). The GM estimates were close to the values assigned by the organizer of the proficiency tests for all of the samples (Table 2).

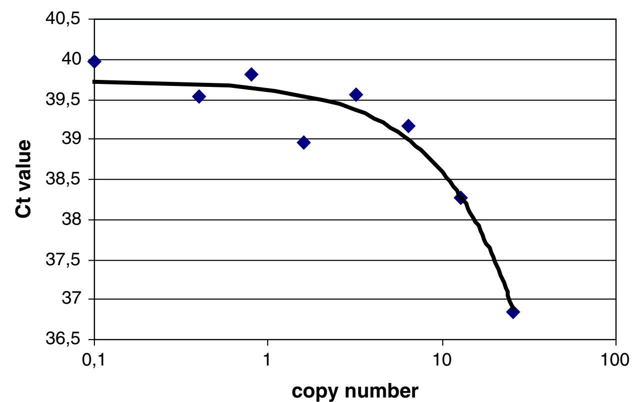

Fig. 1 Ct values depending on the copy number being analyzed (estimated template PFU per PCR ranging from approximately 0.1 to approximately 26). Each data point is the average of the Ct values of the PCR amplifications out of a total of 30 parallels yielding a PCR amplification curve

Performance of quantitative real-time PCR and SIMQUANT when analyzing low copy numbers

To evaluate the effect on the Ct-value, when the template copy number concentration approaches the smallest number of PFU that can result in detectable amplification (theoretically = 1), we performed a total of 30 reactions with each of 9 different dilutions of soybean DNA estimated to contain approximately 0.1 to approximately 26 lectin gene copies per PCR. The observed Ct-values of all positive reactions were successively scored (Fig. 1). The same series was also used to evaluate the effect of number of parallels on the reliability of the positive:negative ratio measurement, i.e., the reliability of DNA copy number estimation. This was achieved by subdividing the results into smaller subsets (Fig. 2). The observed reliability improved with increasing number of parallels.

Table 2 Comparison between quantitative real-time PCR and SIMQUANT on known RRS samples (GeMMA proficiency test samples)

Proficiency test data			SIMQUANT results		
Sample no.	Assigned value (%)	Quantitative real-time PCR	Test 1 (%) (seven parallels %)	Test 2 (%) (seven parallels %)	Pooled data[b] (%) (14 parallels %)
Rt 1	1.2	0.6%[a] (50%)	0.7 (42)	1.1 (8)	0.9 (25)
Rt 2	3.1	<LOQ	7.8 (152)	1.9 (39)	4.1 (32)
Rt 3	2.5	<LOQ	2.5 (0)	1.0 (60)	1.7 (32)
Rt 4	0.5	<LOQ	0.3 (40)	1.6 (220)	0.6 (20)
Rt 5	0.5	<LOQ	0.7 (40)	0.7 (40)	0.7 (40)
Rt 6	7.3	<LOQ	10 (37)	8.9 (22)	[c]

[a] Quantitative estimates and relative errors (absolute values)

[b] The pooled test is based on a statistical evaluation of the pooled experimental results (14 PCRs) from tests 1 and 2 in the cases where the two tests are based on the same dilution

[c] Cannot be calculated because the template DNA was differently diluted in test 1 and test 2

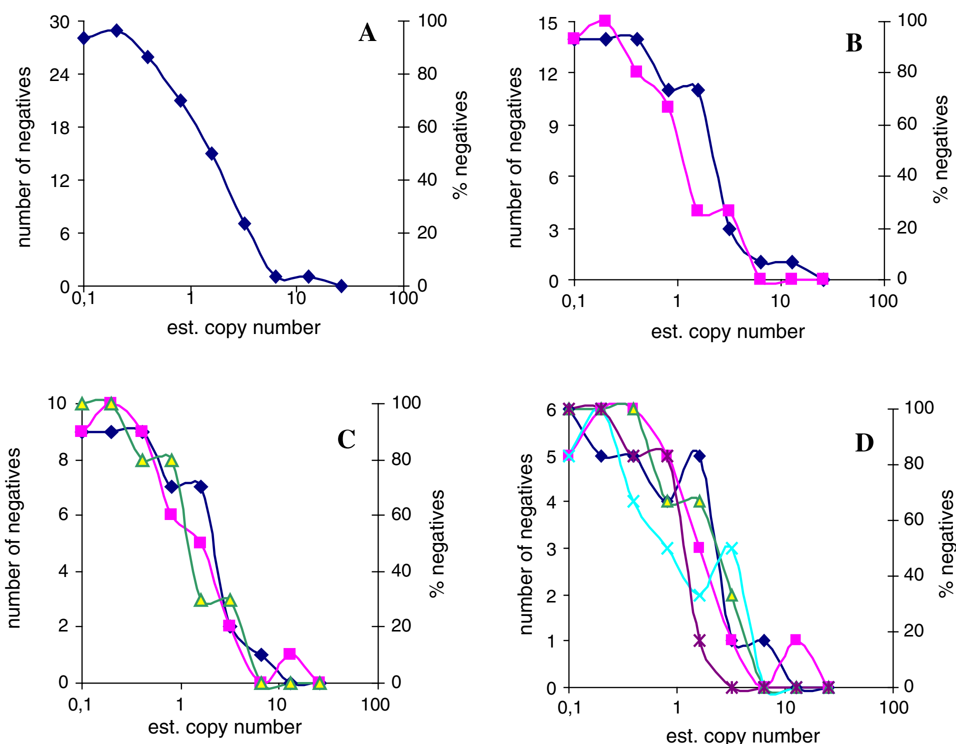

Fig. 2 Effect of the number of parallel PCR tests per concentration on reliability of quantitative estimates. A total of 30 PCR reactions were performed for each of 9 concentrations (estimated template PFU per PCR ranging from approximately 0.1 to approximately 26) of the lectin target sequence. The results were subdivided into subseries of **a** single subseries of 30 reactions per concentration; **b** two subseries of 15 reactions per concentration; **c** three subseries of ten reactions per concentration; and **d** five subseries of six reactions per concentration

Effect of the ratio of negative tests on the confidence interval

Using the MPN statistics it is possible to calculate the confidence interval of individual SIMQUANT measurements. The confidence interval (Y, see "Materials and methods") is influenced by the ratio of positive and negative parallels. The optimal ratio of positive to negative parallel PCRs is the ratio yielding the narrowest confidence interval. With the number of parallel tests set at 20, the confidence intervals were calculated theoretically (Fig. 3) and the optimal ratio was found to be approximately 14 positives for 6 negatives, or 30% negative parallels.

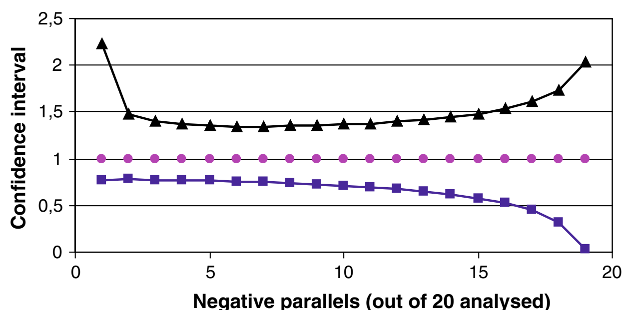

Fig. 3 Confidence interval as a function of the ratio of negative samples (out of 20 analyzed). The confidence interval is relative to MPN estimate normalized to 1. The number of negative tests is indicated along the *horizontal axis*. Upper and lower confidence limits are represented by *triangles* and *squares*, respectively

Testing the general applicability of SIMQUANT

The above use of the SIMQUANT approach was based on the RRS target and lectin reference gene. To evaluate the possibility to use SIMQUANT on other GMO related targets, we also investigated several other PCR methods used for GMO diagnostics on maize in our laboratory (under ISO 17025 accreditation). Table 3 shows the performance of these PCR methods using SIMQUANT. In addition to the *adh*1, we also tested four other presumed single- or low copy maize reference genes. Table 4 shows the observed Ct-values of the five reference genes and the SIMQUANT estimates after dilution to approximately a single haploid maize genome per PCR assay corresponding to one PFU per PCR if these are single copy genes. The observed positive:negative ratios are very similar for all the five genes (Table 4), and close to the predicted ratio if the target is present in (on average) 1 PFU per template DNA volume that is added to the PCR.

Discussion

The principles and statistics underlying SIMQUANT are the same as those used in a number of fields, e.g., in microbiological diagnostics based on MPN quantitation. Limiting dilution has previously been coupled with PCR for several applications in microbiology (e.g., [18, 19]).

Table 3 Calculation of DNA copies based on direct DNA measurement (NanoDrop) and SIMQUANT

Analyte	Copies/μl based on NanoDrop	PFU/μl based on SIMQUANT			PFU/μl average	Rel. SD (%)	Relative error (%)
		A	B	C			
Lec	96,600	86,266	97,058	76,917	86,747	11.6	−0.2
RRS	7,500	5,376	4,707	5,376	5,153	7.5	−1.3
Adh	72,000	51,612	51,612	59,899	54,374	8.8	−4.5
P35S	915	1,098	1,254	769	1,040	23.8	13.7
GA21	829	941	1,491	941	1,124	28.2	35.6
Bt11	990	686	607	867	720	18.5	−7.3
Bt176	3,340	3,650	3,104	2,103	2,952	26.6	−1.6
Mon810	1,995	2,510	2,867	2,510	2,629	7.8	31.8
NK603	1,094	1,412	788	1,872	1,357	40.1	24.1

Table 4 Comparison of gene copy-number of five commonly used endogenous reference genes using SIMQUANT

	Reference gene					Average
	adh	hmga	inv	zein	SSIIb	
Average[a] Ct value	40.7	38.8	43.9	37.1	42.1	40.5
Ratio of negative PCR[b]	10/20	10/20	11/20	11/20	11/20	10.6/20
Number of template PFU per PCR estimated with SIMQUANT	0.69	0.69	0.60	0.60	0.60	0.64

[a] The average Ct value of the positive PCRs

[b] All reference genes were analyzed using the same dilution of the DNA template. 20 parallel PCR reactions per were analyzed for each gene

Multiple qualitative testing of pooled samples is widely used in seed and grain testing for GMO, for certification and control [20], and limiting dilution is used in medicine to quantify e.g., rearranged DNA in leukemic samples [21]. The present study shows that MPN quantitation can be extended to the area of GMO analysis and comparative copy-number determination in general.

Quantitative real-time PCR relies on a linear regressional relationship between observed Ct-values and target copy (PFU) numbers. With decreasing number of PFUs in a quantitative real-time PCR, the Ct-value of positive reactions converge towards an asymptotic value, i.e., similar to the average Ct value produced by a single PFU (Fig. 1). Consequently, the linear regressional relationship is invalid and the Ct value can no longer be used as the quantity estimator. However, in the same concentration range where the Ct value becomes useless as a quantity estimator, the ratio of negative PCRs starts to provide quantitative information (Fig. 2) and MPN statistics can be applied. The principle of quantification (SIMQUANT) is then based on the relationship between positive and negative PCR test results. SIMQUANT is applicable with optimized PCR methods where a single PFU in the PCR will result in detectable amplification. All the PCR methods tested here (Tables 3, 4), which have been developed and validated using internationally accepted criteria for method validation and application in the GMO sector (see e.g. ISO 21570 for more details [10]), were found to be suitable for SIMQUANT.

A recent study on Bt11 maize [14] indicates that the PCR efficiency may be reduced and the repeatability of Ct-values may be decreased, in a real-time PCR with a low concentration of PFUs of the target in a relatively high background of non-target competing or exogenous DNA. This is a realistic situation for many food and feed products. A similar effect on PCR-efficiency and PFU estimation with real-time PCR was observed for the RRS and lectin assays in the present study (Table 1). However and notably, when each volume of the template added to individual PCRs contained on average only a single PFU, then the ratio of positive and negative qualitative PCRs was not influenced by exogenous DNA of standard purity (CTAB extraction) (Table 1), viz both the GM and lectin target were amplified with a similar frequency with and without exogenous DNA. Reduced PCR-efficiency might be caused by the inhibiting impurities or competition from the exogenous DNA, or might be due to the long-lived mismatched binding of primer and probes to the competitive DNA. Experiments have shown that the inhibition by high levels of non-target DNA can be removed by DNase I treatment [22]. Table 1 also shows that the GM assay and the lectin assay have the same sensitivity on pure

homozygous RRS material where both targets were present in equal quantities (PFU ratio 1:1).

The accuracy of the SIMQUANT estimate increases with an increased number of parallels. The actual number of parallels included in the quantitation experiments will have to be a trade-off between required accuracy and cost. Notably, the analysis can be performed in successive modules with increasing accuracy if necessary. To test this empirically, we analyzed 30 parallels of each of nine different concentrations with estimated template copy numbers ranging from 0.1 to 26 PFU per PCR. The 30 parallels were evaluated as 5 subsets of 6, 3 subsets of 10, 2 subsets of 15, and a single subset of 30 and graphs of the corresponding responses were plotted (Fig. 2). We see that the variability in positive:negative ratio resulting from the stochastic variation in distribution of target copies in the template volume increases with decreasing number of reactions per concentration. However, the additive effect of multiple series of few reactions equals the direct effect of a single series of many reactions. One consequence of this is that additional subseries can be added a posteriori when a narrow confidence interval is required, e.g., when analytical results are close to a legal threshold.

There are several ways to influence and estimate confidence intervals or standard deviations with this method. One factor already mentioned is the increased accuracy with increased number of parallels. Yet, it is possible to influence the accuracy of the test and to narrow the confidence interval, without increasing the number of parallels. Figure 3 shows the relationship between the number of negative tests and how the 95% confidence interval responds when the total number of parallel analyses is 20. The optimal ratio of positive to negative PCRs is not 1:1, but rather 7:3. This ratio is achieved when the average concentration of PFUs per PCR is approximately 1. There are also other ways to calculate measurement uncertainty and confidence interval, including theoretical approaches such as Monte Carlo simulation and empirical approaches based on experimental evaluation.

A methodological step that may introduce variability is serial dilutions [3, 23]. However, we have seen that dilution is often not necessary or necessary only to a limited degree using SIMQUANT on real-samples, e.g., as in Table 2 where the DNA solution of the GM-targets was diluted only tenfold. Indeed, SIMQUANT was developed to be used on samples when quantitative real-time PCR cannot be performed because the number of template PFU per PCR is $<LOQ_{abs}$, i.e., when the quantity estimates are associated with unacceptably wide confidence intervals. Thus, serial dilutions may not be a major source of uncertainty in practical applications of SIMQUANT.

The quantitative real-time PCR method for RRS that we used qualitatively in the SIMQUANT analyses in the present study has been used routinely in our laboratory for more than 6 years and on several hundred samples. Some samples previously analyzed in conjunction with our participation in proficiency tests organized by FAPAS/GeMMA carried out in the same period were re-analyzed with SIMQUANT. These samples were originally reported (by us) to be non-quantifiable because PFU_{GM} was less than LOQ_{abs} in the quantitative real-time PCR tests performed on DNA extracted from these samples. The results from the SIMQUANT analyses (Table 2) serve to demonstrate the satisfactory performance of SIMQUANT, as well as to demonstrate that quantitative results can be made available for samples where results cannot be obtained with quantitative real-time PCR as a consequence of the LOQ_{pract}.

Quantitative real-time PCR methods require 30–100 template PFU per PCR volume for statistical reasons [1, 3, 4] while SIMQUANT works on less than one PFU (on average) per analytical volume. Actually, the average PFU per analytical volume could go even less than 0.1 depending on the number of parallels. Furthermore, the billion-fold exponential amplification of the analyte adds considerable measurement uncertainty to quantitative real-time PCR results. SIMQUANT, on the other hand, use presence/absence testing to produce quantitative estimates. We have seen from Table 1 (and experience in the lab) that quantitative real-time PCR is more sensitive to weak PCR inhibition than SIMQUANT PCR. This is due to the fact that the Ct value is more sensitive to moderate inhibition than the qualitative positive/negative score of SIMQUANT. Indeed, this further increases the difference in LOQ_{pract} between the two approaches on samples with inhibition because quantitative real-time PCR may require dilution of the template DNA to reduce possible effects on quantity estimates from soluble inhibitors, while SIMQUANT may not require dilution.

There are two different strategies for quantitation based on multiple quantitative analyses that can be envisaged. The first possibility is to go directly to the SIMQUANT test without a prior quantitative real-time PCR. This approach can be used when prior knowledge about the sample matrix (e.g., oils or lecithin) makes it likely that quantitative real-time PCR is unfeasible, or in situations when there is no access to a real-time PCR machine. When DNA is present in adequate amounts and soluble inhibitors are not likely to be a problem, then quantitative real-time PCR will remain the golden standard for GMO quantification in the foreseeable future. Thus, samples going for GMO quantification will be routinely analyzed using quantitative real-time PCR. If initial analyses using quantitative real-time PCR indicate that the DNA contains too few target PFUs for reliable quantification with real-time PCR, then the analysis can be continued using SIMQUANT as a

second step. In such situations there will already be some useful information available regarding the approximate amount of GM and reference target PFU, how much DNA should be diluted, if necessary, to obtain an approximate concentration of one PFU per volume going to SIMQUANT PCR. The results from quantitative real-time PCR on the reference target may also facilitate control of presence/absence of PCR inhibitors.

A relatively low number of parallels (e.g., 7) will often be sufficient to conclude on the GMO content relative to legal thresholds. However, if the GMO content is close to the legal threshold, or a relatively narrow confidence interval is required, then the number of parallels should be increased or the DNA concentration should be adjusted to approach the optimal positive/negative ratio of 7:3. The parallels may be set up using DNA from a single extraction, but preferably should be set up with DNA from more than one extraction to reduce uncertainty introduced from DNA extraction. We suggest, e.g., performing seven parallel tests from each of two DNA extractions.

Although, the SIMQUANT method has been developed to solve problems related to analyzing GMO samples with few PCR targets, other uses can be foreseen. One such use is the determination of gene copy numbers of different genes in an organism. Currently, the Southern blotting technique is commonly applied to estimate the copy number of a specific gene. A single band in a Southern blot is usually interpreted as evidence that the gene is represented only once in the haploid genome. However, several species including maize have been through evolutionary chromosome duplications and polyploidisation events and in such cases a single band on a Southern blot may be misleading because gene-copies on duplicated chromosomes may yield the same fragment after digestion with restriction enzymes. To test if SIMQUANT could be used to establish the relative gene copy number, we investigated five different maize reference genes presumed to be single or low copy genes [7]. When these genes are analyzed using quantitative real-time PCR methods within their analytical range, the Ct values differs by as much as four cycles at the same DNA concentrations ([7]; Table 4). The large difference in Ct values does not influence quantification based on SIMQUANT (Table 4), and the SIMQUANT data confirmed that all the genes are single copy genes. Moreover, this shows that SIMQUANT also may be used to provide information about the number of gene-copies of different genes in a genome.

The present study describes and evaluates an alternative approach for GMO quantification with the potential for more than a 100-fold reduction in the practical limit of quantification. The SIMQUANT approach is not dependent on expensive real-time PCR equipment and thus may be of particular interest where labor costs are low but equipment availability is limiting, e.g., in developing countries. The approach is based on multiple parallel qualitative tests and on the ratio of positive and negative PCR reactions. This approach yielded acceptable results for the seven GMO related sequences and six reference gene sequence PCR methods investigated in this study. From a theoretical point of view SIMQUANT could also be performed with conventional PCR followed e.g., by gel electrophoresis, but we chose to perform the tests using real-time PCR because (1) this reduced the risk of carry over contamination with amplified DNA considerably, (2) it reduced the need for working with carcinogenic ethidium bromide stains, (3) it improved the detectability of very short amplicons and this is important in highly processed products where the DNA is known to be highly degraded into short fragments, and (4) the real-time assay included a TaqMan probe that discriminates between true and false positive amplification signals.

Acknowledgements This study was financially supported by the European Commission through the FP5 Quality of Life program "Qpcrgmofood" project (contract no. QLK1-1999-01301) and the FP6 Integrated project "CoExtra" (contract no. 007158), and by the Research Council of Norway (grant no. 170363). We would also like to thank Heidi Olsen for technical assistance.

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