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Toward Metrological Traceability for DNA Fragment Ratios in GM Quantification. 3. Suitability of DNA Calibrants Studied with a MON 810 Corn Model

DIANA CHAREL S,* SYLVIA BROEDERS, PHILIPPE CORBISIER, STEFANIE TRAPMANN, HEINZ SCHIMMEL, AND HENDRIK EMONS

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, IRMM, Retieseweg 111, 2440 Geel, Belgium

The quantification of GMOs by real-time PCR relies on an external calibrant. In this paper the suitability of two DNA calibrants, genomic DNA from plant leaves and plasmidic DNA, was investigated. The PCR efficiencies, the correlation coefficients of the calibration curves, and the ratios between PCR efficiencies of transgenic and endogenous sequences were compared for both calibrants using 59 data sets produced by 43 laboratories. There were no significant differences between plasmidic and genomic DNA except for the PCR efficiencies of the calibration curves for the transgene of the construct-specific real-time PCR method. In the GM system investigated, PCR efficiencies of plasmidic calibrants were slightly closer to the PCR efficiencies observed for the unknowns than those of the genomic DNA calibrant. Therefore, plasmidic DNA was the more suitable calibrant for the PCR measurements on genomic DNA extracted from MON 810 seeds. It is shown that plasmidic DNA is an appropriate choice for the calibration of measurements of MON 810 corn with respect to the DNA copy number ratio.

KEYWORDS: Certified Reference Material; CRM; genetically modified organism; GMO; DNA calibrant; calibration; plasmid; real-time PCR; PCR efficiency; measurement uncertainty

INTRODUCTION

Metrological traceability of quantitative measurement results of DNA fragments used for the determination of the genetically modified (GM) content in food and feed is required to achieve a reliable and consequently also comparable quantification of such fragments. Therefore, the general aim of this publication series is to describe systematic studies of metrological traceability in GM measurements and to assess influencing quantities eventually leading to a bias in the measurement results. Metrological traceability refers to "the property of a measurement result relating the result to a stated metrological reference through an unbroken chain of calibrations of a measuring system or comparisons, each contributing to the stated measurement uncertainty" (*1*). Results reported in part 2 indicated that the calibrant plays a major role in the real-time Polymerase Chain Reaction (PCR) measurement of genetically modified organisms (GMOs). Therefore, this paper aims to assess the effect of the type of DNA calibrant, namely, plasmidic DNA (pDNA) or genomic DNA (gDNA), on GM quantification by using them for the calibration of real-time PCR measurements. Different aspects of gDNA and pDNA calibrants have been compared in papers published earlier (*2*, *3*). However, a systematic study of the suitability of a calibrant was not reported until now. In particular, the equivalence of the behavior of the calibrant and the specific analytical sample in a real-time PCR reaction had still to be proven. To assess suitability, the closeness of agreement between the PCR efficiencies of the potential DNA calibrant and the PCR efficiencies obtained for DNA extracted from unknown samples has to be analyzed.

The study had to provide a sufficiently large number of measurement data to establish the basis for testing the statistical significance of various influencing quantities. This also allowed defining a strategy for the certification of the GM reference materials (RMs) with respect to their DNA copy number ratio. In addition, this systematic set of data was exploited for evaluating the expanded combined standard uncertainty associated with a certified value expressed in copy number ratios.

MATERIALS AND METHODS

The reader is referred to part 2 of this publication series for a detailed description of the experimental setup and methodology of the interlaboratory study with 43 participating laboratories and a total of 268 real-time PCR experiments. Four seed powder materials, containing different mass fractions of the GM event MON 810 corn (*Zea mays*) (mass fractions of 0.8, 1.5, 3.8, and 4.5%), were used in this study as unknowns and were analyzed using three DNA extraction and two real-time PCR methods (*4*, *5*).

The GM content of a sample is determined in real-time PCR relative to an endogenous reference. Two calibration curves, namely, transgenic

* Corresponding author (telephone +32 14 571 884; fax +32 14 571 548; e-mail diana.charels@ec.europa.eu).

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Table 1. Comparison of the PCR Efficiencies (ϵ) of gDNA Calibrants, pDNA Calibrants, and gDNA Extracted from Unknown Samples (uDNA) by Means of Two-Sided *t* Tests for the Construct-Specific (**A**) and Event-Specific (**B**) Real-Time PCR Methods[a]

		mean ϵ and SD (%)			variance (%)			*p* for difference in ϵ		
		unknown samples	calibrant		calibrant					
target sequence		uDNA	pDNA	gDNA	pDNA	gDNA	*n*	between pDNA and gDNA	between pDNA and uDNA	between gDNA and uDNA
				(**A**) Construct-Specific Method						
endogenous	*zSSIIb*	96.5 ± 8.0	93.4 ± 4.1	91.1 ± 5.3	0.14	0.23	29	0.05[b]	0.07[c]	3×10^{-3}[b]
transgenic	*hsp70/cryIA(b)*	98.0 ± 6.8	94.3 ± 3.7	90.6 ± 5.4	0.07	0.23	29	8×10^{-4}[c]	0.01[c]	3×10^{-5}[c]
				(**B**) Event-Specific Method						
endogenous	*hmg*	96.0 ± 7.7	95.4 ± 3.7	94.5 ± 4.4	0.11	0.13	30	0.3[b]	0.6[c]	0.2[b]
transgenic	plant/P35S junction	92.1 ± 6.9	90.1 ± 5.3	87.4 ± 5.7	0.15	0.26	30	0.03[b]	0.1[c]	6×10^{-3}[c]

[a] uDNA refers to the PCR efficiency derived from dilution of the unknown samples; *n* indicates the number of data sets; *p* indicates probability. [b] Two-sided *t* tests assuming equal variances were performed as the *F* tests showed no significant differences between variances. [c] Two-sided *t* tests assuming unequal variances were performed as the *F* tests revealed significant differences between variances.

and endogenous calibration curves, were set up for each DNA calibrant as a consequence of this relative quantification. To ensure that the values measured for the unknown samples fall in the range covered by the calibration curves, low- and high-range dilution series were prepared with the gDNA extracted from leaves and with pDNA. The slope and the PCR efficiency are related according to the equation

$$\epsilon = 10^{-1/\text{slope}} - 1 \quad (1)$$

based on the conventional real-time PCR theory (*6*) with ϵ = efficiency of the PCR reaction.

In a first step, the similarity of the transgenic and the endogenous calibration curves obtained with each type of calibrant was investigated by comparing the PCR efficiencies via the slopes of the curves, the correlation coefficients of the two calibration curves, and the ratio between PCR efficiencies of transgenic and endogenous sequences. In a second step, the suitability of the calibrant was investigated by comparing the PCR efficiencies of the calibration curves with the curves obtained from the dilution of the unknown samples under investigation. The PCR efficiencies obtained by diluting the gDNA extracted from ground seeds were compared with the PCR efficiencies obtained by dilution of the pDNA and gDNA calibrants. *F* tests were performed to investigate if variances between groups were similar. When the *F* test revealed significant differences, a *t* test assuming unequal variances was performed. Otherwise, two-sided *t* tests assuming equal variances were used to investigate if the differences between certain groups were significant. In addition, multiple alignment Tukey and Newman−Keuls tests were performed to compare the PCR efficiencies of unknown samples and pDNA and gDNA calibrants.

RESULTS

PCR Efficiencies of the Calibrants. Plasmidic calibrants yielded slightly higher PCR efficiencies for the construct-specific and event-specific real-time PCR detection methods than the gDNA calibrants extracted from leaves (**Table 1**). The PCR efficiencies estimated for the transgenic targets of both PCR methods were slightly less optimal when using gDNA (90.6 and 87.4% versus 94.3 and 90.1% for the gDNA and pDNA calibration curves of the construct- and event-specific detection methods, respectively). No significant differences between PCR efficiencies of gDNA and pDNA calibrants were found for the endogenous targets *zSSIIb* (probability $p = 0.052$) and *hmg* ($p = 0.3$) of both real-time PCR detection methods (**Table 1**). However, there was a significant influence of the type of the DNA calibrant on the PCR efficiency of the transgenic target sequences *hsp70/cryIA(b)* ($p = 8 \times 10^{-4}$) and the plant/P35S junction ($p = 0.03$) specific for the construct- and event-specific detection methods, respectively (**Table 1**).

Table 2. *t* Tests To Compare R^2 Coefficients of pDNA and gDNA Calibration Curves for the Construct-Specific (**A**) and Event-Specific (**B**) Real-Time PCR Methods[a]

	mean R^2		variance			
	pDNA	gDNA	pDNA	gDNA	*n*	*p*
			(**A**) Construct-Specific Method			
zSSIIb	0.998	0.998	1.02×10^{-5}	5.03×10^{-6}	29	0.5[b,c]
hsp70/cryIA(b)	0.997	0.998	1.07×10^{-5}	4.66×10^{-6}	29	0.5[b,c]
			(**B**) Event-Specific Method			
hmg	0.999	0.999	4.02×10^{-7}	1.10×10^{-6}	30	0.5[b,c]
plant/P35S junction	0.998	0.998	2.90×10^{-6}	2.04×10^{-6}	30	0.5[b,c]

[a] *n* indicates the number of data sets and *p* the probability. [b] At a 95% confidence level, two-sided *t* tests assuming unequal variances were performed as the *F* tests revealed significant differences between variances. [c] At a 99% confidence level, two-sided *t* tests assuming equal variances were performed as the *F* tests showed no significant differences between variances.

Comparison of Ratios of PCR Efficiencies. The calibration curves obtained by diluting the transgenic and the endogenous targets for gDNA and pDNA were compared with each other. To allow this comparison the ratio of the PCR efficiencies for both targets was calculated. In theory, this ratio is equal to 1 if the PCR efficiencies of both targets are identical. This ratio should also be equal to 1 if gDNA and pDNA express similar behaviors in the measurement process. Various *t* tests were performed to compare the ratios of PCR efficiencies of the gDNA calibrants with those of the pDNA calibrants. For both detection methods, a comparison of the ratios of the PCR efficiencies did not reveal any significant differences between pDNA and gDNA calibrants ($p = 0.2$ in both cases). For the construct- and event-specific detection methods the transgene to endogene ratios of PCR efficiencies of the unknown samples (1.02, 0.96) were slightly closer to the ratios obtained for pDNA calibrants (1.01, 0.94) than to those of the gDNA calibrant (1.00, 0.93).

Comparison of Correlation Coefficients of DNA Calibration Curves. The correlation coefficient (R^2) provides information about the fitting of data to a linear calibration curve. A comparison of the correlation coefficients of pDNA and gDNA calibration curves did not show significant differences for both PCR methods ($p = 0.5$ for all targeted sequences) (**Table 2**). However, it must be mentioned that entire data sets from single laboratories exhibiting a R^2 value below 0.98 for one of the calibration curves had been excluded beforehand as recommended in the method validation guidelines of the Community

Table 3. Differences in PCR Efficiencies between DNA Calibrants (gDNA, pDNA) and gDNA Extracted from Unknown Samples (uDNA) for the Construct-Specific (**A**) and Event-Specific (**B**) Real-Time PCR Methods[a]

n	DNA extraction method	target	average difference between gDNA and uDNA (%)	SD (%)	average difference between pDNA and uDNA (%)	SD (%)
			(**A**) Construct-Specific Method			
10	CTAB (1)	*zSSIIb*	–4.65	8.91	–3.10	10.31
9	Wizard (2)	*zSSIIb*	–4.30	8.81	–2.85	8.72
10	GENE*Spin* (3)	*zSSIIb*	–6.96	6.08	–3.31	6.78
29	all combined	*zSSIIb*	–5.34	7.81	–3.09	8.41
10	CTAB (1)	*hsp70/cryIA(b)*	–5.67	5.17	–2.40	4.81
9	Wizard (2)	*hsp70/cryIA(b)*	–7.62	9.17	–2.27	7.24
10	GENE*Spin* (3)	*hsp70/cryIA(b)*	–8.88	9.30	–6.09	9.04
29	all combined	*hsp70/cryIA(b)*	–7.38	7.89	–3.63	7.21
			(**B**) Event-Specific Method			
10	CTAB (4)	*hmg*	–4.21	6.32	–2.34	7.06
10	Wizard (5)	*hmg*	1.87	3.56	2.35	3.79
10	GENE*Spin* (6)	*hmg*	–3.75	12.57	–2.44	11.92
30	all combined	*hmg*	–1.96	8.62	–0.76	8.36
10	CTAB (4)	plant/P35S junction	–2.32	9.80	–0.06	6.08
10	Wizard (5)	plant/P35S junction	–4.68	4.83	–2.28	5.61
10	GENE*Spin* (6)	plant/P35S junction	–6.69	11.18	–4.73	11.52
30	all combined	plant/P35S junction	–4.64	8.85	–2.44	8.19

[a] *n*, number of data sets; SD, standard deviation. The method combination number (part 2) is indicated in parentheses (column 2).

Table 4. Probability *p* for Differences in PCR Efficiencies Calculated by Multiple Alignments of PCR Efficiencies (ϵ) of pDNA Calibrants, gDNA Calibrants, and Unknown Samples (uDNA)

	p for difference in ϵ					
	Tukey			Newman–Keuls		
	between pDNA and gDNA	between pDNA and uDNA	between gDNA and uDNA	between pDNA and gDNA	between pDNA and uDNA	between gDNA and uDNA
zSSIIb	0.3	0.1	2×10^{-3}	0.1	0.045	2×10^{-3}
hsp70/cryIA(b)	0.02	0.03	1×10^{-4}	8×10^{-3}	0.01	1×10^{-4}
hmg	0.6	0.8	0.3	0.4	0.6	0.3
plant/P35S junction	0.3	0.2	6×10^{-3}	0.1	0.1	6×10^{-3}

Reference Laboratory for GM Food and Feed (*7*). Such a low correlation coefficient was not accepted within this study as it may reflect erroneous dilutions or inappropriate PCR amplification (part 2). pDNA and gDNA calibrants provided the same good linearity of calibration curves.

Totals of 58 and 60 calibration curves were compared for the construct- and event-specific detection methods, respectively; 95% (pDNA) and 93% (gDNA) of the calibration curves were linear for the construct-specific detection method in the concentration ranges of 10−40000 and 5−5000 cp for pDNA and gDNA calibrants, respectively. The calibration curves for gDNA were linear for higher dilution levels compared to pDNA for the event-specific method. However, such an observation should be interpreted with care as the nominal copy numbers are prone to error due to, for example, mistakes in the estimation of the genome size and the DNA quantification.

Comparison of PCR Efficiencies of Unknown Samples with gDNA and pDNA Calibrants. The average difference of PCR efficiencies between pDNA/gDNA calibrants and unknown seed powder samples was compared for each method combination of DNA extraction and real-time PCR. The differences in PCR efficiencies of the calibration curves between gDNA calibrants and the unknown powder samples were with one exception always slightly larger compared to the difference between the pDNA calibrants and the unknown powder samples (**Table 3**). When two-sided *t* tests were applied, the differences of the PCR efficiencies of each type of calibrant to the unknown powder samples were statistically not significant (results not shown).

In addition, PCR efficiencies of each DNA calibrant and the unknown samples were compared using *t* tests. With the exception of the transgenic target *hsp70/cryIA(b)* ($p = 0.01$) no significant differences were found between PCR efficiencies of pDNA calibrants and unknown samples ($p = 0.07$, 0.6, and 0.1 for *zSSIIb*, *hmg*, and plant/P35S junction, respectively) (**Table 1**). The PCR efficiencies of gDNA calibrants and unknown samples differed significantly for the target sequences *zSSIIb* ($p = 3 \times 10^{-3}$), *hsp70/cryIA(b)* ($p = 3 \times 10^{-5}$), and plant/P35S junction ($p = 6 \times 10^{-3}$), but not for the *hmg* sequence ($p = 0.2$) (**Table 1**). Subsequent multiple alignments of the PCR efficiencies of pDNA, gDNA, and unknown samples are depicted in **Table 4**. Tukey and Newman−Keuls tests confirmed the observations of the two-sided *t* tests summarized in **Table 1** except for two comparisons. For the endogenous *zSSIIb* sequence Newman−Keuls showed a significant difference between the pDNA calibrant and the unknown samples ($p = 0.045$), whereas Tukey ($p = 0.1$) and the *t* test ($p = 0.07$) did not give such an indication (**Tables 1** and **4**). There was no effect of the type of calibrant on the PCR efficiency for the transgenic plant/P35S junction target sequence ($p = 0.3$ and 0.1 for Tukey and Newman−Keuls, respectively), but a significant difference between both calibrants was noted for the *t* test ($p = 0.03$).

The average DNA copy number ratio was determined for each method combination of DNA extraction and real-time PCR. Calibration with gDNA extracted from leaves yielded average values that were 17−27% higher than those obtained with pDNA (**Table 5**). Moreover, it was reported in part 2 of this

Table 5. Overview of the Difference in Copy Number Ratios Obtained with pDNA and gDNA Calibrants for Each PCR Method

detection method	mass fraction (%)	gDNA (cp/cp)	pDNA (cp/cp)	difference[a] (%)
construct-specific	0.8	0.59	0.46	27
	1.5	1.1	0.87	23
	3.8	2.5	2.1	19
	4.5	3.0	2.6	17
event-specific	0.8	0.55	0.45	21
	1.5	0.99	0.81	22
	3.8	2.4	2.1	17
	4.5	2.9	2.4	18

[a] The difference in copy number ratios was calculated according to the following formula: difference = (copy number ratio gDNA) − copy number ratio pDNA/copy number ratio pDNA (eq 2).

series that the measurement results originating from the construct-specific detection method showed for each type of DNA calibrant a dependence of the DNA extraction method. As there was no detectable influence from the DNA extraction method for the event-specific detection method, it was possible to combine the measurement results of the three corresponding DNA extraction methods. It was also reported in part 2 that the results obtained by using plasmids for calibration showed frequently a smaller standard deviation than those originating from genomic calibration curves. Because measurements on the same extracts from the unknowns were calibrated with the two types of DNA, a smaller standard deviation on the results using pDNA calibrants indicates better reproducibility of calibration curves based on pDNA calibrants compared to gDNA calibrants.

The type of DNA calibrant used in this study had a significant impact on the PCR efficiency of the transgenic target *hsp70/cryIA(b)* in the MON 810 model (**Tables 1** and **4**). However, there is no evidence that these findings can be generalized. The calibrant has to behave similarly to the specific analytical sample to obtain measurement results that are close to the true value. The PCR efficiencies of pDNA calibrants were closer to the PCR efficiencies of the unknown sample than the ones for gDNA calibrants (**Table 1**). For the event-specific method, the pDNA calibrant and the unknown sample behave similarly because no statistically significant differences were observed (**Tables 1** and **4**). On the contrary, there was a significant difference between the PCR efficiencies of the transgenic target *hsp70/cryIA(b)* for the pDNA calibrant and the unknown sample for the construct-specific method (**Tables 1** and **4**).

Certification of Materials for Their DNA Copy Number Ratio. The expanded combined standard uncertainty of the certified value is an important quality parameter of certified reference materials. Therefore, the uncertainty, which can be expected for certified values of copy number ratios in matrix RMs, was calculated in a theoretical exercise on the basis of results obtained during this and other studies. As pDNA seemed to be the most suitable calibrant for the MON 810 corn model, the average copy number ratios and their uncertainties were calculated from the data of the event-specific detection method calibrated with pDNA and using all three DNA extraction methods (**Table 6**).

In general, the expanded combined uncertainty is calculated according to the following formula and comprises contributions from the value assignment (characterization), the homogeneity, and the stability (*8*)

$$U_{\mathrm{CRM}} = k(u^2_{\mathrm{char}} + u^2_{\mathrm{bb}} + u^2_{\mathrm{lts}})^{1/2} \qquad (3)$$

with k = coverage factor, u_{char} = uncertainty contribution from the characterization, u_{bb} = uncertainty contribution from the homogeneity study, and u_{lts} = uncertainty contribution from the stability study.

Different matrix powder GM materials from corn have been produced at IRMM and are undergoing regular long-term stability monitoring. It turned out that they possess identical stability properties. Therefore, the uncertainty contribution for the long-term stability could be taken from a previous certification study (relative uncertainty contribution of 1−8% for a time interval of 24 months and GM fractions between 0.8 and 4.5% m/m) (*9*). A further contribution to the combined uncertainty comes from the homogeneity assessment. Its relative value was estimated between 9.8 and 3.2% for GM mass fractions between 1 and 10% (*10*). Therefore, the expanded uncertainty U_{CRM} for certified values can be expected to be 0.2−0.3, which corresponds to a relative uncertainty of 9−27% for the different GM concentrations (**Table 6**).

DISCUSSION

Higher copy number ratios were obtained in several cases when gDNA was used for the calibration of real-time PCR measurements. Therefore, it has been investigated which type of calibrant has properties that are closest to the properties of the DNA extracted from the unknown samples. As there are currently no matrix RMs available that are certified for their DNA copy number ratio, the absolute true copy number cannot be determined and can be approached only in an iterative process. An estimation of copy number ratios in different corn varieties has been published (*12*). These data are based on one DNA extraction method. Therefore, the assumptions made cannot be generalized as the application of a different DNA extraction method may influence the relative extractability of different tissue types and could therefore shift the estimated copy number ratio. As it has been shown in our study (part 2) that the copy number ratio determined by PCR may depend on the DNA extraction method applied when different DNA extraction methods are used, a bias cannot be excluded. In the MON 810 corn model, calibration with pDNA provided values closer to the estimated copy number ratio according to Holst-Jensen et al. (*12*) compared to calibration with gDNA (part 2).

The question of whether a certain calibrant leads to measurement results close to the true value can be answered only on the basis of an investigation of well-characterized known samples. In the classic metrological approach for complex samples (e.g., for element quantifications in foodstuff) the trueness would preferably be assessed through the use of more than one independent method based on different measurement principles for quantification. This is, however, not possible in the case of measurements for the MON 810 corn model as it has been shown here that only one quantitative event-specific real-time PCR method can be used. Consequently, the true value can be approached only in a stepwise manner by trying to identify and eliminate any bias. A suitable DNA calibrant has to behave similarly to the sample under investigation, which is a prerequisite for correct measurement results. For GM quantification by real-time PCR, suitability of the calibrant can be assessed, on the one hand, by comparing the calibration curves with the curves obtained for the diluted unknown samples. On the other hand, several characteristics such as the slope, correlation coefficient, and ratios of PCR efficiencies of calibration curves need to be compared. Through the use of a multiparametric approach the properties of the DNA calibrant can be determined. A comparison of the aforementioned

Table 6. Average Copy Number Ratios and Expanded Combined Uncertainties (U_{CRM}, $k = 2$) for the Event-Specific Detection Method, Obtained with a pDNA Calibrant[a]

mass fraction (%)	average copy no. ratio	relative standard uncertainty contribution			U_{rel} (%)	U_{CRM}[e] (copy no. ratio)
		homogeneity[b] (%)	stability[c] (%)	characterization[d] (%)		
0.8	**0.45**	9.8	8.3	3.9	27	**0.2**
1.5	**0.81**	9.8	4.4	2.7	22	**0.2**
3.8	**2.1**	3.2	1.8	3.4	10	**0.2**
4.5	**2.4**	3.2	1.5	2.9	9	**0.3**

[a] U_{rel} refers to the relative expanded combined uncertainty. [b] The uncertainty contribution from the homogeneity is relative to a mass fraction and was determined using data from a previous study (*10*). [c] This uncertainty contribution was estimated using data from previous long-term stability studies (*9*) and is expressed relative to a mass fraction. [d] The uncertainty contribution was assessed using data from this study and is expressed relative to a copy number ratio. [e] U_{CRM} is calculated according to the following formula: $U_{CRM} = k(u^2_{char} + u^2_{bb} + u^2_{lts})^{1/2}$ (eq 3), with $k = 2$ corresponding to a level of confidence of about 95% (*11*).

characteristics of DNA calibration curves has been performed previously by Taverniers et al. (*2*), but their finding that the *t* test did not reveal any significant differences related to the comparison of slopes, *y* intercepts, and correlation coefficients between gDNA and pDNA calibration curves may not be representative due to the limited number of calibration curves compared ($n = 4$).

In the study reported here, statistical analyses have shown that pDNA and gDNA calibrants behave in a similar way for the parameters studied (**Tables 2** and **3**) with the exception of the PCR efficiency of the transgenic target sequence *hsp70/cryIA(b)* (**Tables 1** and **4**). However, the DNA copy number ratio of transgenic to endogenous target sequences needs also to be taken into account because the GM content is expressed relative to an endogenous reference. There was no significant difference between both calibrants with respect to the ratios of PCR efficiencies of the transgenic to the endogenous target sequence. However, even though the ratios of the PCR efficiencies for the two target sequences might be identical, the individual PCR efficiency of each target sequence has a significant impact on GM quantification by real-time PCR. There is a significant difference, with respect to the PCR efficiency of the transgenic target sequences, between gDNA and pDNA calibrants for the construct-specific real-time PCR detection method (**Tables 1** and **4**). The effect of just a 4% difference in PCR efficiencies of the transgenic target sequence on GM quantification by real-time PCR can generate a much larger difference in copy number due to the exponential nature of the amplification within PCR measurements (*13*). Consequently, such a small difference in PCR efficiencies of pDNA and gDNA calibrants may explain the range of overestimation (17−27%) observed when the average values obtained are compared (**Table 5**). There are, however, two prerequisites to identify the impact of the PCR efficiency on the measurement results. A large systematic set of data has to be available for calculations, and the calibration curves should be linear.

Nogva and Rudi (*14*) hypothesized that the copy number quantification could be influenced by a difference in PCR amplification efficiencies of different lengths of fragments during the first cycles of PCR. They suggested that smaller DNA fragments generated during the amplification are more easily accessible for the polymerase. This is apparently not the case in the current study. Indeed, the PCR efficiencies obtained from dilution of gDNA extracted from unknown seed powder samples were higher than the PCR efficiencies of the similarly sized gDNA calibrant extracted from leaves (**Table 1**). The lower PCR efficiency noted for the gDNA calibrant extracted from leaves may be due to the influence of substances coextracted with DNA and/or components of DNA isolation buffers (*15*). A possible reason for the difference in PCR efficiencies of gDNA and pDNA calibrants could be the solution used for the dilution of DNA calibrants. The presence of background DNA for pDNA calibrants could prevent the unspecific interaction of target DNA sequences with the walls of the vial, whereas gDNA diluted in nuclease-free water could be prone to such effects for the lower concentration levels.

Acknowledging the significance of the PCR efficiencies for the quantification by real-time PCR, the PCR efficiencies of the two types of DNA calibrants were compared with the PCR efficiencies of the gDNA extracted from unknown seed powder samples (**Tables 3** and **4**). The PCR efficiencies of the extracts from the unknowns were estimated from a dilution series of the extracts (2× and 5× dilutions). The differences in PCR efficiencies between the pDNA calibrant and unknown samples were with one exception smaller than those between gDNA and the unknowns. The smallest difference between the PCR efficiencies of the calibrant and the analytical sample is likely to give copy number ratios close to the true value. Although tempting, it is not possible to select the best-suited DNA extraction method on the basis of the results displayed in **Table 3**. Judging from the smallest difference in PCR efficiencies, one would choose the cetyl trimethyl ammonium bromide (CTAB) DNA extraction method (*16*) for the extraction of gDNA from unknown samples when the event-specific real-time PCR detection method and the pDNA calibrant are applied (**Table 3**). A comparison of the copy number ratios for this PCR method obtained with each DNA extraction method, however, shows that all three DNA extraction methods perform equally well (part 2). It is not possible to select the "ideal" DNA extraction method that gives copy number ratios closest to the true value on the basis of the difference between the PCR efficiencies of the calibrant and the unknown sample within the measurement variability. Because the number of data sets for each DNA extraction method ($n = 9-10$) is too limited, the differences in PCR efficiencies between the pDNA calibrant and the unknown sample are statistically not significant. One would have to investigate the suitability of the different calibrants and extraction methods on a case by case basis. Considering all data from one PCR method, the differences in PCR efficiencies between the gDNA calibrant and the unknown sample are always larger [−5.3 versus −3.1%, −7.4 versus −3.6%, −2.0 versus −0.8%, and −4.6 versus −2.4% for the *zSSIIb*, *hsp70/cryIA(b)*, *hmg*, and plant/P35S junction targets of the construct- and event-specific methods, respectively] than those between the pDNA calibrant and the unknown sample (**Table 3**).

In this study a significant difference between the PCR efficiencies of the transgenic target sequence from pDNA and gDNA calibrants was encountered when all data sets from the construct-specific PCR method were pooled (**Table 1**). Moreover, it was shown that the gDNA calibrant behaves in a different way compared to the sample under investigation for

three of four target sequences (**Table 4**). Altogether our results indicate that pDNA is a suitable calibrant for the event-specific PCR method for the MON 810 corn model. The data from the construct-specific method were not taken into account because of the dependence of the copy number ratio on the DNA extraction method applied and the significant difference between the PCR efficiencies of the pDNA calibrant and the analytical sample for the transgenic target sequence *hsp70/cryIA(b)* (**Tables 1** and **4**). Consequently, the uncertainty on the DNA copy number ratio to be expected for a MON 810 certified reference material was estimated by applying the data obtained within this and other studies and on the basis of calibration with pDNA. The relative expanded combined standard uncertainty amounted up to 27% for a CRM with a GM mass fraction in the range of 0.8−4.5%. For this estimation, contributions to the combined uncertainty from the characterization of the material by real-time PCR, the homogeneity, and the stability of the material were considered and are almost equally distributed. It turns out that an increase of the relative uncertainty of a certified value stating the DNA copy number ratio in the matrix RM will be unavoidable in comparison to the certified value for the mass fraction in the same RM.

This study has addressed the measurement unit for the expression of results of GM quantification. The certified value of existing GM RMs is currently expressed as a mass fraction. After characterization and certification of the existing RMs with respect to their copy number ratio, the corresponding CRMs will carry information about their GM content expressed in both measurement units. This would support also the European Commission Recommendation 2004/787/EC (*17*).

There are various reasons why the values for the DNA copy number ratio will need to be established for each RM on a case by case basis. For instance, there is a dependence of the GM content of corn expressed as a mass fraction on the genetic composition of the seed samples (*18*, *19*). The endosperm of corn is a triploid tissue. The GM content of this tissue will depend on the parental origin of the donor (i.e., ♂ or ♀) of the GM trait. In addition, the DNA content of the endosperm changes during the ripening process of the kernel (*20*). Moreover, it has been shown that processing of the materials can cause DNA degradation resulting in the measurement of different copy number ratios (*21*, *22*). Anyhow, the results of the current study have revealed the influence of the type of DNA calibrant on the measurement result of GM quantification by real-time PCR. The data are used to define a detailed strategy for the certification of GM RMs with respect to their copy number ratio. Moreover, the data indicate that studies have to be carried out for each RM and its associated real-time PCR detection method, to allow the selection of an appropriate DNA extraction method and to choose the suitable type of DNA calibrant.

ABBREVIATIONS USED

CRM, Certified Reference Material; CTAB, cetyl trimethyl ammonium bromide; Ct, cycle threshold; DNA, deoxyribonucleic acid; ϵ, efficiency of the PCR reaction; gDNA, genomic DNA; GM, genetically modified; GMO, genetically modified organism(s); *hmg*, high mobility group gene; *hsp70/cryIA(b)*, junction region between the no. 1 intron sequence of the heat shock protein 70 gene and the *Bacillus thuringiensis* gene encoding CryIA(b); IRMM, Institute for Reference Materials and Measurements; *n*, number of data sets; plant/P35S junction, junction between the integration-border region of the plant genomic sequence and the inserted sequence element originating from the cauliflower mosaic virus 35S promoter; pDNA, plasmidic DNA; *k*, coverage factor; *p*, probability; PCR, Polymerase Chain Reaction; RM, reference material; R^2, correlation coefficient, SD, standard deviation; u_{bb}, uncertainty contribution from the homogeneity study; u_{char}, uncertainty contribution from the characterization; u_{lts}, uncertainty contribution from the stability study; U_{CRM}, expanded combined uncertainty; uDNA, gDNA extracted from unknown samples; *zSSIIb*, *Zea* starch synthase IIb gene.

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