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CERTIFICATION REPORT

Certification of a Maize 98140 Reference Material for its DNA Copy Number Ratio

Certified Reference Material ERM®-BF427c

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CERTIFICATION REPORT

Certification of a Maize 98140 Reference Material for its DNA Copy Number Ratio

Certified Reference Material ERM®-BF427c

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ABSTRACT

This report describes the certification of the Certified Reference Material (CRM) ERM®-BF427c for its DNA copy number ratio. This CRM is a powder processed from seeds of genetically modified 98140 maize and conventional maize.

ERM-BF427c is part of a set of maize CRMs containing different mass fractions of GM maize 98140. The CRM was processed and originally certified for its mass fraction by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE. An interlaboratory comparison was conducted in 2009 to additionally certify the existing CRM for its DNA copy number ratio.

The CRM and its certified copy number ratio is intended to be used for quality control of measurements of the DNA copy number ratios of maize event 98140 in genetically modified food and feed. During the certification, the plasmid DNA (pDNA) ERM®-AD427 was used for calibration together with the event-specific 98140 real-time Polymerase Chain Reaction (PCR) method validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) (available on http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). ERM-AD427 contains a 80 bp fragment of the 5' insert-to-plant junction specific for the 98140 maize event. Additionally, the plasmid carries a 79 bp fragment of the maize endogeneous *high mobility group* (*hmg*) gene, specific for the maize taxon.

The CRM ERM-BF427c is available in glass bottles containing 1 g of maize seed powder, closed under argon atmosphere, and is certified for its DNA copy number ratio.

The minimum amount of sample to be used is 100 mg.

ERM-BF427c	**Certified value**	**Uncertainty** [3]
98140 maize mass fraction [1]	20.0 g/kg [2]	0.8 g/kg
98140 maize DNA copy number ratio [4]	1.75 % [5]	0.13 %

[1] Mass fraction of 98140 maize, based on the masses of mixed dried genetically modified 98140 maize seed powder and dried non-modified maize seed powder, and their respective water content.

[2] The certified value is traceable to the International System of Units (SI).

[3] The certified uncertainty is the expanded uncertainty with a coverage factor $k = 2$, corresponding to a level of confidence of about 95 %, estimated in accordance with the ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

[4] The copy number ratio of 98140 maize is defined by the 98140 maize real-time Polymerase Chain Reaction quantification method validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF, available on http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) and calibrated with the 98140 plasmid DNA Certified Reference Material ERM®-AD427.

[5] The certified DNA copy number ratio is the unweighted mean of 20 accepted data sets. It is traceable to the International System of Units (SI).

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GLOSSARY

α	error probability
ALS[1]	acetolactate synthase protein
ANOVA	analysis of variance
bp	base pair
cp	copy numbers
CRM	Certified Reference Material
CTAB	cetyltrimethylammonium bromide
Ct value	cycle threshold, number of PCR cycles to pass a set threshold
DNA	deoxyribonucleic acid
ε	PCR efficiency
EDTA	ethylenediaminetetraacetic acid
ERM®	trademark of European Reference Materials
EURL-GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed, formerly referred to as Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF)
g	standard gravity
gat4621[1]	gene encoding a glyphosate acetyltransferase (GAT) protein
gDNA, gDNA$_\ell$, gDNA$_s$	genomic DNA, genomic DNA extracted from leaves, genomic DNA extracted from seeds
GM	genetically modified
GM-HRA[1]	modified acetolactate synthase enzyme
GMO	genetically modified organism
hmg	*high mobility group* gene (taxon-specific gene)
IQR	interquartile range
IRMM	Institute for Reference Materials and Measurements
k	coverage factor
n	number of replicates
N	number of bottles analysed
N_d	number of data sets
N_{ds}	number of data sub-sets
NTC	non-template control
p	probability
PCR	polymerase chain reaction
pDNA	plasmid DNA
R^2	coefficient of determination
rel	relative (subscript used to describe relatively expressed values)
RM	Reference Material
RSD	relative standard deviation
s	standard deviation
s_{bb}	standard deviation between bottles
SI	International System of Units
TE	Tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
U	expanded uncertainty
u^*_{bb}	standard uncertainty related to the between-bottles inhomogeneity that can be hidden by the method repeatability
u_{char}	standard uncertainty of characterisation
U_{CRM}	expanded uncertainty of the certified value
u_{lts}	standard uncertainty of the long-term stability
$\overline{x}$	mean
$\overline{x}_i$	mean obtained for data set i
zm-hra[1]	gene coding for a modified acetolactate synthase

[1] Following international nomenclature guidelines, three-letter non italic capital codes refer to the protein, whereas lower case italic letters are used for genes.

1 INTRODUCTION

Legislation in the European Union demands the labeling of food products consisting of or containing "more than 0.9 % genetically modified organisms" (GMOs), provided the GMO has been placed on the market in accordance with Community legislation [1]. This enforces the necessity to develop and validate reliable quantitative methods, as well as the need to produce reference materials (RMs) to calibrate and control the application of these methods. This threshold of 0.9 % is commonly understood as a mass fraction and the Institute for Reference Materials and Measurements (IRMM) certifies gravimetric mixtures of genetically modified (GM) seed powder with non-GM seed powder for their GM mass fraction.

In October 2004, the European Commission recommended to express the content of GM material in food and feed as the percentage of GM haploid genomes in relation to the total of haploid genomes in a DNA mixture obtained for a certain plant species [2]. This percentage, known as DNA copy number ratio, is determined according to the following formula:

$$\text{DNA copy number ratio [\%]} = \frac{\text{GM DNA copy number}}{\text{Target taxon specific DNA copy number}} \cdot 100 \qquad (1)$$

As a consequence, it has been decided to certify the existing CRMs, additionally to their GM mass fraction, for their GM DNA copy number ratio.

The maize 98140 event is registered in the Organisation for Economic Co-operation and Development Unique Identification Registry (available via http://bch.cbd.int/database/lmo-registry/), as specified in the Regulation (EC) No 65/2004 of 14/01/2004 [3] establishing a system for the development and assignment of unique identifiers for GMOs. The maize 98140 event received the unique identifier DP-Ø9814Ø-6.

The GM *Zea mays* L. line 98140 was developed by Pioneer Hi-Bred International Inc. (Johnston, IA, US). The 98140 maize has been genetically modified by insertion of *gat4621* and *zm-hra* genes [4], along with the necessary regulatory elements for gene expression in the maize plant. The expressed GAT4621 protein is a glyphosate acetyltransferase (GAT), encoded by an optimised form of the *gat* gene from *Bacillus licheniformis*, that confers tolerance to the glyphosate. The ZM-HRA protein is an acetolactate synthase (ALS), encoded by an optimised form of the endogenous *als* gene from *Zea mays*, that confers tolerance to ALS-inhibiting herbicides, such as chlorimuron and thifensulfuron. The commercial name assigned to the genetically modified 98140 maize in the US market is Optimum™ GAT™ Corn[2].

CRM ERM-BF427c, consisting of non-GM and GM maize 98140 seed powders, was certified in 2009 for its GM mass fraction [5]. In 2009, an interlaboratory comparison was conducted to certify ERM-BF427c for its DNA copy number ratio. An event-specific maize 98140 real-time Polymerase Chain Reaction (PCR) quantification method validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) [6] was used in this study. The measurements were calibrated with the pDNA ERM-AD427 [7], containing DNA sequences specific for the maize event 98140 and for the taxon in a 1:1 ratio. The plasmid contains a 80 bp fragment of the 5' insert-to-plant junction [4], as well as a 79 bp fragment of the maize endogenous *high mobility group* (*hmg*) gene [8]. Both fragments are specifically targeted by the event-specific maize 98140 real-time PCR quantification method used [6].

[2] Optimum™ and GAT™ are trademarks of Pioneer Hi-Bred International, Inc. Johnston, IA, US

The certification of ERM-BF427c for its DNA copy number ratio was based on real-time PCR measurements and includes:

- characterisation of ERM-BF427c with respect to the DNA copy number ratio of the 98140 event;
- assessment of homogeneity, short- and long-term stability of ERM-BF427c.

ERM-BF427c certified for the DNA copy number ratio is intended to be used for quality control of measurements of the DNA copy number ratio of maize event 98140 in GM food and feed using the event-specific 98140 detection method and the ERM-AD427 calibrant.

2 PARTICIPANTS

Characterisation of ERM-BF427c for its DNA copy number ratio

Agencia Española de Seguridad Alimentaria - Centro Nacional de Alimentación, Madrid, ES* (ENAC 178/LE397)
Danish Plant Directorate, Laboratory for Diagnoses in Plants, Food and Feed, Lyngby, DK* (DANAK, 330)
Danmarks Tekniske Universitet, DTU - New Technical University of Denmark, Fødevareinstituttet, Søborg, DK* (DANAK, 350)
Ente Nazionale Delle Sementi Elette ENSE, Tavazzano, IT
Eurofins Genescan GmbH, Freiburg, DE* (DACH, DAC-PL-0526-07-03)
European Commission, Joint Research Centre, Institute for Health and Customer Protection (IHCP), Ispra, IT* (DACH, DAC-PL-0459-06-00)
European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE* (BELAC, 268-TEST)
Groupe d'études et de contrôle des variétés et des semences GEVES (BioGEVES), Surgeres, FR* (COFRAC, 1-1540)
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Institut für Hygiene und Umwelt, Behörde für Umwelt und Gesundheit, Hamburg, DE* (DACH, DAC-PL-0137-01-10)
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Livsmedelsverket - National Food Administration, Biology Division, Uppsala, SE* (SWEDAC, 1457)
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Nederlandse Organisatie voor toegepast-natuurwetenschappelijk Onderzoek (TNO), TNO quality of life - Food & Biotechnology Innovations - GMO foods, Zeist, NL* (Dutch Accreditation Council RvA, L027)
Nestlé Research Center, Lausanne, CH* (SAS, STS 188)
Tullilaboratorio - Finnish Customs Laboratory, Espoo, FI* (FINAS, T006)
Umweltbundesamt Wien, Wien, AT* (Federal Ministry of Economics and Labour, 200)

Certification

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE*,§ (BELAC 268-TEST)

* Laboratory holds accreditation ISO/IEC 17025 for DNA based GM measurements (accreditation body and registration number are mentioned).
§ Laboratory holds accreditation ISO Guide 34 for the production of reference materials (accreditation body and registration number are mentioned).

3 CRM PROCESSING

ERM-BF427c is a gravimetrically prepared dried maize seed powder containing a certified mass fraction of 20.0 g/kg of the GM maize 98140 with an expanded uncertainty (U_{CRM}, $k = 2$) of 0.8 g/kg. ERM-BF427c was processed in 2008 as part of a set of four CRMs certified for their mass fraction of maize 98140. For a detailed description of the processing of ERM-BF427c, the reader is referred to an earlier report [5].

4 HOMOGENEITY

4.1 HOMOGENEITY STUDY

The homogeneity and minimal sample intake of ERM-BF427c were investigated at the time of the production and certification of the CRM in 2008. The GM content determined in the frame of the certification of the matrix CRM is expressed as a mass fraction and not as a DNA copy number ratio. However, this has no impact on the conclusion whether or not the material is sufficiently homogeneous.

The between-bottle standard deviation (s_{bb}) and the maximum standard uncertainty related to the inhomogeneity that can be hidden by the method repeatability (u^*_{bb}) were converted into relative uncertainties, *i.e.* $s_{bb, rel}$ and $u^*_{bb, rel}$, respectively, by dividing by the mean value of the study (Table 1). The largest of the two values was included into the calculation of the uncertainty for the certified value (Section 7.3).

Table 1: Mean mass fraction, standard deviation due to inhomogeneity between bottles, and maximum heterogeneity of ERM-BF427c analysed by real-time PCR using 150 mg sample intake[1]. *N* is the number of analysed samples, and *n* is the number of replicates measured for each sample.

Measured mass fraction mean [%]	Number of samples analysed and replicates	Between bottle heterogeneity		Maximum hidden heterogeneity	
		s_{bb} [%]	$s_{bb, rel}$	u^*_{bb} [%]	$u^*_{bb, rel}$
2.427	$N = 18$, $n = 5$	0.035	0.014	0.033	0.014

[1] The values correspond to the homogeneity data obtained during the certification for mass fraction.

For more detailed information, the reader is referred to the mass fraction certification report [5].

4.2 MINIMUM SAMPLE INTAKE

A sample intake of 150 mg of powder was used for DNA extraction by the CTAB method in the homogeneity study. The assumption that this amount ensures a sufficient homogeneity was investigated and proved to be correct [5].

Different sample intakes of 200 mg (GENE*Spin*) and 100 mg (CTAB and Qiagen/DNeasy) were used for DNA extraction during characterisation. The variances of the measurements using 200 mg sample intake and of the measurements using 100 mg do not differ statistically, therefore the minimum sample intake was set to 100 mg. For the validity of the certified values of ERM-BF427c, sample intakes not smaller than 100 mg have to be used.

5 STABILITY

5.1 SHORT-TERM STABILITY

The stability of ERM-BF427c was investigated at the time of the certification of the material for its mass fraction (the change of unit of expression of the values has no impact on stability). It could be concluded that ERM-BF427c can be shipped under ambient conditions. More details on the short-term stability study can be found in an earlier report [5].

5.2 LONG-TERM STABILITY

Extensive stability monitoring has been carried out for all dry-mixed maize GMO CRMs processed in the same way as ERM-BF427c. There is no reason to expect a different behaviour of ERM-BF427c compared to other maize matrices upon long-term storage at 4 ºC under controlled conditions. Therefore, the long-term stability assessment of the maize 98140 CRMs relies on the stability observed for various maize GMO CRMs that were identically processed.

The long-term stability of maize CRMs during storage has been monitored at IRMM for more than seven years, using, among various methods, event-specific real-time PCR methods (Figure 1, based on unpublished results). Initially, the GM content of powders was only expressed as a mass fraction. The stability of a particular CRM is not affected by the kind of quantity that expresses the GM content (*i.e.* mass fraction or copy number ratio). In addition to that, the data being compared during long-term stability studies consists of ratios between the results from samples stored at 4 ºC and reference samples kept at -70 ºC ($\bar{x}_{4\,°C} / \bar{x}_{-70\,°C}$), and the final results have the dimension one. Therefore, the previous long-term stability and post-certification monitoring data of all maize CRMs is used to assess the stability over long storage times, and to calculate the uncertainty contribution of the stability.

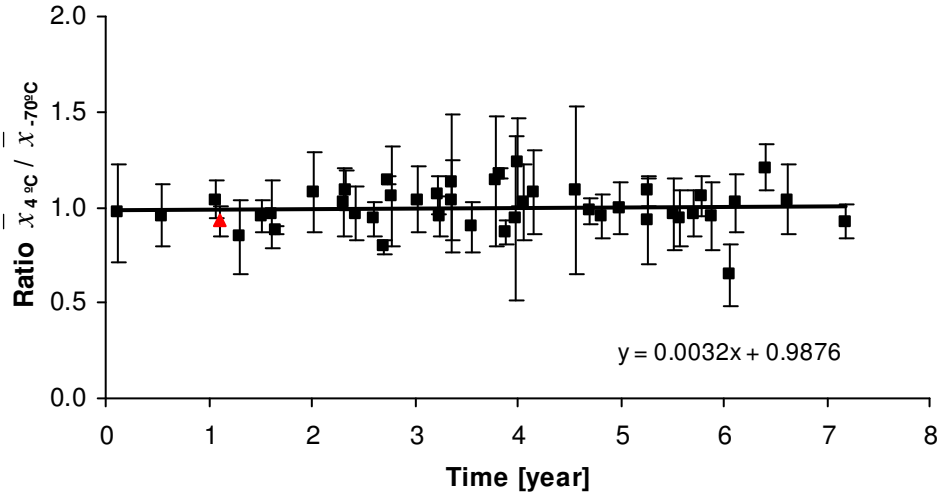

Figure 1: Long-term stability of different dried maize seed powder (not only 98140 (▲)) stored at 4 ºC for various time periods, based on real-time PCR measurements. The stability of the GM content is assessed via the ratio between the values corresponding to samples stored at 4 ºC and samples stored for the same time period at the reference temperature -70 ºC (■), with bars indicating the expanded uncertainty interval ± *U* (*k* = 2). Each point corresponds to the mean value of 3 to 9 measurements. The bold line is the regression line generated on the basis of all data points. The stability monitoring was performed using the nominal 1 % GM maize powders of ERM-BF411 (Bt176), BF412 (Bt11), BF413 (MON 810), BF414 (GA21), BF415 (NK603), BF416 (MON 863), BF417 (MON 863×MON 810), BF418 (1507), BF420 (3272), BF423 (MIR604) and BF424 (59122), the nominal 2 % GM maize powder of ERM-BF413k (MON 810) and BF427 (98140), and the 0.57 % MON 810 DNA copy number ratio certified maize powder ERM-BF413.

The statistical analysis revealed that there was no significant trend at 95 % confidence level for the stability regression line generated as described above. The relative standard uncertainty of the long-term stability ($u_{lts,\ rel}$) [9], estimated from the available maize post-certification monitoring data, was approximately 0.9 % for a shelf life of one year and was used as the contribution due to the instability of CRMs upon storage in the uncertainty budget (Table 8, Section 7.3).

In conclusion, the results demonstrate that the storage conditions for maize seed powder CRMs at IRMM are well suited for long-term storage. Moreover, post-certification monitoring is being carried out at regular time intervals in order to further investigate the stability of ERM-BF427c.

6 CHARACTERISATION STUDY

6.1 STUDY SET-UP

Twenty-one laboratories were selected on the basis of proven experience and quality management system in place. For the characterisation of ERM-BF427c with respect to its DNA copy number ratio, a total of 36 analyses were requested. To detect a possible dependence of the real-time PCR quantification method on the DNA extraction method, three different protocols were applied to extract DNA from the 'unknown' maize seed powders: i) a modified cetyltrimethylammonium bromide (CTAB) method [10], ii) the DNeasy plant mini kit (Qiagen, Benelux B.V., Venlo, NL), and iii) the GENE*Spin* DNA kit (GeneScan Analytics GmbH, Freiburg, DE). The CTAB method (Annex 1) was adopted from ISO 21571:2005, whereas the commercially available kits were applied according to the manufacturer's specifications, using a sample intake of 100 and 200 mg maize, for ii) and iii) respectively. Twelve data sets were allocated to each DNA extraction method.

Matrix CRM ERM-BF427c, DNA extraction kits, DNA calibrants, and real-time PCR consumables were shipped to the participating laboratories on dry ice.

For each independent analysis, two bottles of ERM-BF427c were provided. From each bottle, three samples were extracted and analysed ($N = 2$, $n = 3$), further referred to as 'unknowns' (U1-U3 for bottle 1, and U4-U6 for bottle 2). The extraction of DNA from the seed powder provided with the two bottles, as well as the real-time PCR measurements were performed on two different days. The DNA concentration was estimated by UV spectrometry and/or by fluorometry. The DNA concentration of each extract was adjusted to 20 ng/µL with nuclease-free water, and these "undiluted extracts" were further diluted 1.5, 3.0, and 4.0 times. The dilution series were analysed in triplicate by event-specific real-time PCR using primer pairs and labelled TaqMan® probes specific for the maize 98140 event [6, 7] and maize endogenous *hmg* gene (Figure 2). Two DNA calibrants were used for all real-time PCR experiments, namely gDNA and pDNA calibrant. The gDNA calibrant was prepared from gDNA extracted from leaves of hybrid maize 98140 plants, which were individually tested for the presence of 98140 trangene. The pDNA calibrant is the specific 98140 maize pDNA CRM ERM-AD427 [7]. Dilution series were prepared by the participating laboratories for both calibrants, as follows: i) gDNA was diluted in nuclease-free water to yield DNA concentrations ranging from 10 to 1.6×10^4 cp/µL, and ii) pDNA ERM-AD427 was diluted in TE buffer (1 mmol/L Tris, 0.01 mmol/L EDTA, pH 8.0) to concentration levels ranging from 10 to 10^5 cp/µL. Thus, one set of analysis generated two data sets, *i.e.* one that was calibrated with pDNA ERM-AD427, and one that was calibrated with gDNA from leaves.

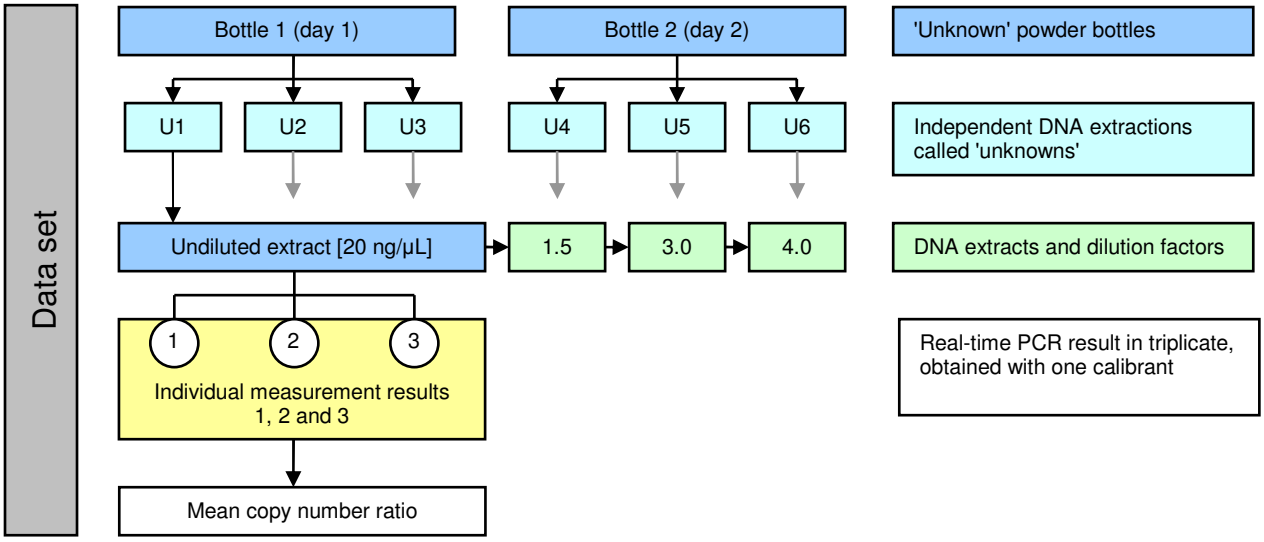

Figure 2: Analysis scheme for one calibrant followed by the participating laboratories during the copy number study. Undiluted extract U1 is given as an example. The same steps are also followed for all other samples and their dilutions. The same pipetted DNA extracts, *i.e.* the triplicates that are shown in the yellow box, are also analysed using the other calibrant. One data set comprises six DNA extractions and their dilutions analysed in triplicates by real-time PCR, using one type of calibrant.

Real-time PCR experiments using TaqMan Universal PCR mastermix were carried out according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, US) on 96 well microtiter plates, according to the method validated by EURL-GMFF for the event specific quantification of the maize event 98140 [6]. The real-time PCR reaction volume used in this certification study was 25 µL and all runs were performed for 45 cycles; the baseline and threshold of the individual real-time PCR measurements were set automatically.

6.2 ACCEPTANCE CRITERIA FOR DATA SETS

Data acceptance criteria for this study were established. Most of them are described by Charels *et al.* [11] as outlined below. Data sets that did not fulfil all acceptance criteria were excluded. The number of data sets that failed a particular acceptance criterion is also given. An overview of all rejections per data set is given in Table 2.

As the real-time PCR measurements were carried out on two different days, one data set comprises a total of four calibration curves consisting of the detection of the endogeneous and transgenic targets, using either the gDNA or pDNA calibrant. The results obtained on the two days of analysis were considered separately for the two calibrants. This means that the rejection of a data set calibrated with pDNA ERM-AD427 did not trigger the exclusion of the data set that was calibrated with gDNA from leaves and vice versa.

6.2.1 Technical acceptance criteria

One laboratory did not provide any measurement result due to technical reasons.

Only data sets with negative non-template controls (NTC) were accepted. Five data sets were not accepted for the gDNA calibration and four for the pDNA calibration because the corresponding NTCs were positive or missing.

Data sets were rejected if any inconsistency in the dilution series was noticeable as an abnormal trend of reported threshold cycles (Ct) (a higher dilution factor was expected to

result in increasing Ct). One data set from each calibration sequence, *i.e.* gDNA and pDNA, was rejected due to dilution inconsistencies of DNA extracted from the 'unknown' powder samples.

Mean values of triplicate measurements were not accepted if the Ct variability between one replicate was higher than 1.5 of the mean Ct of the remaining replicates within the particular dilution point. Dilution points were also excluded if the amplification signal was absent. However, this did not trigger the rejection of the complete data set, unless the remaining number of acceptable dilution points within the dilution series was insufficient. On the basis of this exclusion criterion, no complete data set was rejected.

6.2.2 Calibration curves

The calibration curves for this copy number study needed to fulfil the minimal performance requirements, which were set by the method validation guidelines of EURL-GMFF [12]. In particular, these selection criteria apply to the slope and linearity of the calibration curves.

Firstly, calibration curves with a coefficient of determination (R^2) of minimum 0.98 were accepted. Only one data set of the event-specific method was excluded because of a coefficient of determination of 0.96 obtained for the pDNA calibrant.

Secondly, control limits for PCR efficiencies (ε) were applied. For both calibrants, transgene and endogene mean PCR efficiencies and their standard deviations were calculated. Control limits were calculated per calibrant, transgene and endogene as the mean plus or minus one standard deviation. The resulting upper and lower control limits applied in this study are 87 % and 106 %. These efficiencies correspond to a slope of -3.68 and -3.19, respectively. For the gDNA calibrant, five data sets were not accepted as their efficiencies were outside the acceptance range (87-106 %). For the pDNA calibrant ERM-AD427, six data sets were rejected due to efficiencies below 87 % and two data sets due to efficiencies above 106 %.

6.2.3 Working interval

DNA extracts and dilutions exhibiting a Ct value outside the working range of the calibration curve were excluded for further calculations. Based on this criterion, no data sets of the gDNA calibration and no data sets of the pDNA calibration were excluded.

6.2.4 Copy number ratios, DNA extracts and their dilutions

The mean Ct values for the undiluted DNA extracts and their dilutions for each 'unknown' were converted into transgene and endogene copy numbers of which copy number ratios were calculated for each 'unknown', for each day, and each calibrant. Only the copy number ratios of the undiluted extracts were used for each accepted 'unknown' to calculate the certified value.

Two criteria were applied for the complete acceptance of the data set. Firstly, the copy number ratio of an undiluted extract was considered for calculation if at least one more copy number ratio from its corresponding dilution series could be calculated. Secondly, for a data set to be accepted, a minimum of two DNA extracts per day needed to fulfil this criterion, *i.e.* maximum six copy number ratios and minimum four copy number ratios per data set and calibrant were accepted.

Two data sets of the pDNA calibration and one data set of the gDNA calibrations were rejected due to insufficient data points per day (inconsistencies within the dilution series were observed for two DNA extracts analysed in the same day).

6.2.5 Relative standard deviation

The relative standard deviation (RSD) was calculated for the copy number ratios per day and for both days, per data set. Only data sets that had an RSD under repeatability conditions per day and for the whole data set below 25 % were accepted. This criterion led to the rejection of one data set calibrated with the gDNA calibrant.

Table 2: Overview of rejected data sets and reasons for their exclusion. Blank cells indicate that the data sets were accepted; data sets accepted for both pDNA and gDNA calibrations are not given.

Data set	DNA extraction method	ERM-AD427 pDNA calibrant	gDNA calibrant from leaves
4	CTAB[1]	calibration curve (ε)	calibration curve (ε)
6	CTAB[1]	calibration curve (ε)	technical (NTC)
7	CTAB[1]		RSD
10	CTAB[1]	calibration curve (ε)	
13	Qiagen/DNeasy[2]		technical (NTC)
14	Qiagen/DNeasy[2]	calibration curve (ε)	technical (NTC)
15	Qiagen/DNeasy[2]	calibration curve (ε)	calibration curve (ε)
16	Qiagen/DNeasy[2]	technical	technical
18	Qiagen/DNeasy[2]	insufficient data points	calibration curve (ε)
19	Qiagen/DNeasy[2]	technical (NTC)	calibration curve (ε)
21	Qiagen/DNeasy[2]	insufficient data points	insufficient data points
25	GENE*Spin*[3]	calibration curve (R^2)	technical
27	GENE*Spin*[3]	technical (NTC)	technical (NTC)
29	GENE*Spin*[3]	technical (NTC)	technical (NTC)
30	GENE*Spin*[3]	calibration curve (ε)	
32	GENE*Spin*[3]	calibration curve (ε)	calibration curve (ε)
33	GENE*Spin*[3]	calibration curve (ε)	
36	GENE*Spin*[3]	technical (NTC)	

[1] DNA was extracted using a modified CTAB method and a sample intake of 100 mg (Annex 1).
[2] DNA was extracted using the DNeasy plant mini kit (Qiagen, Benelux B.V., Venlo, NL) according to the specifications of the manufacturer for a sample intake of 100 mg.
[3] DNA was extracted using the GENE*Spin* extraction method carried out according to the manufacturer's protocol (GeneScan Analytics GmbH, Freiburg, DE) with a sample intake of 200 mg.

6.3 ACCEPTED DATA SETS

For the pDNA calibrant ERM-AD427 and for the gDNA calibrant from leaves, 20 and 22 data sets were accepted, respectively.

For three data sets from the gDNA calibrant and for two data sets from the pDNA calibrant only five of the six 'unknowns' per data set were accepted. For all other data sets, all six 'unknowns' were used to calculate the mean copy number ratio.

The results of the copy number ratios per data set are summarised in Table 3. The mean copy number ratios are given in Table 4.

Table 3: Overview of accepted data sets; mean DNA copy number ratio and standard deviation (*s*). Blank cells indicate excluded data sets; data sets excluded for both pDNA and gDNA calibrations are not given.

Data set	DNA extraction method	**DNA copy number ratio ± *s*** [%]	
		ERM-AD427 pDNA calibrant	gDNA calibrant from leaves
1	CTAB[1]	1.44 ± 0.24	1.71 ± 0.25
2	CTAB[1]	1.55 ± 0.22	1.75 ± 0.22
3	CTAB[1]	1.80 ± 0.17	1.88 ± 0.18
5	CTAB[1]	1.59 ± 0.09	1.81 ± 0.12
7	CTAB[1]	1.64 ± 0.26	
8	CTAB[1]	1.65 ± 0.14	1.86 ± 0.13
9	CTAB[1]	1.74 ± 0.13	1.98 ± 0.15
10	CTAB[1]		2.38 ± 0.31
11	CTAB[1]	1.74 ± 0.16	2.04 ± 0.10
12	CTAB[1]	2.10 ± 0.25	2.56 ± 0.15
13	Qiagen/DNeasy[2]	2.23 ± 0.26	
17	Qiagen/DNeasy[2]	1.30 ± 0.08	1.57 ± 0.04
20	Qiagen/DNeasy[2]	1.50 ± 0.19	1.87 ± 0.11
22	Qiagen/DNeasy[2]	1.92 ± 0.14	2.10 ± 0.20
23	Qiagen/DNeasy[2]	2.19 ± 0.28	2.46 ± 0.28
24	Qiagen/DNeasy[2]	2.09 ± 0.22	2.25 ± 0.25
26	GENE*Spin*[3]	1.80 ± 0.16	2.06 ± 0.17
28	GENE*Spin*[3]	1.83 ± 0.09	1.97 ± 0.10
30	GENE*Spin*[3]		1.86 ± 0.12
31	GENE*Spin*[3]	1.69 ± 0.14	1.68 ± 0.14
33	GENE*Spin*[3]		1.70 ± 0.11
34	GENE*Spin*[3]	1.66 ± 0.15	1.78 ± 0.15
35	GENE*Spin*[3]	1.60 ± 0.10	1.91 ± 0.13
36	GENE*Spin*[3]		1.90 ± 0.11

[1] DNA was extracted using a modified CTAB method and a sample intake of 100 mg (Annex 1).
[2] DNA was extracted using the DNeasy plant mini kit (Qiagen, Benelux B.V., Venlo, NL) according to the specifications of the manufacturer for a sample intake of 100 mg.
[3] DNA was extracted using the GENE*Spin* extraction method carried out according to the manufacturer's protocol (GeneScan Analytics GmbH, Freiburg, DE) with a sample intake of 200 mg.

Table 4: Means of results and *s* from the ERM-BF427c Copy Number Certification Study. Mean DNA copy number ratios obtained with pDNA calibrant ERM-AD427 and gDNA calibrant from leaves are calculated as the unweighted mean of means. N_d is the number of data sets used in the evaluation.

	DNA copy number ratio ± *s* [%]	
	ERM-AD427 pDNA calibrant	gDNA calibrant from leaves
ERM-BF427c	1.75 ± 0.25 (N_d = 20)	1.96 ± 0.26 (N_d = 22)

6.4 STATISTICAL EVALUATION OF DNA CALIBRANTS

The mean DNA copy number ratios of the accepted data sets were compared per extraction method and calibrant (Figure 3). The number of accepted data sets per DNA extraction method and calibrant was low, and a normality test was, therefore, not performed [13]. Alternatively, DNA copy number ratios within the intervals created by the subtraction and addition of the standard deviation were compared. The DNA copy number ratios per extraction method when calibrated with ERM-AD427 had overlapping $\bar{x}$ ± s intervals. This

was also true for DNA copy number ratios obtained when gDNA from leaves was used as a calibrant. Therefore, the data of the three different DNA extraction methods were pooled per calibrant.

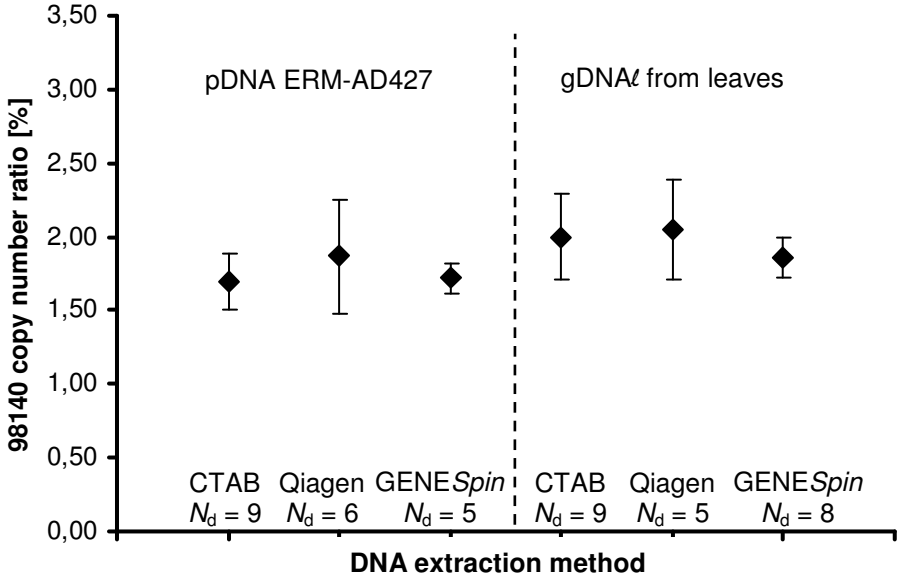

Figure 3: The influence of the DNA extraction method on the measured copy number ratios. Copy number ratio (♦) ± standard deviation *s* for gDNA and pDNA calibrant and each extraction method are shown. N_d is for the number of data sets used for evaluation, from each calibrant and extraction method.

DNA copy number ratios obtained by real-time PCR calibrated with the pDNA calibrant ERM-AD427 or gDNAℓ from leaves followed a normal distribution. No outliers with the Grubbs and Dixon tests (95 % confidence level) were detected for copy number ratios when calibrated with either the pDNA calibrant or gDNAℓ extracted from leaves. One outlier was detected for data calibrated with the pDNA calibrant ERM-AD427 by the Nalimov *t*-test, at 95 % confidence level. However, no outlying result was found using the same statistical test, at 99 % confidence level. For the DNA copy number ratios obtained when calibrated with gDNAℓ from leaves, the Nalimov *t*-test revealed two outliers at 95 %, but not at 99 % confidence level.

The mean DNA copy number ratio obtained when experiments were calibrated with gDNA extracted from leaves is higher than the value obtained with ERM-AD427 as calibrant (Table 4 and Figure 3). A subsequent single factor ANOVA confirmed that the calibrant has a significant influence on the DNA copy number ratio ($p = 1.27 \times 10^{-2}$, $\alpha = 0.05$).

6.5 ANALYTICAL BEHAVIOUR OF PLASMID AND GENOMIC DNA

As there is a significant influence of the type of calibrant on the measured copy number ratio (Table 4 and Figure 3), the data could not be pooled. Consequently, the copy number ratio is defined by the calibrant. In this section, properties of the calibration curves, *i.e.* PCR efficiency and linearity of the regression line of both calibrants are compared to those of the maize seed powder (the 'unknowns' resemble a typically food or feed sample) in order to select the most appropriate calibrant.

The PCR efficiencies as well as the linearity of the regression lines were calculated on the basis of serial dilutions in plasmid dilution buffer of the pDNA calibrant ERM-AD427, dilutions of gDNA extracted from leaves from germinated 98140 maize seeds (gDNAℓ), as well as dilutions of gDNA extracted from the ERM-BF427c maize seed powder (gDNA$_S$). To

evaluate the analytical behaviour of the plasmid and genomic DNA, these two parameters have been compared and statistically analysed.

The first parameter was the PCR efficiency (ε) estimated for both transgenic and endogenous targets, using the three DNA types. The PCR efficiencies were only compared if the respective data sets passed the selection criteria defined beforehand, namely the coefficient of determination (R^2) of the calibration curve, and PCR efficiency (estimated on the basis of the slope of the calibration curve). These selection criteria were applied in order to avoid interferences of technically weak results generated by the participating laboratories [11, 12]. Firstly, a R^2 below 0.98 was not accepted within the study as it may reflect erroneous dilutions or inappropriate PCR amplification. Secondly, the PCR efficiencies interval was defined on the basis of pDNA and gDNA efficiencies (both materials used as calibrants in the study). The mean values of the PCR efficiencies were calculated for the endogeneous and transgenic targets using either the pDNA or gDNA calibrant. Four intervals were accordingly generated based on mean ± 1 *s*. The minimum and maximum values of the resulting cut-off points were then used to define the lower and higher limits of the PCR efficiency of the study, *i.e.* 87 and 106 % respectively. These values were consistent with the performance of the simplex real-time PCR method for quantification of the 98140 maize event observed with in-house performed studies and from results obtained by collaborations with external laboratories.

The PCR efficiencies estimated for both endogeneous and transgenic targets were comparable when using pDNA and gDNAℓ calibrants (96.9 and 95.2 % versus 96.5 and 95.6 % for the pDNA and gDNA calibration curves of the taxon- and event-specific detection methods, respectively) (Table 5). No significant differences between PCR efficiencies of pDNA and gDNAℓ calibrants were found for the endogeneous and transgenic targets, *hmg* (p = 0.49, α = 0.05) (Figure 4A) and 98140 (p = 0.58, α = 0.05) (Figure 4B) on simplex real-time PCR detection methods.

The distributions of the various PCR efficiencies for pDNA/gDNAℓ calibrant and gDNAs were also compared and showed a large overlap for both targets for the three DNA types (Figure 4). The PCR efficiencies of the gDNAs calibration curves were always larger than the PCR efficiencies obtained with pDNA and gDNAℓ. Except for the difference between the PCR efficiencies of the transgenic target for gDNAℓ and gDNAs, noted as statistically not significant (p = 0.054, α = 0.05), all other comparisons led to significantly different results for the 98140 target sequence (Figure 4 and Table 5).

Table 5: Comparison of the real-time PCR efficiencies of gDNA extracted from leaves and seeds, and pDNA. N_{ds} is the number of accepted data sub-sets under repeatability conditions, *s* is the standard deviation.

Target sequence	Mean PCR efficiency ± *s* [%]		
	ERM-AD427 pDNA	Seeds gDNAs	Leaves gDNAℓ
hmg	96.9 ± 2.9 (N_{ds} = 61)	98.5 ± 4.0 (N_{ds} = 84)	96.5 ± 3.2 (N_{ds} = 66)
98140	95.2 ± 4.2 (N_{ds} = 58)	97.3 ± 5.6 (N_{ds} = 72)	95.6 ± 3.9 (N_{ds} = 56)

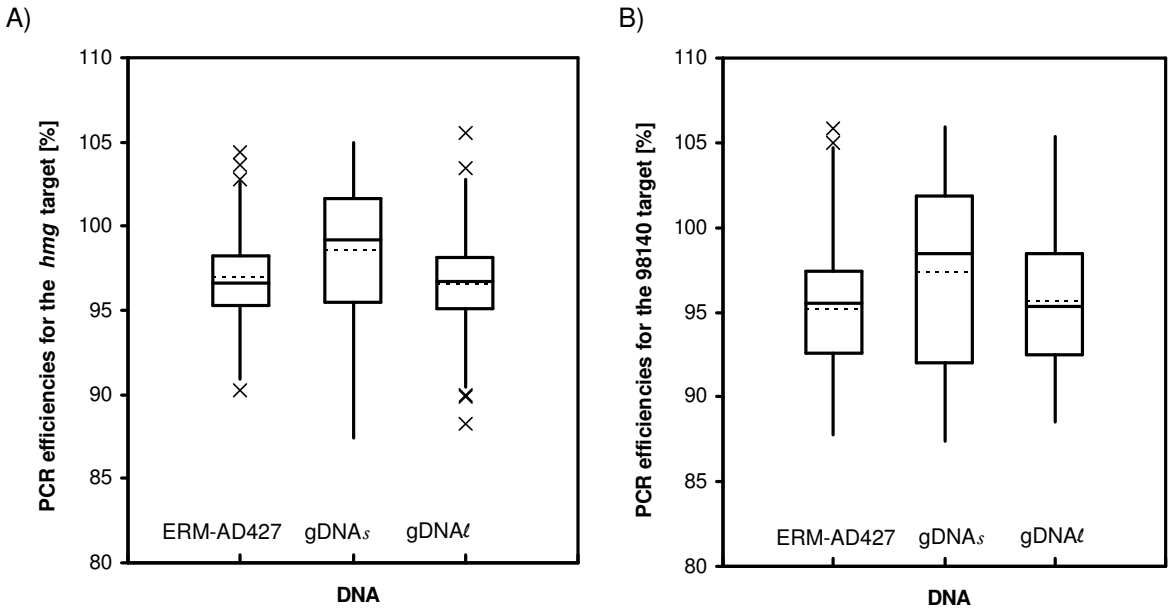

Figure 4: Box-and-whisker diagram illustrating the PCR efficiencies for the *hmg* (A) and 98140 (B) targets, based on the calibration curves performed using either ERM-AD427 calibrant or gDNA extracted from 98140 seeds or 98140 leaves. The bottom and top of the box are the 1st and 3rd quartiles, respectively; the line near the middle of the box is the median. The ends of the whiskers are determined by subtracting 1.5 times the interquartile range (IQR) from the 1st quartile, and adding 1.5 times the IQR to the 3rd quartile, respectively. The dashed line corresponds to the mean value of each group, whereas outlier values (95 % confidence level) are marked with x.

The second parameter studied was the coefficient of determination (R^2) that provides information about the fitting of data to a linear calibration curve obtained for both targets, using the three types of DNA (Figure 5 and Table 6).

Table 6: Comparison of the coefficients of determination (R^2) of gDNA extracted from leaves or seeds and pDNA; N_{ds} is the number of accepted data sub-sets under repeatability conditions, *s* is the standard deviation.

Target sequence	Mean $R^2 \pm s$		
	ERM-AD427 pDNA	Seeds gDNA*s*	Leaves gDNA*ℓ*
hmg	0.999 ± 0.001 (N_{ds} = 61)	0.997 ± 0.004 (N_{ds} = 84)	0.999 ± 0.001 (N_{ds} = 66)
98140	0.994 ± 0.004 (N_{ds} = 58)	0.995 ± 0.004 (N_{ds} = 72)	0.999 ± 0.002 (N_{ds} = 56)

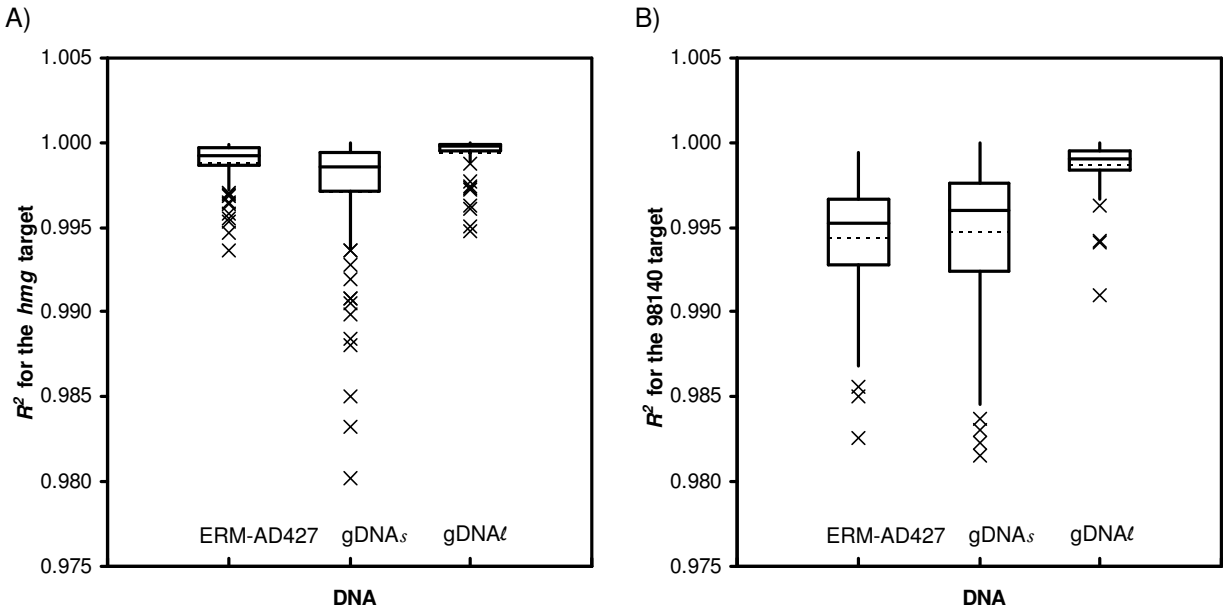

Figure 5: Box-and-whisker diagram representing the coefficients of determination (R^2) for *hmg* (A) and 98140 (B) targets, based on the calibration curves performed using either ERM-AD427 calibrant or gDNA extracted from 98140 seeds or 98140 leaves. A description of a box-and-whisker diagram is given in Figure 4.

A comparison of the coefficients of determination of pDNA and gDNA*ℓ* calibration curves showed no major influence of the type of the DNA calibrant in the case of the endogenous target (Figure 5A). For the transgenic target, the distribution of the coefficients of determination displayed a large variation (Figure 5B). However, the coefficients of determination of gDNA*s* dilution curves were closer to those of the pDNA calibrant in the case of both targets.

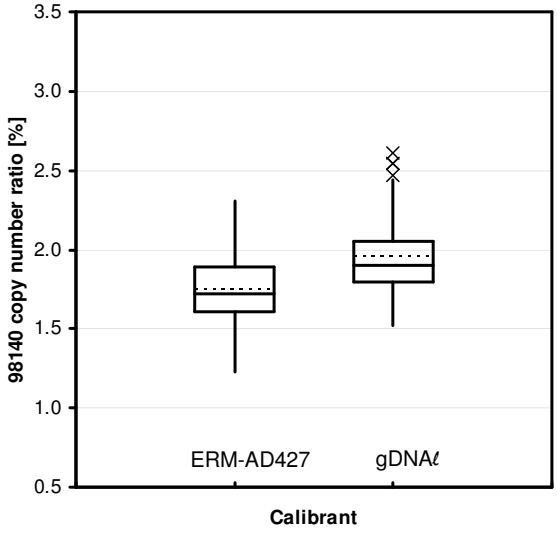

Figure 6: Box-and-whisker diagram representing the 98140 copy number ratio in ERM-BF427c using either ERM-AD427 or gDNA extracted from 98140 leaves as calibrant. A description of a box-and-whisker diagram is given in Figure 4.

Finally, the 98140 GM copy number ratio of ERM-BF427c (20.0 g/kg) has been compared using either ERM-AD427 or gDNA*ℓ* as calibrants (Figure 6 and Table 5). For this study, copy number ratios obtained per day from accepted data sets were compared. The data sets from both calibrants follow a normal distribution. Although the GM copy number ratios obtained for ERM-BF427c by the two different calibrants overlap, the mean GM copy number ratios are very different. Single factor ANOVA confirms that the data set calibrated

with ERM-AD427 is significantly different from the data set calibrated with gDNAℓ from leaves ($p = 7.6 \times 10^{-4}$, $\alpha = 0.05$). Therefore the GM copy number data calibrated with ERM-AD427 and gDNAℓ cannot be pooled.

Table 7: GM content in ERM-BF427c expressed in copy number ratio and calibrated with either ERM-AD427 or gDNAℓ extracted from 98140 leaves; N_{ds} is the number of accepted data sub-sets, *s* is the standard deviation.

	DNA copy number ratio ± *s* [%]	
	ERM-AD427 pDNA	Leaves gDNAℓ
ERM-BF427c	1.75 ± 0.27 (N_{ds} = 40)	1.96 ± 0.27 (N_{ds} = 44)

In the study reported here, statistical analyses have shown that pDNA and gDNAℓ calibrants behave in a similar way with respect to the PCR efficiencies of the transgenic and endogeneous target sequence (Figure 4). The individual PCR efficiency of each target sequence has, however, a significant impact on GM quantification by real-time PCR. Therefore, the effect of a small difference in PCR efficiencies of the transgenic and endogeneous targets on GM quantification by real-time PCR can generate a rather large difference in copy number due to the exponential nature of the PCR amplification. Consequently, such a small difference in PCR efficiencies of gDNAℓ and pDNA calibrants may explain the difference between the mean values (Tables 4 and 7).

An additional measurement of the gDNA extracted from ERM-BF427c was done by digital PCR [14]. Digital PCR is a real-time PCR method that is independent of a calibrant but dependent on the same PCR chemistry, primers and probes as real-time PCR. Digital PCR involves distributing the PCR solution containing template nucleic acid molecules across a large number of individual partitions prior to amplification. Following PCR amplification, a count of the proportion of partitions containing a detectable number of PCR amplicons can be used to estimate the total number of template DNA copies in the original DNA extract. The DNA copy number ratio was then calculated from the total number of template DNA copies measured for both targets. The DNA copy number ratio and its expanded uncertainty (k = 2) obtained by simplex digital PCR for gDNA extracted from ERM-BF427c was 1.54 ± 0.13 % (N = 3, n = 3, with each n consisting of 5 replicate measurements), and is closer to the copy number ratio obtained when pDNA calibrant ERM-AD427 is used.

Based on studying the analytical behaviour of pDNA and gDNA, it can be concluded that both calibrants, *i.e.* pDNA ERM-AD427 and gDNAℓ extracted from maize 98140 leaves are suitable to calibrate the real-time PCR method applied here. However, the user should be aware that the choice of calibrant influences the measured copy number ratio and can lead to significantly different results (Table 4). Furthermore, the DNA copy number ratio measured using either the pDNA ERM-AD427 or gDNAℓ as a calibrant is different than the one obtained by digital PCR. Studies have indicated that the most suitable approach is to set a reference system based on pDNA, as described in the certification report of the pDNA calibrant ERM-AD427 [7], as primary calibrant for DNA copy number ratio measurements, together with the approved GM quantification method published by the EURL-GMFF [1].

7 CERTIFIED VALUE AND UNCERTAINTY BUDGET

7.1 METROLOGICAL TRACEABILITY

The certified DNA copy number ratio is defined as the 98140 DNA copy numbers divided by maize-specific DNA copy numbers calculated in terms of haploid genomes [2]; it is expressed in percent. These DNA copy number ratios are determined using the maize 98140 event-specific real-time PCR method calibrated with the pDNA calibrant ERM-AD427. Therefore, and given the interlaboratory comparison comprising 20 accepted data sets for the pDNA calibrant, the identity of the measurand is defined by the event-specific real-time PCR detection method validated by EURL-GMFF [6] and calibrated with the 98140 maize pDNA ERM-AD427 [7]. The measurement results from the interlaboratory comparison were shown to be independent of the DNA extraction method applied (Section 6).

The certified value is traceable to the International System of Units (SI); the DNA copy number ratio is defined by the event-specific real-time PCR detection method and calibrant as described above.

7.2 CERTIFIED VALUE

The certified value is a DNA copy number ratio based on the 98140 DNA copy numbers and maize-specific DNA copy numbers, expressed in percent. The DNA copy number ratio obtained for each data set, and expressed in %, is calculated as follows:

$$\text{DNA copy number ratio}\,[\%] = \frac{\text{GM DNA copy numbers [cp]}}{\text{maize specific DNA copy numbers [cp]}} \cdot 100$$

Because the data are normally distributed (Section 6), the certified value ($\bar{x}$) is calculated as the unweighted mean of data set means (Equation 3).

$$\bar{x} = \frac{\sum_{i=1}^{N_d} \bar{x}_i}{N_d} \qquad (3)$$

($\bar{x}_i$ = mean DNA copy number ratio obtained for data set i; N_d = number of data sets).

7.3. UNCERTAINTY BUDGET

The expanded combined uncertainty of the certified value (U_{CRM}) comprises standard uncertainty contributions from the homogeneity, stability and characterisation. The uncertainty of the calibrant is negligible [7] and, therefore, not considered. It is estimated according to equation 4 [15]:

$$U_{CRM} = k \cdot \bar{x} \cdot \sqrt{u_{bb,rel}^2 + u_{lts,rel}^2 + u_{char,rel}^2} \qquad (4)$$

(k = coverage factor; $\bar{x}$ = certified value; $u_{bb,\ rel}$ = relative uncertainty contribution from the heterogeneity; $u_{lts,\ rel}$ = relative uncertainty contribution from the long-term stability; $u_{char,\ rel}$ = relative uncertainty contribution from the characterisation).

The relative standard uncertainty from the characterisation ($u_{char,\ rel}$) was assessed during the interlaboratory comparison by estimating the standard deviation of the normally distributed data, and is calculated using equation 5:

$$u_{\text{char, rel}} = \frac{s}{\bar{x} \cdot \sqrt{N_{\text{d}}}} \quad (5)$$

(s = standard deviation; $\bar{x}$ = certified value; N_{d} = number of data sets)

The relative standard uncertainty introduced by the inhomogeneity at 150 mg level has been estimated on the basis of a normally distributed population of real-time PCR measurements. The relative uncertainty contribution from the stability (u_{lts}) was estimated on the basis of real-time PCR results following long-term monitoring of other maize CRMs certified for their GMO content. A coverage factor of 2 (k = 2) was used to calculate the expanded uncertainty corresponding to a level of confidence of about 95 % (Table 8).

Table 8: Uncertainty budget for the DNA copy number ratio of 98140 maize in ERM-BF427c.

CRM certified value DNA copy number ratio [%]	**Relative standard uncertainty contributions**			**Expanded uncertainty U_{CRM} (k = 2) [%]**
	$u_{\text{bb, rel}}$ [1]	$u_{\text{lts, rel}}$ [2]	$u_{\text{char, rel}}$ [3]	
1.754	0.014	0.009	0.032	0.127

[1] Relative standard uncertainty introduced by the inhomogeneity measured with a sample intake of 150 mg.
[2] Relative standard uncertainty related to the long-term stability, estimated for a shelf life of 12 months.
[3] Relative standard uncertainty introduced by the characterisation.

7.4. COMMUTABILITY

ERM-BF427c is prepared from non-GM and GM maize seed powders. The certified value is a DNA copy number ratio based on the 98140 DNA copy numbers and maize-specific DNA copy numbers, expressed in percent; the DNA copy number ratio is defined by the real-time PCR method for detection of 98140 [6] and calibrated with the plasmid DNA Certified Reference Material ERM-AD427. However, as the two calibrants tested during certification, which are pDNA ERM-AD427 and genomic DNA extracted from plant leaves (gDNAℓ), led to significant differences in the measured copy number ratio value of ERM-BF427c, commutability [16] problems should be considered.

Both calibrants, pDNA ERM-AD427 as well as gDNAℓ, differed from the value obtained by the calibrant-independent digital PCR measurements. It could not be proven during the analytical behaviour study that one of the calibrants behaves more similar to the gDNA extracted from maize seed powder. However, calibration with ERM-AD427 leads to lower copy number ratio values compared to the calibration with gDNAℓ extracted from leaves.

For practical reasons, ensuring for instance full characterisation and reproduction of additional batches of calibrants, pDNA ERM-AD427 was selected to be the highest point of the calibration chain. The copy number ratios measured by the 98140 real-time PCR method and using the ERM-AD427 calibrant on ERM-BF427c and food and feed samples are reproducible and expected to be the same.

8 INSTRUCTIONS FOR USE

8.1 INTENDED USE

The material ERM-BF427c is certified for both its mass fraction of the GM event 98140 [5] and its DNA copy number ratio when applying event-specific 98140 real-time PCR and calibration with pDNA ERM-AD427 (this report). The DNA copy number ratio is based on the 98140 copy numbers and maize-specific DNA copy numbers, expressed in percent.

The material certified for its DNA copy number ratio is intended to be used for quality control of measurements of the 98140 DNA copy number ratios in GM food and feed. Genomic DNA can be extracted from the powder applying a validated method of choice for further use in real-time PCR measurements. Experiments should be performed exclusively with the event-specific 98140 detection method [6], calibrated with the pDNA calibrant ERM-AD427 [7], as the certified value is obtained using this detection method and calibrant.

8.2 HANDLING

The bottles containing the CRM should be allowed to reach the room temperature prior to opening. The dry maize powder is hygroscopic. Users are therefore advised to close bottles immediately after taking a sample.

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ANNEX 1

Modified protocol for DNA extraction using the CTAB method

Required reagents:

Reagent	Composition, specifications
H_2O	sterile nuclease-free water
RNase A	100 mg/mL, ready-to-use solution
Proteinase K	20 mg/mL, ready-to-use solution
Chloroform	p.a. quality
Ethanol	96 %, p.a. quality
NaCl solution	1.2 mol/L NaCl
CTAB extraction buffer	1.4 % mass concentration CTAB 1.0 mol/L NaCl 0.1 mol/L Tris-HCl pH 8.0 15.0 mmol/L Na_2EDTA
CTAB precipitation buffer	0.5 % mass concentration CTAB 40 mmol/L NaCl 50 mmol/L Tris-HCl pH 8.0
TE Buffer	1 mmol/L Tris pH 8.0 0.01 mmol/L EDTA

Procedure:

1. Transfer 100 mg of plant powder into each of three 2 mL microcentrifuge tubes.
2. Add 1 mL pre-heated (65 °C) CTAB extraction buffer (per 2 mL tube) and mix thoroughly by shaking or tapping the tube.
3. Add 10 µL of RNase A (100 mg/mL) and mix shortly by shaking.
4. Incubate minimum 15 min at 65 °C, mixing the constituents a few times by shaking.
5. Add 20 µL Proteinase K (20 mg/mL) to the tube, mix by shaking.
6. Incubate minimum 15 min at 65 °C with occasional shaking.
7. Spin down the cell debris 10 min (12 000 × *g*).
8. Transfer the supernatant to a 1.5 mL microcentrifuge tube containing 500 µL chloroform.
9. Vortex or shake minimum 10 s and then centrifuge 10 min at 12 000 × *g* to separate the phases.
10. Transfer the upper phase to a new 1.5 mL tube containing roughly an equal volume of chloroform (this can be estimated from step 8).
11. Mix minimum 10 s and then centrifuge 5 min at 12 000 × *g* to separate the phases.
12. Transfer the upper layer to a 2 mL tube, carefully determining the volume transferred.
13. Add 2 volumes CTAB precipitation buffer and mix by pipetting up and down.
14. Incubate 1 hour at room temperature to precipitate the DNA.
15. Centrifuge 10 min at maximum speed; carefully decant the supernatant and discard it.
16. Suspend the (invisible) precipitate in 400 µL 1.2 M NaCl; mix gently.
17. Add 400 µL chloroform, mix minimum 10 s.
18. Centrifuge 5 min at 12 000 × *g* to separate the phases.
19. Transfer the supernatant to a 1.5 mL tube, carefully determining the volume transferred.

20. Add 2 volumes cold (-20 °C) ethanol (p. a. grade) and mix by gently inverting the tube several times. Strands of precipitated DNA should become visible, otherwise incubate for 30 min at -20 °C.
21. Spin down the DNA precipitate minimum 10 min at 12 000 × *g*.
22. Carefully decant the supernatant and wash the pellet by addition of 500 µL cold (-20 °C) 70 % (volume fraction) ethanol; mix shortly.
23. Centrifuge 5 min at 12 000 × *g*, carefully remove the supernatant using a 1 mL pipette or by decanting it (optionally spin again shortly and remove the remaining fluid with a low volume pipette).
24. Allow the pellet to dry a few min at room temperature.
25. Suspend the pellet in 100 µL nuclease-free water by overnight incubation at 4 °C.

European Commission

EUR 24718 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Certification of a Maize 98140 Reference Material for its DNA Copy Number Ratio – Certified Reference Material ERM®-BF427c
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Abstract

This report describes the certification of the Certified Reference Material (CRM) ERM®-BF427c for its DNA copy number ratio. This CRM is a powder processed from seeds of genetically modified 98140 maize and conventional maize.

ERM-BF427c is part of a set of maize CRMs containing different mass fractions of GM maize 98140. The CRM was processed and originally certified for its mass fraction by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE. An interlaboratory comparison was conducted in 2009 to additionally certify the existing CRM for its DNA copy number ratio.

The CRM and its certified copy number ratio is intended to be used for quality control of measurements of the DNA copy number ratios of maize event 98140 in genetically modified food and feed. During the certification, the plasmid DNA (pDNA) ERM®-AD427 was used for calibration together with the event-specific 98140 real-time Polymerase Chain Reaction (PCR) method validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) (available on http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). ERM-AD427 contains a 80 bp fragment of the 5' insert-to-plant junction specific for the 98140 maize event. Additionally, the plasmid carries a 79 bp fragment of the maize endogeneous *high mobility group* (*hmg*) gene, specific for the maize taxon.

The CRM ERM-BF427c is available in glass bottles containing 1 g of maize seed powder, closed under argon atmosphere, and is certified for its DNA copy number ratio.

The minimum amount of sample to be used is 100 mg.

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