Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of a Maize NK603 Reference Material for its DNA Copy Number Ratio

Certified Reference Material ERM®-BF415e

EUR 24699 EN – 2011

JRC EUROPEAN COMMISSION

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European Commission
Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
Reference materials sales
Retieseweg 111
B-2440 Geel, Belgium
E-mail: jrc-irmm-rm-sales@ec.europa.eu
Tel.: +32 (0)14 571 705
Fax: +32 (0)14 590 406

http://irmm.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

JRC 62825

EUR 24699 EN
ISBN 978-92-79-19074-2
ISSN 1018-5593
doi:10.2787/38148

Luxembourg: Publications Office of the European Union

Printed in Belgium

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Certification of a Maize NK603 Reference Material for its DNA Copy Number Ratio

Certified Reference Material ERM®-BF415e

E. de Andrade, B. Jeynov, P. Corbisier,
A.M. Kortekaas, S. Trapmann, S. Vincent, H. Emons

European Commission, Joint Research Centre
Institute for Reference Materials and Measurements (IRMM), Geel (BE)

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ABSTRACT

This report describes the certification of the Certified Reference Material (CRM) ERM®-BF415e for its DNA copy number ratio. This CRM is composed of genetically modified NK603 maize seed and conventional maize seed powders.

ERM-BF415e is part of a set of maize CRMs containing different mass fractions of genetically modified NK603 maize. The CRM was processed and originally certified for its mass fraction by the European Commission, Directorate General Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE.

An interlaboratory comparison was conducted in 2009 to certify the existing CRM additionally for its DNA copy number ratio. The CRM is intended for quality control of measurements of the DNA copy number ratios of maize event NK603 in genetically modified food and feed. Hereby, the plasmid DNA (pDNA) ERM-AD415 was used for calibration together with the event-specific NK603 real-time Polymerase Chain Reaction (PCR) method validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm), modified as described in Section 6.1. ERM-AD415 contains a 108 bp fragment of the 3' insert-plant junction specific for the NK603 maize event. Additionally, the plasmid carries a 79 bp fragment of the maize endogenous high mobility group gene (*hmg*), specific for the maize taxon.

The CRM ERM-BF415e is available in glass bottles containing 1 g of maize powder, closed under argon atmosphere and was certified for its DNA copy number ratio.

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

MAIZE SEED POWDER		
	Certified value	Uncertainty [3]
NK603 maize mass fraction [1]	19.6 g/kg [2]	0.9 g/kg
NK603 maize DNA copy number ratio [4]	0.95 % [5]	0.11 %

[1] Mass fraction of maize NK603 based on the masses of genetically modified NK603 maize seed powder and 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 ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.
[4] The copy number ratio of maize NK603 is defined by the maize NK603 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, modified to target the taxon specific *high mobility group* (*hmg*) gene according to Broothaerts et al. (2008) J Agric Food Chem 56(19): 8825-31 and calibrated with the maize NK603 plasmid DNA Certified Reference Material ERM®-AD415.
[5] The certified DNA copy number ratio is the unweighted mean of 21 accepted data sets. It is traceable to the International System of Units (SI).

GLOSSARY

α	error probability
ANOVA	analysis of variance
bp	base pair
cp	copy number
CRM	Certified Reference Material
CTAB	cetyltrimethylammonium bromide
Ct	number of PCR cycles to pass a set threshold
DNA	deoxyribonucleic acid
$\mathcal{E}$	PCR efficiency
EDTA	ethylenediaminetetraacetic acid
ERM®	trademark of European Reference Materials
EPSPS[1]	5-enolpyruvlyshikimate-3-phosphate synthase
EURL-GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed, formerly named Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF)
g	standard gravity
gDNA	genomic DNA
gDNAℓ	genomic DNA extracted from NK603 maize leaves
gDNAs	genomic DNA extracted from the NK603 seed powder ERM-BF415e
GM	genetically modified
GMO	genetically modified organism
hmg[1]	*high mobility group* gene from *Zea mays* L. (taxon specific gene)
IQR	interquartile range
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
$T_1E_{0.01}$	1 mmol/L Tris, 0.01 mmol/L EDTA, pH 8.0
Tris	tris(hydroxymethyl)aminomethane
U	expanded uncertainty
u_{bb}	standard uncertainty related to the between-bottle inhomogeneity
u^*_{bb}	maximum standard uncertainty related to inhomogeneity that can be hidden by the method repeatability
u_{char}	standard uncertainty of characterisation
U_{CRM}	expanded uncertainty of the CRM
u_{lts}	standard uncertainty of the long-term stability
$\overline{x}$	mean
$\overline{x}_i$	mean obtained for data set *i*

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

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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 measurement methods as well as the need to produce reference materials (RMs) to calibrate and control the application of these quantification 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 and 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 from a certain plant species [2]. This percentage, known as DNA copy number ratio, is determined according to the following formula:

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

As a consequence, the existing CRMs certified for their GM mass fraction need additionally to be certified for their DNA copy number ratio.

The maize NK603 event is registered in the Organisation for Economic Co-operation and Development Unique Identification Registry (available through the Biosafety Clearing-House database for living modified organisms: http://bch.cbd.int/database/lmo-registry/) as specified in Regulation (EC) No 65/2004 of 14/01/2004, establishing a system for the development and assignment of unique identifiers for genetically modified organisms [3]. The maize NK603 event received the unique identifier maize MON-ØØ6Ø3-6.

Maize line NK603 was developed by Monsanto Company (St. Louis, MO, US) to allow the use of glyphosate containing herbicides (Roundup®) as a weed control option for maize crops. The line was genetically engineered to contain a bacterial form of the gene *epsps*, encoding a glyphosate-tolerant form of the enzyme 5-enolpyruvlyshikimate-3-phosphate synthase (EPSPS). The recombinant gene was isolated from the soil bacterium *Agrobacterium tumefaciens* strain CP4. The maize line NK603 was produced by biolistic transformation of the inbred maize line LH82xB73 with a 6706 bp DNA fragment containing two adjacent EPSPS expression cassettes, each containing a single copy of the *cp4 epsps* gene [4].

CRM ERM®-BF415e, consisting of a mixture of a non-modified maize and the NK603 GM maize powder, was certified in 2004 for its GM mass fraction [5]. In 2009, an interlaboratory comparison was conducted to certify ERM-BF415e for its DNA copy number ratio. The event-specific NK603 real-time Polymerase Chain Reaction (real-time PCR) quantification method, validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) [6], could not be integrally used. It was modified for the endogenous target as a consequence of the results of the research carried on by Broothaerts et al. [7]. The quantitative measurements were calibrated with the plasmid DNA (pDNA) ERM®-AD415 [8], containing DNA sequences specific for the maize event NK603 and for the taxon in an equimolar ratio. This plasmid contains a 108 bp fragment of the 3' insert plant junction (NK603 event) [6] as well as a 79 bp fragment of the maize endogenous *high mobility group* gene (*hmg*) [9]. The NK603 fragment is targeted by the event-specific NK603 detection method used [6].

The certification of ERM-BF415e for its DNA copy number ratio was based on real-time PCR measurements and includes:

- characterisation of ERM-BF415e with respect to the DNA copy number ratio of event NK603;
- assessment of the homogeneity and the long-term stability of ERM-BF415e.

With respect to the copy number ratio certification the material ERM-BF415e is intended to be used for quality control of measurements of the DNA copy number ratio of maize event NK603 in GM food and feed using the modified event-specific NK603 detection method and the ERM-AD415 calibrant.

2 PARTICIPANTS

Homogeneity

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

Characterisation of ERM-BF415e for its DNA copy number ratio

- Central Agricultural office, Food and Feed Safety Directorate, Laboratory for GMO Food, Budapest, HU* (DAP GmbH, DAP-PL-3101.00)
- Central Control and Testing Institute of Agriculture, Bratislava, SK* (Osvedčenie Akreditácii č.S-200)
- Crop Research Institute, Prague, CZ* (Czech accreditation institute, No 8/2007)
- Danish Plant Directorate, Laboratory for Diagnoses in Plants, Food and Feed, Lyngby, DK* (DANAK 330)
- Ente Nazionale Delle Sementi Elette (ENSE), Laboratorio Analisi Sementi, Tavazzano, IT (ISTA IT03)
- Eurofins Analytik GmbH, WEJ Dept Biology 135, Hamburg, DE* (DACH, DAC-PL-0526-07-04)
- Eurofins GeneScan GmbH, Freiburg, DE* (DACH, DAC-PL-0526-07-03)
- European Commission, Joint Research Centre, Institute for Health and Consumer Protection (IHCP), Ispra, IT* (DACH, DAC-PL-0459-06-00)
- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), BE* (BELAC, 268-TEST)
- Finnish Customs Laboratory, Espoo, FI* (FINAS, T006)
- Hainaut Vigilance Sanitaire - Institut Provincial d'Information et d'Analyses Sanitaires, Mons, BE* (BELAC 068-TEST)
- Institut für Hygiene und Umwelt, Behörde für Umwelt und Gesundheit, Hamburg, DE* (DACH, DAC-PL-0137-01-10)
- Instituto Superiore di Sanità, Centro nazionale per la Qualità degli Alimenti e per i Rischi Alimentari, Roma, IT
- Landeslabor Schleswig-Holstein Lebensmittel-, Veterinär- und Umwelt-Untersuchungsamt, Neumünster, DE* (AKS, AKS-P-10101-EU)
- Lifeprint GmbH, Illertissen, DE* (DACH, DAP-PL-3515.00)
- Livsmedelsverket, National Food Administration, Biology Division, Uppsala, SE* (SWEDAC, 1457)
- National Food and Veterinary Risk Assessment Institute (former National Veterinary Laboratory), Vilnius, LT* (DAP, DAP-PL-3328.99)
- National Institute of Biology, Department of Biotechnology and Systems Biology, Ljubljana, SI* (Slovenska akreditacija, LP-028)
- Staatliches Gewerbeaufsichtsamt Hildesheim, Dez 33 Gentechnik, Hildesheim, DE* (DACH, DAC-PL-0360-05-00)
- TNO Nutrition and Food Research - GMO foods, Zeist, NL* (RvA, L027)
- USDA, Grain Inspection, Technical Service Division, Kansas City, MO, US

Certification

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

* The laboratory holds ISO/IEC 17025 accreditation for DNA-based GMO measurements (accreditation body and registration number are mentioned).

§ Laboratory holds accreditation ISO Guide 34 for the production of reference materials.

3 CRM PROCESSING

ERM-BF415e is a gravimetrically prepared dried maize powder containing a certified mass fraction of 19.6 g/kg of the GM maize event NK603 with an expanded uncertainty (U_{CRM}, $k = 2$) of 0.9 g/kg. ERM-BF415e was processed in 2003 as part of a set of six CRMs certified for their mass fraction of NK603 [5].

4 HOMOGENEITY

4.1 HOMOGENEITY STUDY

The homogeneity study dedicated to this certification was performed under repeatability conditions using 15 bottles randomly taken from the entire batch and tested in random order. From each bottle 3 extracts were made and each analysed in triplicate. The DNA extraction method used was obtained with the GENE*Spin* kit (GeneScan Analytics GmbH, Freiburg, DE) and required a sample intake of 200 mg [2]. The measurements were done using the NK603 pure GM seed powder material for calibration and the maize NK603 event-specific real-time PCR quantification method [6] modified for the reference gene target as described in Section 6.1. As a consequence the measurement result obtained within this study is expressed as GM mass fraction percent and not as a DNA copy number ratio. However, this has no impact on the conclusion whether or not the material is homogeneous at a given sample intake.

In a first step it was checked whether the data followed a normal distribution using normal probability plots and histograms. The individual data and the bottle means from the homogeneity study measured for ERM-BF415e were normally distributed. No outliers were detected for these data applying the single and double Grubbs tests (95 % confidence level). Regression analysis was used to evaluate a potential trend in results related to the filling sequence. No significant trend was observed. The presence of a trend in the analytical sequence could not be investigated as the study setup did not allow for this – in a real-time PCR plate samples are not measured in a sequence but in the same run. As there were only two plates per PCR target no trend could be established.

ANOVA was used to assess the between bottle standard deviation (s_{bb}) and the maximum standard uncertainty due to a inhomogeneity that can be hidden by the method repeatability (u^*_{bb}), using the equations [10]:

$$s_{bb} = \sqrt{\frac{MS_{bb} - MS_{wb}}{n}} \qquad u^*_{bb} = \sqrt{\frac{MS_{wb}}{n}} \cdot \sqrt[4]{\frac{2}{df_{wb}}}$$

(MS_{bb} = mean sum of squares between bottles; MS_{wb} = mean sum of squares within bottles; n = number of replicates; df_{wb} = degrees of freedom within bottles).

Both values were converted into relative uncertainties by dividing to the mean of the study. The results of the homogeneity testing are summarised in Table 1. The larger of the two values (here, $s_{bb,rel}$) was used in the estimation of the uncertainty of the certified value (Section 7.3).

[2] Application of the GENE*Spin* extraction method in the homogeneity study required the higher sample intake of 200 mg maize seed powder to allow for a sufficient yield of extracted DNA.

Table 1: Relative standard uncertainty linked to the inhomogeneity between bottles of the dry-mixed NK603 maize ERM-BF415e analysed by real-time PCR ($N = 15$, $n = 3$).

mean NK603 mass fraction Mean measured mass fraction [%]	s_{bb} mass fraction [%]	$s_{bb, rel}$	u^*_{bb} mass fraction [%]	$u^*_{bb, rel}$
1.920	0.092	0.048	0.051	0.027

4.2 MINIMUM SAMPLE INTAKE

Different sample intakes, 200 mg (GENE*Spin*) and 100 mg (CTAB/CTAB-Tip20), 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 estimated to be 100 mg. For the validity of the certified values of ERM-BF415e, sample intakes not smaller than 100 mg have to be used.

5 STABILITY

5.1 SHORT-TERM STABILITY

During certification of other dry-mixed maize GMO CRMs they proved to be stable at ambient and elevated temperatures (18 and 60 °C) for a period of 2-4 weeks [11, 12, 13, 14, 15, 16, 17]. As those results have been obtained for maize materials processed in the same way as the ERM-BF415 series there is no reason to expect a different pattern in the stability of ERM-BF415e. It can be concluded that ERM-BF415e can be shipped under ambient conditions.

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-BF415. There is no reason to expect a different behaviour of ERM-BF415e compared to the other maize matrices upon long-term storage at 4 °C and under controlled conditions. Therefore, the long term-stability of the maize NK603 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 °C and reference samples kept at -70 °C ($\bar{x}_{4\,°C}$ / $\bar{x}_{-70\,°C}$), 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 estimate the uncertainty contribution of the stability.

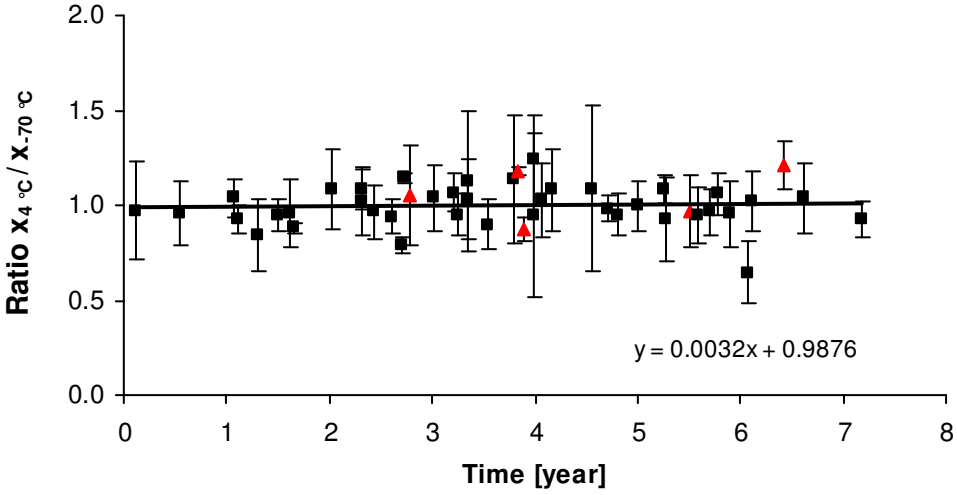

Figure 1: Long-term stability of different dried maize seed powder (not only NK603 (▲)) stored at 4 ºC for various time periods, based on real-time PCR measurements. The stability of the GM content is assessed via the ratio between values corresponding to samples stored at 4 ºC and samples stored for 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 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.

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 ($u_{lts,rel}$) [18], 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-BF415e.

6 CHARACTERISATION

6.1 STUDY SET-UP

Twenty-one laboratories were selected on the basis of proven experience and quality management systems in place. For the characterisation of ERM-BF415e with respect to its DNA copy number ratio, a total of 42 analyses were requested. To detect a possible dependence of the real-time PCR quantification method from the DNA extraction method, three different DNA extraction methods were tested:

- A modified cetyltrimethylammonium bromide (CTAB) extraction method [19], 100 mg sample intake (Annex 1, adopted from ISO 21571:2005);
- The CTAB method referred above followed by Genomic tip-20/G purification step (Qiagen Benelux BV, Venlo, NL), 100 mg sample intake (Annex 2);
- 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.

Fourteen data sets were allocated to each DNA extraction method. IRMM performed three independent analyses, applying one of the tested DNA extraction methods in each. One laboratory applied the CTAB extraction method when the analysis request specified DNA extraction with CTAB tip-20. Therefore, during data analysis, the number of labs performing the CTAB DNA extraction method was set to 15 and the number of labs performing the CTAB tip-20 extraction method was set to 13.

Matrix CRM ERM-BF415e, DNA calibrants (pDNA and gDNA extractred from leaves) and real-time PCR primers and probes were shipped to the participating laboratories on dry ice.

For each independent analysis two bottles of ERM-BF415e 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 was performed on two different days. The DNA concentration was estimated by UV spectrometry and/or by fluorometry. The DNA concentration of each DNA extract was adjusted to 20 ng/µL with nuclease-free water and the extracts were further diluted 1.5, 3, 4 and 6 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 event NK603 [6] and maize endogenous *hmg* gene [7] (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 NK603 plants, which were individually tested for the presence of NK603 trangene. The pDNA calibrant is the specific NK603 maize pDNA CRM ERM-AD415. 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 $1.6 \cdot 10^4$ cp/µL, and ii) pDNA ERM-AD415 was diluted in $T_1E_{0.01}$ buffer (1 mmol/L Tris, 0.01 mmol/L EDTA, pH 8.0) to yield DNA concentrations 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-AD415 and one that was calibrated with gDNA from leaves.

Real-time PCR experiments using TaqMan Universal PCR MasterMix were carried out according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, US) in 96 well microtiter plates, according to the method validated by the CRL-GMFF for the event-specific quantification of the maize NK603 event [6] with exception of the following changes: the reference gene target was changed from *adh1* to *hmg* to avoid a possibility for erroneous quantification of the copy number of the reference target as reported by Broothaerts et al. [7]; the real-time PCR reaction volume used in this certification study was 25 µL; the baseline and threshold of the individual real-time PCR measurements were set automatically. One laboratory performed the real-time PCR measurements in 384-well

microtiter plates with adjusted real-time PCR assay volume of 20 µL. A table summarising deviations from the CRL protocol is available in Annex 3.

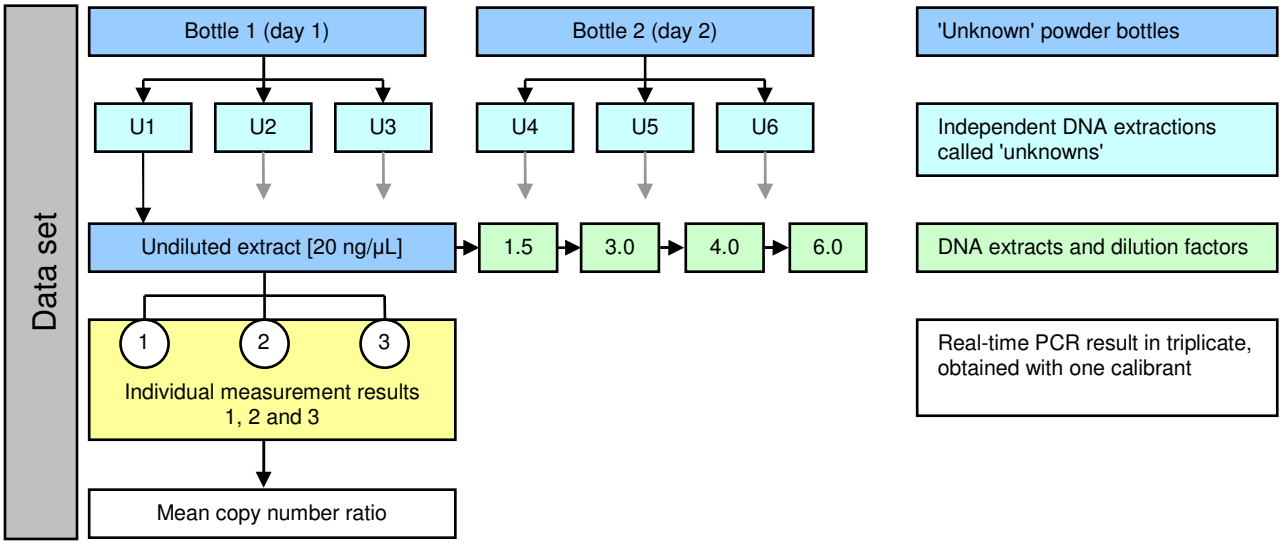

Figure 2: Analysis scheme followed by the participating laboratories during the copy number study. Undiluted U1 is given as an example. The same steps are also followed for all other samples and their dilutions. Please note that the same pipetted DNA extracts, *i.e.* the triplicates that are shown in the yellow box, are also analysed using the other calibrant.

6.2 ACCEPTANCE CRITERIA FOR DATA SETS

Data acceptance criteria for this study were established. Most of them are described by Charels et al. [20] 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 (E) and transgenic (T) targets using either the pDNA calibrant ERM-AD415 or gDNA calibrant from leaves. The results obtained on the two days of analysis were considered separately for the two calibrants. This means that within the set of an independent analysis the rejection of a data set calibrated with the pDNA calibrant ERM-AD415 was no reason to reject the data set calibrated with the gDNA calibrant from plant leaves and vice versa.

6.2.1 Technical acceptance criteria

Only data sets with negative non-template controls (NTCs) were accepted. Six data sets were not accepted for the gDNA calibrant and five for the pDNA calibrant because their NTCs were positive.

Data sets were rejected if any inconsistency in the dilution series was noticeable in the trend of reported threshold cycles (Ct) (in a dilution series, increasing dilution factors were expected to result in increasing Cts). Two data sets from each calibrant sequence, i.e. gDNA and pDNA, were rejected due to inconsistencies between the dilutions and reported Cts.

Means of triplicate measurements were not accepted if the Ct difference within one triplicate was higher than 1.5 for any of the replicates subtracted from the mean Ct of the remaining replicates within the particular dilution point. Dilution points were also excluded if amplification signal was absent. 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 the EURL-GMFF [21]. In particular, these selection criteria apply to the slope and linearity of the calibration curves.

Calibration curves with a coefficient of determination (R^2) of minimum 0.98 were accepted. Two data sets for the gDNA calibrant and four data sets for the pDNA calibrant were rejected that did not fulfil this requirement.

Control limits for PCR efficiencies *(ε)* were applied. For both calibrants, the transgene and endogene mean PCR efficiencies and their standard deviations were calculated. Control limits were calculated per calibrant, transgene and endogene as the mean PCR efficiency plus or minus one standard deviation, *i.e.* four intervals were generated. The minimum and maximum of those calculated values were then applied as the lower and upper limits of the PCR efficiency. In this study the resulting control limits were 77 % and 99 %. These efficiencies correspond to a slope of -4.03 and -3.35, respectively. For data sets calibrated with gDNA calibrant from leaves five data sets were rejected as their PCR efficiencies were outside the control limits (77-99 %). For data sets calibrated with the pDNA calibrant ERM-AD415, seven data sets were rejected due to PCR efficiencies outside the control limits (77-99 %).

6.2.3 Working interval

DNA extracts and dilutions exhibiting a Ct outside the working range of the calibration curve were excluded for further calculations. Based on this criterion, one data set calibrated with the pDNA calibrant ERM-AD415 was excluded and no data set calibrated with the gDNA calibrant from leaves was excluded.

6.2.4 Copy number ratios, DNA extracts and their dilutions

The mean Cts 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 within its 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.* a maximum of six copy number ratios and a minimum of four copy number ratios per data set and calibrant were accepted.

No data set was rejected due to insufficient data points per day.

6.2.5 Relative Standard Deviation

The relative standard deviation (RSD) was calculated for the copy number ratios per calibrant, 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.

No data sets calibrated with the gDNA calibrant from leaves were rejected due to the criterion described above. One data set calibrated with the pDNA calibrant was rejected due to an RSD per day exceeding 25 %. One data set calibrated with the pDNA calibrant was rejected as the RSD from the whole data set exceeded 25 %.

Table 2: Overview of rejected data sets and reasons for their exclusion. Blank cells indicate that data set was accepted; data sets accepted for both pDNA and gDNA calibrations are omitted.

Data set	DNA extraction method	ERM-AD415 pDNA calibrant	gDNA calibrant from leaves
2	CTAB[1]	calibration curve (ε)	
5	CTAB[1]		calibration curve (ε)
7	CTAB[1]	calibration curves (R^2, ε)	
10	CTAB[1]	technical (NTC)	calibration curves (R^2, ε)
11	CTAB[1]	calibration curve (ε)	technical (NTC)
12	CTAB[1]	calibration curve (ε)	
13	CTAB[1]	calibration curves (R^2, ε)	
15	CTAB-Tip20[2]	calibration curve (ε)	
16	CTAB-Tip20[2]	working interval	technical (NTC)
21	CTAB-Tip20[2]	technical (NTC)	
22	CTAB-Tip20[2]	technical (NTC)	technical (NTC)
23	CTAB-Tip20[2]	calibration curve (ε)	calibration curve (ε)
24	CTAB[1]		calibration curve (ε)
25	CTAB-Tip20[2]	technical	
26	CTAB-Tip20[2]	calibration curve (ε)	
27	CTAB-Tip20[2]	RSD	technical (NTC)
28	CTAB-Tip20[2]	technical (NTC)	technical (NTC)
35	GENE*Spin*[3]	calibration curves (R^2, ε)	
37	GENE*Spin*[3]	RSD	technical
38	GENE*Spin*[3]	technical	calibration curves (ε)
39	GENE*Spin*[3]	calibration curves (R^2, ε)	technical
40	GENE*Spin*[3]		calibration curve (ε)
41	GENE*Spin*[3]	calibration curve (ε)	technical (NTC)
42	GENE*Spin*[3]	technical (NTC)	calibration curves (R^2, ε)

[1] DNA was extracted using a modified CTAB method and a sample intake of 100 mg (Annex 1).
[2] DNA was extracted using a modified CTAB method followed by a Genomic tip-20/G purification step and a sample intake of 100 mg (Annex 2).
[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-AD415 and for the gDNA calibrant from leaves 21 and 27 data sets were accepted, respectively.

For three data sets from the gDNA calibrant and for one data set from the pDNA calibrant five of the six unknowns per data set were accepted. For all other data sets all 6 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 ± standard deviation (*s*). Blank cells indicate excluded data sets; data sets excluded for both pDNA and gDNA calibrations are omitted.

		DNA copy number ratio ± *s* [%]	
Data set	DNA extraction method	ERM-AD415 pDNA calibrant	gDNA calibrant from leaves
1	CTAB[1]	0.99 ± 0.01	1.40 ± 0.07
2	CTAB[1]		1.10 ± 0.09
3	CTAB[1]	0.81 ± 0.06	1.10 ± 0.10
4	CTAB[1]	0.85 ± 0.06	1.06 ± 0.05
5	CTAB[1]	1.10 ± 0.01	
6	CTAB[1]	0.73 ± 0.06	1.07 ± 0.00
7	CTAB[1]		1.21 ± 0.01
8	CTAB[1]	0.96 ± 0.03	1.17 ± 0.01
9	CTAB[1]	0.88 ± 0.04	1.20 ± 0.03
12	CTAB[1]		1.15 ± 0.02
13	CTAB[1]		1.16 ± 0.16
14	CTAB[1]	0.92 ± 0.13	1.33 ± 0.13
15	CTAB-Tip20[2]		1.21 ± 0.02
17	CTAB-Tip20[2]	0.94 ± 0.01	1.21 ± 0.01
18	CTAB-Tip20[2]	0.98 ± 0.05	1.23 ± 0.03
19	CTAB-Tip20[2]	0.91 ± 0.11	1.23 ± 0.07
20	CTAB-Tip20[2]	0.92 ± 0.05	1.29 ± 0.02
21	CTAB-Tip20[2]		1.28 ± 0.08
24	CTAB[1]	1.14 ± 0.33	
25	CTAB-Tip20[2]		1.11 ± 0.20
26	CTAB-Tip20[2]		1.10 ± 0.07
29	GENE*Spin*[3]	0.95 ± 0.03	1.11 ± 0.12
30	GENE*Spin*[3]	0.90 ± 0.00	1.08 ± 0.01
31	GENE*Spin*[3]	0.84 ± 0.10	1.12 ± 0.17
32	GENE*Spin*[3]	1.07 ± 0.07	1.20 ± 0.08
33	GENE*Spin*[3]	1.14 ± 0.04	1.33 ± 0.09
34	GENE*Spin*[3]	0.98 ± 0.16	1.24 ± 0.04
35	GENE*Spin*[3]		1.30 ± 0.01
36	GENE*Spin*[3]	0.93 ± 0.04	1.19 ± 0.02
40	GENE*Spin*[3]	0.89 ± 0.01	

[1] DNA was extracted using a modified CTAB method and a sample intake of 100 mg (Annex 1).
[2] DNA was extracted using a modified CTAB method followed by a Genomic tip-20/G purification step and a sample intake of 100 mg (Annex 2).
[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 standard deviations (*s)* from the ERM-BF415e Copy Number Certification Study. Mean DNA copy number ratios obtained with pDNA ERM-AD415 calibrant and gDNA calibrant from leaves (gDNA*ℓ*) 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-AD415 pDNA	gDNA*ℓ*
ERM-BF415e	0.95 ± 0.10 (N_d = 21)	1.19 ± 0.09 (N_d = 27)

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 [22]. 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-AD415 had overlapping $\bar{x} \pm s$ 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.

DNA copy number ratios obtained by real-time PCR calibrated with the pDNA calibrant ERM-AD415 or gDNA from leaves followed a normal distribution. No outliers were detected with those data using the Grubbs and Dixon outlier tests (95 % confidence level). When using the Nalimov *t*-test, in each case (data calibrated with pDNA or gDNA from leaves) one outlying result was detected at 95 % confidence level. However, no outlying results were found using the same statistical test at 99 % confidence level. The results detected as outliers by the Nalimov *t*-test at 95 % confidence level were retained and used in the evaluation of the certified value and its uncertainty.

The mean DNA copy number ratio obtained when experiments were calibrated with gDNA extracted from leaves is higher than the value obtained with ERM-AD415 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 ($p = 1.74 \times 10^{-11}$, 95 % confidence level).

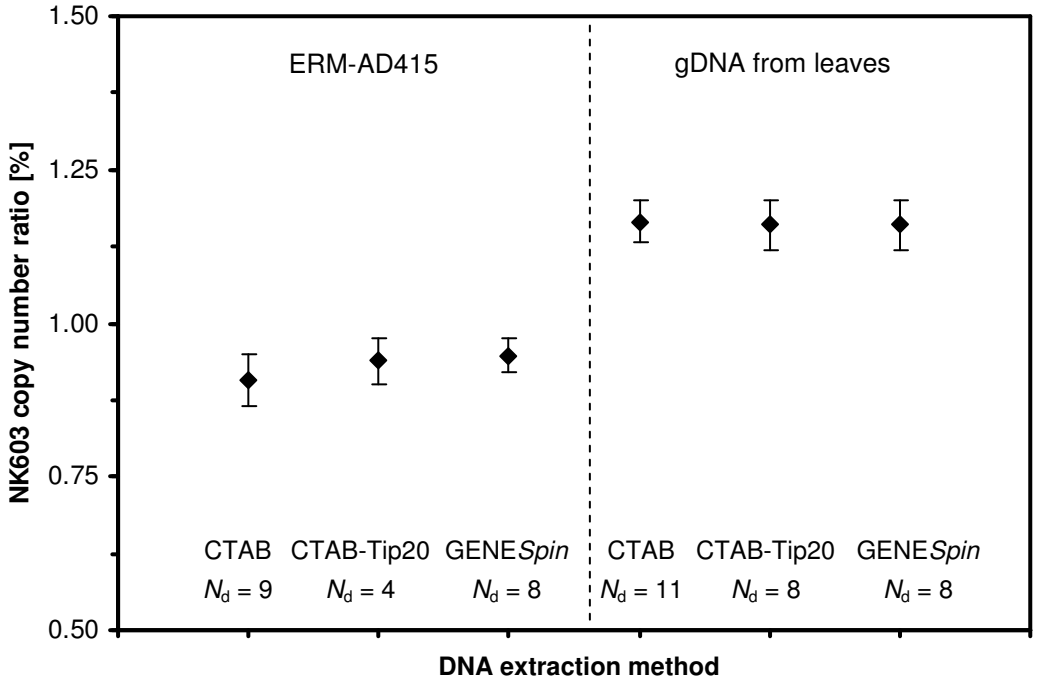

Figure 3: The influence of the DNA extraction method on the measured copy number ratios. Copy number ratios (♦) ± standard deviation *s* for gDNA and pDNA calibrant and each extraction method are shown. N_d is the number of data sets used for evaluation, from each calibrant and extraction method.

6.5 ANALYTICAL BEHAVIOUR OF PLASMID AND GENOMIC DNA

As there is a significant influence of the type of calibrant on the copy number ratio, the data could not be pooled. Consequently, the certified value 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 unknows resemble typically a 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-AD415, dilutions of gDNA extracted from leaves from germinated NK603 maize seeds (gDNAℓ), as well as dilutions of gDNA extracted from the ERM-BF415e maize seed powder (gDNAs). To evaluate the analytical behaviour of the plasmid and genomic DNA, these two parameters were 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 [20, 21]. Firstly, a R^2 value 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 gDNAℓ efficiencies (both materials used as calibrants in the study). The means of the PCR efficiencies were calculated for the endogenous and transgenic targets using either the pDNA or gDNAℓ calibrant. Four intervals were generated accordingly based on the mean ± 1 standard deviation (1 s) (not shown). The minimum and maximum values of the resulting eight cut-off points were then used to define the lower and higher limits of the PCR efficiency of the study, *i.e.* 77 and 99 % respectively. These values were consistent with the performance of the simplex real-time PCR method for quantification of the NK603 maize event observed with in-house performed studies and from results obtained by collaborations with external laboratories.

The means of the PCR efficiencies varied between 83.0 % and 95.0 % (Table 5). For both targets, PCR efficiencies were highest using the pDNA and lowest for the gDNAℓ calibrant. Comparing the targets, PCR efficiencies were higher for the endogenous gene *hmg* than for the transgene NK603 (Table 5).

A difference in PCR efficiencies between the pDNA and the gDNAℓ calibrants was observed (2.8 % for the endogenous and 4.6 % for the transgenic targets) (Table 5). The PCR efficiencies of pDNA and gDNAℓ calibrants were found to be significantly different in the cases of both targets (ANOVA: *hmg:* $p = 2.2 \times 10^{-8}$, $\alpha = 0.05$; NK603: $p = 3.6 \times 10^{-14}$, $\alpha = 0.05$) using a simplex real-time PCR detection method.

The distributions of the various PCR efficiencies for pDNA/gDNAℓ and gDNAs were compared and showed a large overlap for both targets for the three DNA types (Figure 4). There was no significant difference between the PCR efficiencies of both the endogenous and transgenic targets for pDNA and gDNAs (Kruskal-Wallis test[3] for the endogenous target: $p = 0.59$, $\alpha = 0.05$; ANOVA for the transgenic target: $p = 0.25$, $\alpha = 0.05$). All other comparisons led to significantly different results in the cases of both (endogenous and transgenic) real-time PCR targets (Figure 4 and Table 5).

[3] Kruskal-Wallis test was applied as the compared data did not follow a normal distribution.

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

Target sequence	**Mean PCR efficiency ± *s*** [%]		
	ERM-AD415 pDNA	Seeds gDNA gDNA*s*	Leaves gDNA gDNA*ℓ*
hmg	95.0 ± 2.3 (N_{ds} = 66)	93.1 ± 4.8 (N_{ds} = 107)	92.2 ± 3.1 (N_{ds} = 66)
NK603	87.6 ± 3.5 (N_{ds} = 74)	87.1 ± 5.3 (N_{ds} = 110)	83.0 ± 3.3 (N_{ds} = 75)

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

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 5). For the transgenic target the distribution of the coefficient of determination displayed a large variation. However, the coefficients of determination of gDNA*s* dilution curves distributed closer to those of the pDNA calilbrant in the case of both targets.

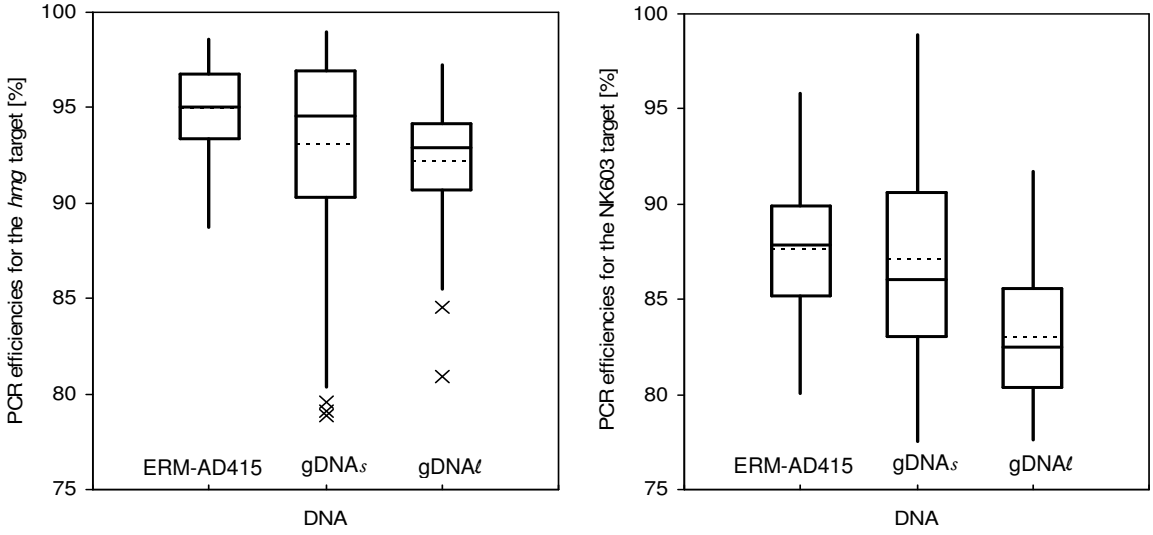

Figure 4: Box-and-whisker diagram illustrating the PCR efficiencies for *hmg* and NK603 based on the dilution series performed using either ERM-AD415 calibrant or gDNA extracted from NK603 seeds or NK603 leaves. The bottom and top of the box are the 1st and 3rd quartiles, respectively; the full line 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 of each group, whereas values outside the 95 % confidence interval are marked with x.

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

Target sequence	Mean R^2 ±*s*		
	ERM-AD415 pDNA	Seeds gDNA gDNA*s*	Leaves gDNA gDNA*ℓ*
hmg	0.998 ± 0.003 (N_{ds} = 66)	0.997 ± 0.004 (N_{ds} = 107)	0.998 ± 0.003 (N_{ds} = 66)
NK603	0.994 ± 0.004 (N_{ds} = 74)	0.992 ± 0.005 (N_{ds} = 110)	0.998 ± 0.002 (N_{ds} = 75)

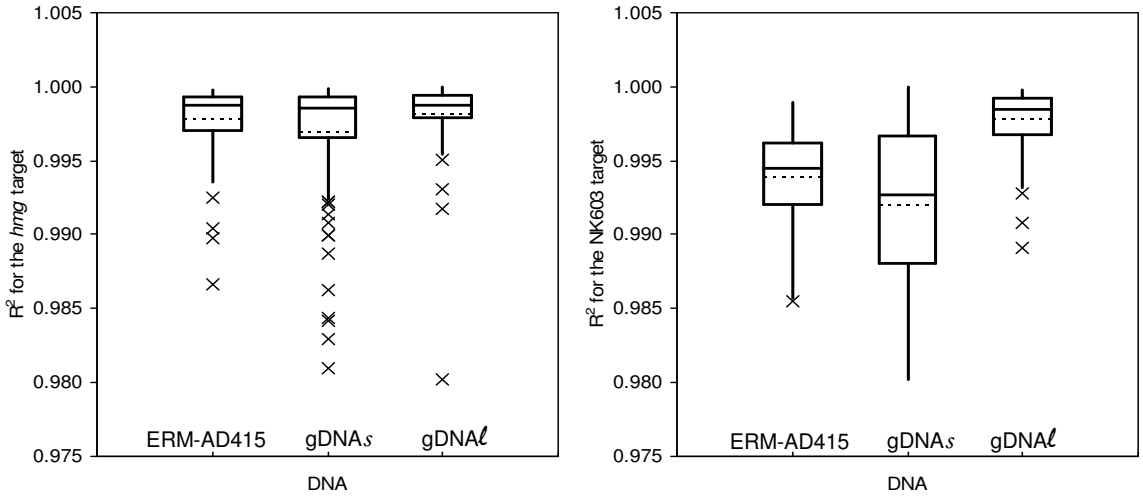

Figure 5: Box-and-whisker diagram representing the coefficients of determination (R^2) for *hmg* and NK603 based on the dilution curves performed using either ERM-AD415 calibrant or gDNA extracted from NK603 seeds or NK603 leaves. A description of a box-and-whisker diagram is given in Figure 4.

Finally, the NK603 GM copy number ratio of ERM-BF415e (19.6 g/kg by mass fraction) was compared using either ERM-AD415 or gDNA*ℓ* as calibrants (Figure 6 and Table 7). 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. Though the GM copy number ratios obtained for ERM-BF415e by the two different calibrants overlap, the mean GM copy number ratios are very different. Single factor ANOVA analysis confirms that the data set calibrated with ERM-AD415 is significantly different from the data set calibrated with gDNA from leaves ($p = 1.9 \times 10^{-17}$, $\alpha = 0.05$). Therefore the GM copy number data calibrated with ERM-AD415 or gDNA from leaves cannot be pooled.

In the study reported here, statistical analyses have shown that pDNA and gDNA*ℓ* calibrants behave in a different way with respect to the PCR efficiencies of the transgenic and endogenous target sequences (Figure 4). The individual PCR efficiency of each target sequence has a significant impact on GM quantification by real-time PCR. Therefore, the effect of a small difference in PCR efficiencies of the transgenic and endogenous 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 significant differences in PCR efficiencies of the calibrants may explain the difference of GM copy number ratios observed when the mean values are compared (Table 7).

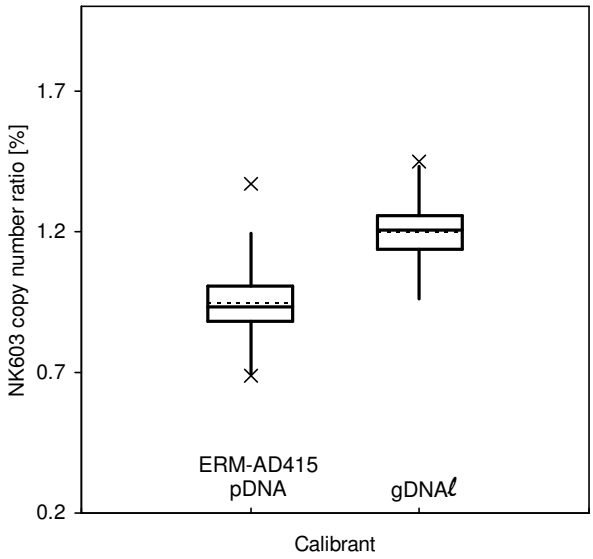

Figure 6: Box-and-whisker diagram and distribution graph of the NK603 copy number ratio in ERM-BF415e using either ERM-AD415 or gDNA extracted from NK603 leaves as calibrant. A description of a box-and-whisker diagram is given in Figure 4.

Table 7: DNA copy number ratio in ERM-BF415e calibrated either with the ERM-AD415 or genomic DNA extracted from NK603 leaves; N_{ds} is the number of accepted data sub-sets under reproducibility conditions, *s* is the standard deviation.

	DNA copy number ratio ± *s* [%]	
	ERM-AD415 pDNA	Leaves gDNAℓ
ERM-BF415e	0.95 ± 0.12 (N_{ds} = 42)	1.19 ± 0.11 (N_{ds} = 54)

An additional measurement of the gDNA extracted from ERM-BF415e was done by digital PCR [23-24]. 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 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-BF415e was 0.96 ± 0.08 % (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-AD415 is used.

Based on studying the analytical behaviour of pDNA and gDNA, it can be concluded that both calibrants, i.e. pDNA ERM-AD415 and gDNAℓ extracted from maize NK603 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). Using the pDNA calibrant ERM-AD415, the estimated DNA copy number ratio was in agreement with results obtained by digital PCR (about 1 % difference). When gDNAℓ is used as a calibrant, the DNA copy number ratio might be overestimated by about 19 % compared to results 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-AD415 [8], as primary calibrant for DNA copy number ratio measurements, together with the approved GM quantification method published by the EURL-GMFF [6].

7 CERTIFIED VALUE AND UNCERTAINTY BUDGET

7.1 METROLOGICAL TRACEABILITY

The certified DNA copy number ratio is defined as the NK603 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 NK603 event-specific real-time PCR method calibrated with the pDNA CRM. Therefore, and given the interlaboratory comparison comprising 21 accepted data sets, the certified value for the DNA copy number ratio is defined by the event-specific real-time PCR detection method validated by the EURL GMFF [6] modified as described in this report (Section 6.1) and calibrated with the NK603 maize pDNA Certified Reference Material ERM-AD415 [8]. 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 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 NK603 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 percent, is calculated as follows:

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

Because the data are normally distributed (Section 6), the mean ($\overline{x}$) is calculated as the unweighted mean of data set means:

$$\overline{x} = \frac{\sum_{i=1}^{N_d} \overline{x}_i}{N_\mathrm{d}}$$

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

7.3 UNCERTAINTY BUDGET

The expanded uncertainty of the certified value (U_{CRM}) comprises the relative standard uncertainty contributions from the homogeneity, stability and characterisation. The uncertainty of the calibrant is negligible and therefore not considered [8]. The expanded uncertainty of the certified value is estimated according to the following equation [25]:

$$U_{CRM} = k \cdot \overline{x} \cdot \sqrt{u_{\mathrm{bb,rel}}^2 + u_{\mathrm{lts,rel}}^2 + u_{\mathrm{char,rel}}^2}$$

(k = coverage factor; $u_{\mathrm{bb,rel}}$ = realative uncertainty contribution from the homogeneity, $u_{\mathrm{lts,rel}}$ = relative uncertainty contribution from the long-term stability, $u_{\mathrm{char,rel}}$ = relative uncertainty contribution form 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. It is calculated using the equation:

$$u_{\text{char, rel}} = \frac{s}{\bar{x} \cdot \sqrt{N_d}} .$$

The relative standard uncertainty related to the homogeneity at 200 mg level was estimated on the basis of a normally distributed population of real-time PCR measurement results. 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).

The standard uncertainty contribution introduced by the homogeneity is larger than the standard uncertainty contributions from the characterisation and from the long-term stability of the maize powders (Table 8).

Table 8: Uncertainty budget for the DNA copy number ratio of NK603 maize in ERM-BF415e**.**

CRM certified value [%]	**Relative standard uncertainty contributions**			**Expanded uncertainty** U_{CRM} [%]
	$u_{bb, rel}$ [1]	$u_{lts, rel}$ [2]	$u_{char, rel}$ [3]	U_{CRM} (k = 2)
0.945	0.048	0.009	0.024	0.103

[1] Relative standard uncertainty related to the homogeneity measured with a sample intake of 200 mg.
[2] Relative standard uncertainty related to the long-term stability, estimated on the basis of a shelf life of 12 months.
[3] Relative standard uncertainty related to the characterisation.

7.4 COMMUTABILITY

ERM-BF415e is prepared from non-GM and GM maize seed powder. The certified value is a DNA copy number ratio based on the NK603 copy numbers and maize specific DNA copy numbers, expressed in percent; it is defined by the real-time PCR method for detection of NK603 described in this certification report, calibrated with the plasmid DNA CRM ERM-AD415 [8]. The two calibrants tested during certification, pDNA ERM-AD415 and genomic DNA extracted from plant leaves (gDNAℓ), led to significant differences in the measured copy number ratio of ERM-BF415e and commutability [26] problems should be considered.

While results obtained during certification using the pDNA ERM-AD415 calibrant were in agreement with the value obtained by the calibrant-independent digital PCR measurements (1 % difference), calibration using gDNAℓ differed from that value by 19 %. However, during the analytical behaviour study it could not be proven that one of the calibrants behaves more similar to the gDNA extracted from maize seed powder.

For practical reasons, ensuring for instance full characterisation and reproduction of additional batches of calibrants, pDNA ERM-AD415 was selected to be the highest point of the calibration chain. The copy number ratios measured by the NK603 real-time PCR method and using the ERM-AD415 calibrant on ERM-BF415e and food and feed samples are reproducible and expected to be the same.

8 INSTRUCTIONS FOR USE

8.1 INTENDED USE

The material ERM-BF415e is certified for both its mass fraction of the GM event NK603 [5] and the DNA copy number ratio when applying event-specific NK603 real-time PCR [this report]. The DNA copy number ratio is based on the NK603 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 the quality control of measurements of the NK603 DNA copy number ratios in GM food and feed. Genomic DNA may 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 NK603 detection method [6] modified according to method used in this report, calibrated with the pDNA calibrant ERM-AD415 [8] 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.

ACKNOWLEDGEMENTS

The authors would like to thank W. Broothaerts, M. Caprioara-Buda, W. Meyer and T. Linsinger from IRMM for sharing technical expertise in the analysis of results, M. Contreras and S. Mazoua from IRMM for their technical assistance and L. Deprez, A. Held and M. Potalivo from IRMM for the reviewing of the certification report, as well as the experts of the Certification Advisory Panel "Biological Macromolecules and Biological/Biochemical Parameters": R. Dybkaer (Frederiksberg Hospital, DK), A. Heissenberger (Umweltbundesamt/Environment Agency Austria, Vienna, AT) and U. Örnemark (Emendo Dokumentgranskning Handelsbolag, Hillared, SE) for their critical comments.

REFERENCES

[1] Regulation (EC) No 1830/2003 of 22.09.2003 concerning the traceability and labeling of genetically modified organisms and the traceability of food and feed products from genetically modified organisms and amending Directive 2001/18/EC. Off J Eur Union L 268 (2003) 24-28.

[2] Commission Recommendation (EC) No 787/2004 of 4 October 2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003. Off J Eur Union L 348 (2004) 18-26.

[3] Regulation (EC) No 65/2004 of 14 January 2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms. Official J Eur Union L(10) (2004) 5-10.

[4] Scientific Opinion of the Panel on Genetically Modified Organisms on applications (EFSA-GMO-NL-2005-22 and EFSA-GMO-RX-NK603) for the placing on the market of the genetically modified glyphosate tolerant maize NK603 for cultivation, food and feed uses and import and processing, and for renewal of the authorisation of maize NK603 as existing product. The EFSA Journal 1137 (2009) 1-50.

[5] S. Trapmann, P. Conneely, P. Corbisier, D. Gancberg, S. Gioria, M. Van Nyen, H. Schimmel and H. Emons. The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of NK603 Maize. Certified Reference Materials ERM-BF415. (2005) ISBN 92-894-8063-7. EC certification report EUR Report 21270.

[6] Event-specific method for the quantitation of maize line NK603 using real-time PCR available on the homepage of the Community Reference Laboratory for GM Food and Feed (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) published on 10/01/2005.

[7] W. Broothaerts, P. Corbisier, H. Schimmel, S. Trapmann, S. Vincent, H. Emons. A single nucleotide polymorphism (SNP839) in the adh1 reference gene affects the quantitation of genetically modified maize (Zea mays L.). J Agric Food Chem 56(19) (2008) 8825-31.

[8] B. Jeynov, E. De Andrade Silva, W. Broothaerts, S. Mazoua, S. Vincent, A. Merveillie, S. Trapmann, P. Corbisier, H. Emons. Certification of plasmid DNA containing NK603 DNA maize fragments. Certified Reference Material ERM®-AD415. (2010) ISBN 972-92-79-19071-1**.** EC certification report EUR 24696 EN.

[9] A.B. Krech, A. Wurtz, C. Stemmer, G. Feix, K.D. Grasser. Structure of genes encoding chromosomal HMG1 proteins from maize. Gene 234 (1999) 45-50.

[10] T.P.J. Linsinger, J. Pauwels, A.M.H. Van der Veen, H. Schimmel, A. Lamberty Homogeneity and stability of reference materials. Accred Qual Assur 6 (2005) 20-25.

[11] S. Trapmann, L. Le Guern, J. Prokisch, P. Robouch, G.N. Kramer, H. Schimmel, J. Pauwels. The Certification of Reference Materials of Dry Mixed Maize Powder with different Mass Fractions of MON 810 Maize. Certified Reference Materials IRMM-413. (IRMM-413-0 / IRMM-413-1 / IRMM-413-2 /IRMM-413-3 / IRMM-413-4 / IRMM-413-5). (2001) ISBN 92-894-1978-4. EC certification report EUR 20111 EN.

[12] S. Trapmann, J. Charoud-Got, P. Conneely, M. Contreras, P. Corbisier, D. Gancberg, E. Hannes, S. Gioria, A. Muñoz-Pineiro, M. Van Nyen, H. Schimmel, S. Szilagy, H. Emons. The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of MON 863 Maize Certified Reference Material ERM®-BF416 (ERM®-BF416a/ERM®-BF416b/ERM®-BF416c/ERM®-BF416d). (2001) ISBN 92-894-9195-7. EC certification report EUR 21574 EN.

[13] S. Trapmann, P. Conneely, P. Corbisier, D. Gancberg, E. Hannes, S. Gioria, M. Van Nyen, H. Schimmel, S. Szilágyi, K. Teipel, H. Emons. The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of MON 863 x MON 810 Maize Certified Reference Materials ERM®-BF417 (ERM®-BF417a / ERM®-BF417b / ERM®-BF417c / ERM®-BF417d). (2005) ISBN 92-894-6735-5. EC certification report EUR 21567 EN.

[14] S. Trapmann, P. Conneely, M. Contreras, P. Corbisier, D. Gancberg, E. Hannes, S. Gioria, A. Muñoz-Pineiro, M. Van Nyen, H. Schimmel, S. Szilagy, H. Emons. The

Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of 1507 Maize Certified Reference Materials ERM®-BF418 (ERM®-BF418a / ERM®-BF418b / ERM®-BF418c / ERM®-BF418d). (2005) ISBN 92-894-9748-3. EC certification report EUR 21689 EN.

[15] S. Trapmann, P. Conneely, M. Contreras, P. Corbisier, S. Gioria, M. Van Nyen, S. Vincent, H. Emons. The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of MIR604 Maize. Set of Certified Reference Materials ERM®-BF423a, ERM®-BF423b, ERM®-BF423c, ERM®-BF423d. (2006) ISBN 92-79-01635-0. EC certification report EUR 22175 EN.

[16] W. Broothaerts, J. Charoud-Got, P. Conneely, M. Contreras, P. Corbisier, S. Gioria, M. Van Nyen, S. Vincent, S. Trapmann. The certification of reference materials of maize seed powder with different mass fractions of the maize event 3272 Certified Reference Materials ERM®-BF420 (ERM®-BF420a, ERM®-BF420b, ERM®-BF420c). (2007) ISBN 978-92-79-05219-4. EC certification report EUR 22698 EN.

[17] D. Gancberg, S. Trapmann, S. Broeders, K. Teipel, P. Conneely, M. Contreras, P. Corbisier, E. Hannes, S. Gioria, M. Van Nyen, H. Schimmel, H. Emons. The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractionsof 59122 Maize Certified Reference Materials ERM®-BF424 (ERM®-BF424a, ERM®-BF424b, ERM®-BF424c, ERM®-BF424d). (2006) ISBN 92-79-02755-7. EC certification report EUR 22333 EN.

[18] A. Lamberty, H. Schimmel, J. Pauwels. The study of the stability of reference materials by isochronous measurements. Fresenius J Anal Chem 360 (1998) 359-361.

[19] M.G. Murray, W.F. Thompson. Rapid isolation of high molecular weight plant DNA. Nucl Acids Res 8 (1980) 4321-4325.

[20] D. Charels, S. Broeders, P. Corbisier, S. Trapmann, H. Schimmel, T. Linsinger, H. Emons. Toward metrological traceability for DNA fragment ratios in GM quantification Part 2: Systematic Study of Parameters Influencing the Quantitative Determination of MON 810 Corn by Real-Time PCR. J Agric Food Chem 55 (2007) 3258-3267.

[21] Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

[22] J. Schwarze. ed. Grundlagen der Statistik 2: Wahrscheinlichkeitsrechnung und induktive Statistik. (2009) Verlag Neue Wirtschafts-Briefe, GmbH & Co. KG, Herne 1986.

[23] S. Bhat, J. Herrmann, P. Armishaw, P. Corbisier, K.R. Emslie. Single molecule detection in nanofluidic digital array enables accurate measurement of DNA copy number. Anal Bioanal Chem 394 (2009) 457-67.

[24] P. Corbisier, S. Bhat, L. Partis, V.R. Xie, K.R. Emslie. Absolute quantification of genetically modified MON810 maize (Zea mays L.) by digital polymerase chain reaction. Anal Bioanal Chem 396 (2010) 2143-50.

[25] EURACHEM/CITAC, EURACHEM/CITAC Guide - Quantifying uncertainty in analytical measurement, second ed., EURACHEM (2000) http://www.eurachem.org

[26] International vocabulary of metrology - Basic and general concepts and associated terms (VIM) JCGM 200 (2008). Available via http://www.bipm.org/en/publications/guides/vim.html.

ANNEX 1

Modified protocol for DNA extraction using the CTAB method

Required reagents:

Reagent	Composition
H_2O	Sterile nuclease-free water
RNase A	100 mg/mL
Proteinase K	20 mg/mL
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 mol/L NaCl 0.1 mol/L Tris-HCl pH 8.0 15 mmol/L Na_2EDTA
CTAB precipitation buffer	0.5 % mass concentration CTAB 40 mmol/L NaCl 50 mmo/L Tris-HCl pH 8.0
$T_1E_{0.01}$ Buffer	1 mmol/L Tris pH 8.0 0.01 mmol/L EDTA

Procedure:

1. Weigh out 100 mg of plant powder into 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 minutes 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 minutes at 65 °C with occasional shaking.
7. Spin down the cell debris 10 minutes at 12000 *g*.
8. Transfer the supernatant to a 1.5 mL microcentrifuge tube containing 500 µL chloroform.
9. Vortex or shake minimum 10 seconds and then centrifuge 10 min at 12000 *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 seconds and then centrifuge 5 minutes at 12000 *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 minutes at 12000 *g*; carefully decant the supernatant and discard it.
16. Suspend the precipitate in 400 µL 1.2 mol/L NaCl; mix gently.
17. Add 400 µL chloroform, mix minimum 10 seconds.

18. Centrifuge 5 minutes at 12000 *g* to separate 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 minutes at -20 °C.
21. Spin down the DNA precipitate minimum 10 minutes at 12000 *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 minutes at 12000 *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 minutes at room temperature.
25. Suspend the pellet in 100 µL nuclease-free water by overnight incubation at 4 °C.

ANNEX 2

Protocol for DNA extraction using the CTAB-Tip20 method

The CTAB-Tip20 DNA extraction procedure applies the steps described in the CTAB protocol (Annex 1) followed by additional purification using a QIAGEN Genomic-tip 20/G purification kit.

The DNA preparation obtained after CTAB extraction is subjected to further purification using a Genomic-tip 20/G column kit according to the manufacturer's instructions described in the "Genomic-tip Protocol".

ANNEX 3

Changes to the real-time PCR protocol published in the method validated by the EURL GMFF [6].

	CRL protocol	**Protocol used during certification**
Reference target	70 bp amplicon of the *adh1* endogenous gene	79 bp amplicon of the *hmg1* gene
PCR primers and probes used in real-time PCR	Adh1 primer F: 5'-CCAGCCTCATGGCCAAAG-3' Adh1 primer R: 5'-CCTTCTTGGCGGCTTATCTG-3' Adh1 probe: 5'-(6-FAM)-CTTAGGGGCAGACTCCCGTGTTCCCT-(TAMRA)-3'	ZM1-F: 5'-TTGGACTAGAAATCTCGTGCTGA-3' ZM1R: 5'-GCTACATAGGGAGCCTTGTCCT- 3' Probe ZM1: 5'-(6-FAM)-CAATCCACACAAACGCACGCGTA-(TAMRA)-3'
Amount of substance concentration of the reference target primers used in real-time PCR	Adh1 primer F: 150 nmol/L Adh1 primer R: 150 nmol/L Adh1 probe: 50 nmol/L	ZM1-F: 300 nmol/L ZM1R: 300 nmol/L Probe ZM1: 160 nmol/L
Real-time PCR reaction volume	50 µL	25 µL
Data analysis	Manual setting of baseline and threshold	Automatic setting of baseline and threshold

European Commission

EUR 24699 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Certification of a Maize NK603 Reference Material for its DNA Copy Number Ratio – Certified Reference Material ERM®-BF415e
Author(s): E. de Andrade, B. Jeynov, P. Corbisier, A.M. Kortekaas, S. Trapmann, S. Vincent, H. Emons
Luxembourg: Publications Office of the European Union
2011 – 32 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-19074-2
doi:10.2787/38148

Abstract

This report describes the certification of the Certified Reference Material (CRM) ERM®-BF415e for its DNA copy number ratio. This CRM is composed of genetically modified NK603 maize seed and conventional maize seed powders.

ERM-BF415e is part of a set of maize CRMs containing different mass fractions of genetically modified NK603 maize. The CRM was processed and originally certified for its mass fraction by the European Commission, Directorate General Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE.

An interlaboratory comparison was conducted in 2009 to certify the existing CRM additionally for its DNA copy number ratio. The CRM is intended for quality control of measurements of the DNA copy number ratios of maize event NK603 in genetically modified food and feed. Hereby, the plasmid DNA (pDNA) ERM-AD415 was used for calibration together with the event-specific NK603 real-time Polymerase Chain Reaction (PCR) method validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm), modified as described in Section 6.1. ERM-AD415 contains a 108 bp fragment of the 3' insert-plant junction specific for the NK603 maize event. Additionally, the plasmid carries a 79 bp fragment of the maize endogenous high mobility group gene (*hmg*), specific for the maize taxon.

The CRM ERM-BF415e is available in glass bottles containing 1 g of maize powder, closed under argon atmosphere and was certified for its DNA copy number ratio.

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

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Publications Office

ISBN 978-92-79-19074-2