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Certification of different mass fractions of DP23211 in maize seed powder: ERM®-BF445a and ERM®-BF445b

A.M. Kortekaas, J. Seghers, S. García-Ruiz, M.C. Contreras López, M. Khaled-Gijón, P. Corbisier, S. Trapmann

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Contact information
Name: EC, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Reference Materials Unit
Address: Retieseweg 111, 2440 Geel, Belgium
Email: JRC-RM-DISTRIBUTION@ec.europa.eu
Tel.: 0032/14/571722
Website: https://crm.jrc.ec.europa.eu

EU Science Hub
https://joint-research-centre.ec.europa.eu

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Contents

Abstract

ERM®-BF445a and ERM®-BF445b are matrix materials certified for the mass fraction of DP23211 maize (unique identifier DP-Ø23211-2), produced within the scope of ISO 17034 accreditation [1] and in accordance with ISO Guide 35:2017 [2].

Genetically modified (GM) maize seeds of the DP23211 event and seeds from a non-GM maize variety were milled, sieved and dried to obtain GM and non-GM seed powders. The CRMs are available in glass vials containing at least 1 g of dried maize powder, which were sealed under an atmosphere of argon.

The purity of the non-GM and GM maize seeds and the respective maize powders was quantified. The stability during transport and storage was assessed in accordance with ISO Guide 35:2017 [2]. The minimum sample size for one measurement is 200 mg.

The certified values were obtained, taking into account the genetic purity of the base materials with respect to the DP23211 maize. The certified values were confirmed by event-specific real-time polymerase chain reaction (PCR) (measurements were within the scope of accreditation to ISO/IEC 17025:2017 [3]).

Technically invalid results were removed but no outlier was eliminated unless a technical reason for the deviation was found.

Uncertainties of the certified values were calculated in accordance with ISO 17034:2016 [1] and ISO Guide 35:2017 [2] and include uncertainties related to the characterisation, based on the genetic purity assessment.

The materials are intended for calibration or quality control for DP23211 maize detection methods, or they can be used for establishing control charts or validation studies for these methods.

Before release of the CRMs, the certification project was subjected to peer-review involving internal experts.

Acknowledgements

The authors would like to acknowledge the support received from colleagues of JRC for the processing, organising of stability studies, measuring, reviewing of the certification project and distribution of these CRMs.

Authors

A. M. Kortekaas [1)], J. Seghers [1)], S. García-Ruiz [1)], M.C. Contreras López [1)], M. Khaled-Gijón [1)], P. Corbisier [1)], S. Trapmann [1)]

[1)] European Commission, Joint Research Centre, Directorate F, Geel, BE

1 Introduction

1.1. Background

The European Union has legislation which regulates the placing on the market of any food or feed which consists of, contains, or is produced from genetically modified organisms (GMOs). These products are referred to as genetically modified food and feed and require authorisation for marketing in the European Union. They also require labelling if they contain more than 0.9 % of GMOs [4]. This labelling threshold is applicable for the adventitious or technically unavoidable presence of GMOs, whilst GMOs that are intentionally added need to be labelled independently from any threshold. However, feed may contain up to 0.1 % (m/m) of a GMO for which an authorisation process is pending, or for which authorisation in the EU has expired [5]. The implementation of these thresholds require the availability of validated methods for GMO quantification and of certified reference materials.

Pioneer Hi-Bred International, Inc. (Johnston, IA, US) developed the genetically modified DP23211 maize event (unique identifier code DP-Ø23211-2, following Commission Regulation (EC) No 65/2004 [6]). This transgenic maize was modified for insect resistance and herbicide tolerance. The two genetic modifications to control maize for corn rootworm pests consists of the expression of DvSSJ1 double-stranded RNA (dsRNA), targeting the *smooth septate junction protein 1* gene (*DvSSJ1*) of the western corn rootworm (*Diabrotica virgifera virgifera*) and additionally the expression of the insecticidal IPD072Aa protein derived from *Pseudomonas chlororaphis*. For herbicide tolerance this maize expresses the phosphinothricin acetyltransferase (PAT) protein from *Streptomyces viridochromogenes* which inactivates phosphinothricin, the active ingredient in glufosinate herbicides. The expression of the phosphomannose isomerase (PMI) protein was used as selection marker during transformation [7, 8].

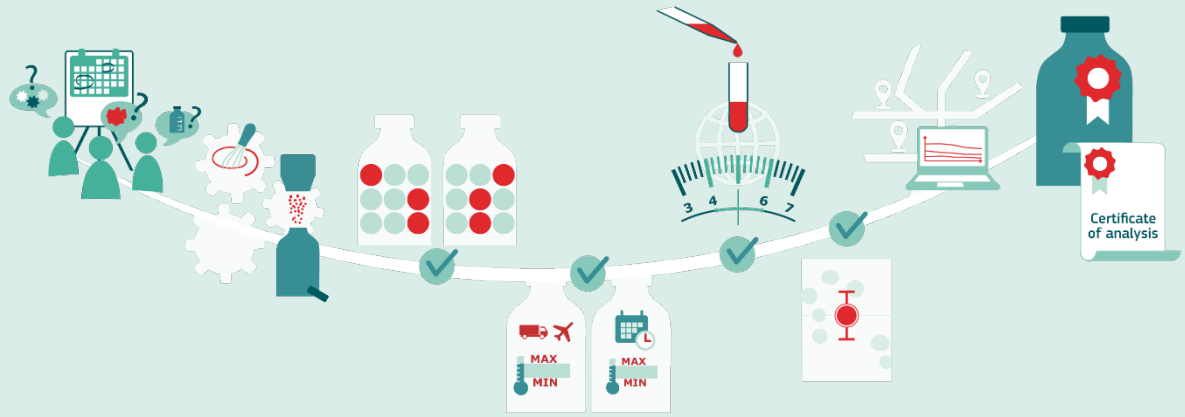
Pioneer Hi-Bred International, Inc. (Johnston, IA, US) commissioned the European Commission's Joint Research Centre (JRC), Directorate F – Health, Consumers and Reference Materials, to produce a certified reference material (CRM) for the quantification of DP23211 maize. The CRM produced by the JRC, received the code ERM-BF445 and is composed of two CRMs containing different mass fractions of DP23211 in maize seed powder. Like previous GMO CRM productions, the codes used for the different concentrations of the mass fraction of DP23211 maize followed the labelling pattern where ERM-BF445a and ERM-BF445b are the pure non-GM and GM materials, respectively.

1.2. Choice of the material

The set of CRMs of ERM-BF445 consists of milled GM and non-GM seeds. Seeds (in contrast to grains) were selected as the source of raw material because of their high degree of purity. The seeds were milled to obtain a maize powder of which DNA can be extracted effectively. ERM-BF445a and b were prepared to serve as calibrant or quality control for the quantitative polymerase chain reaction (qPCR) method.

1.3. Outline of the CRM project

Box 1. Reference material production



Reference material (RM) production is defined in ISO 17034 [1] as a project comprising planning and processing of the material, followed by homogeneity and stability testing, characterisation and assigning of one or more property values. Depending on the intended use of the RM a commutability study is carried out.

For certified reference materials (CRMs) a certificate is issued while for RMs a product information sheet is issued by the reference material producer (RMP).

CRMs and RMs are distributed globally and the stability of their assigned values is monitored throughout the life-time of the material.

For this set of CRMs only the pure non-GM material, ERM-BF445a, and the pure GM material, ERM-BF445b, were produced.

The event-specific qPCR method for DP23211 maize, developed by Pioneer Hi-Bred International, Inc. (Johnston, IA, US) will be published by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) (Ispra, IT) after validation in an international inter-laboratory collaborative study in accordance with EC Regulation [4, 9].

The ERM-BF445 CRMs and the validated method set the reference point for the quantification of GMO in food/feed samples.

The genetic purity with respect to the DP23211 maize event of the non-GM and DP23211 maize seeds was investigated by Pioneer Hi-Bred International, Inc. (Johnston, IA, US) and the JRC (Geel, BE). The results were pooled for the calculations of the certified values and their uncertainties.

Uncertainties of certified values were estimated in compliance with ISO 17034 [1], which implements the basic principles of ISO/IEC Guide 98 (GUM) [10].

The CRM project, including the certification approach and the evaluation of the obtained measurement data, was subjected to peer-review involving internal experts.

Certain commercial equipment, instruments, and materials are identified in this report to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

2 Participants

2.1 Project management, Data evaluation, Processing, Homogeneity and Stability measurements

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE (accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM)

2.2 Provider of raw material and quantification method

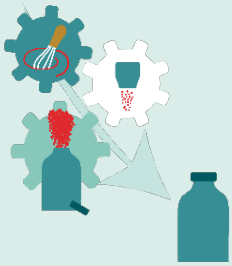
Pioneer Hi-Bred International, Inc. (Johnston, IA, US) provided the raw materials, the non-GM and GM DP23211 maize seeds and the DP23211 event-specific quantitative PCR method.

2.3 Characterisation measurements (Purity)

- European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, (Geel, BE) (measurements under the scope of ISO/IEC 17025:2017 accreditation BELAC No. 268-TEST)
- Pioneer Hi-Bred International, Inc. (Johnston, IA, US)

3 Material processing and processing control

Box 2. Reference material processing



RM processing covers the raw material conversion into a homogenous and stable material. It typically includes processing steps such as grinding or sieving and drying steps to enhance stability. When the processed material fulfils the specifications, the final material is filled into individual containers, referred to as RM units, such as bottles or ampoules and is labelled.

3.1 Purity of the starting material

Pioneer Hi-Bred International, Inc. (Johnston, IA, US) supplied the Joint Research Centre, Directorate F – Health, Consumers and Reference Materials (JRC, Geel, BE) with non-GM maize seeds and DP23211 maize seeds to prepare the ERM-BF445 CRMs. According to the information provided by Pioneer Hi-Bred International, Inc. (Johnston, IA, US), the DP23211 maize seeds are hemizygous and the DP23211 maize event was inherited from the male parent. Upon arrival, the seeds were stored at (4 ± 3) °C in the dark until processing.

The genetic purity with respect to the DP23211 maize event of the GM maize seeds was assessed at the JRC (Geel, BE) by analysing 54 randomly selected seeds for the presence of the DP23211 maize GM event. Genomic DNA was extracted from the leaf tissue of 54 plants grown from the individual seeds, applying a validated extraction method using the DNeasy Plant Mini kit (Qiagen, Antwerp, BE). Genomic DNA extracted from pure DP23211 and pure non-GM maize powder was used as positive control and negative control, respectively. Each DNA extract was then analysed in three replicates by PCR. An event specific PCR method, validated in-house, showed the presence of the DP23211 maize target in 53 of the 54 tested GM seedlings. The PCR method provided by Pioneer Hi-Bred International, Inc. (Johnston, IA, US) followed the protocol for SsoAdvanced™ Universal Probes (Bio-Rad Laboratories N.V., Temse, BE) however, at the JRC, the PCR reaction volume was adapted to 25 µL. Detection was performed on a QuantStudio 7 PCR system (Life Technologies Europe BV, Gent, BE).

These results deviated slightly from the level of purity provided by Pioneer Hi-Bred International, Inc. (Johnston, IA, US) where 325 seeds were tested and found all positive for DP23211.

For statistical analysis, the results from the 54 plants tested at the JRC (Geel, BE) were pooled with the results from 325 seeds, tested by Pioneer Hi-Bred International, Inc. (Johnston, IA, US). This revealed that the GM maize batch had a genetic purity of 98.7 % (Poisson distribution for rare events, 95 % level of confidence).

The genetic purity of the non-GM seed batch with respect to the DP23211 maize event was investigated using the processed seed powder and analysed by the JRC (Geel, BE) (Section 3.4). Ten units of ERM-BF445a were randomly selected and the DNA was extracted from two samples (extraction replicates) taken from each unit (N = 10, n = 2). Each DNA extract was then analysed in three replicates by PCR. This analysis did not detect the DP23211 event and confirmed the purity data from Pioneer Hi-Bred International, Inc. (Johnston, IA, US) where 3200 non-GM maize seeds, in eight seed lots of 400 seeds were ground and tested. All lots confirmed the absence of the DP23211 event which resulted in a purity value of 99.9 %.

3.2 Processing

All maize seeds received by the JRC (Geel, BE) were rinsed with water, drained and dried on trays in the drying chamber of a freeze-dryer at 20 °C for 20 h (Epsilon DF 2-100D, Martin Christ, Osterode, DE). Approximately 30 kg of non-GM maize seeds and 10 kg of DP23211 maize seeds were used for the production of ERM-BF445. The non-GM and GM base materials were processed separately into powders.

Cross-contamination and contamination with foreign DNA was avoided by using clean and cotton-free laboratory clothing and treating all the contact surfaces with a decontamination solution (DNA Erase™, MP Biomedicals, Irvine, CA, US) before exposure to the maize materials. An in-house validation study had previously proven that the solution degraded DNA effectively under the used conditions.

The maize seeds were frozen overnight in liquid nitrogen in stainless steel containers and were subsequently milled using a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Cologne, DE). The milling chamber was kept below -90 °C throughout the process. The feeding speed of the mill was adjusted to ensure that the seeds were milled to the required particle size. The powder was sieved with a 710 µm stainless steel mesh on a sieving machine (Russell Finex, London, UK). After sieving, the coarse fraction (> 710 µm) was immediately re-milled. The remaining powder from each base material was cold mixed in a DynaMIX CM200 (WAB, Muttenz, CH) for one hour to homogenise the distribution of the different types of maize seed tissues, since it is known that the milling and sieving processes result in separation of the various seed tissues from one another. After mixing, the powder was maintained at (4 ± 3) °C in an airtight container.

To facilitate homogenous mixing of the powders, the water content of the powders was reduced further by drying them overnight under vacuum in a freeze-dryer at 20 °C. The dried powders were subsequently mixed for one hour to homogenise. The final water mass fraction of the non-GM powder and the GM powder was measured as (31.1 ± 3.9) g/kg and (17.5 ± 2.2) g/kg, respectively (N = 1, n = 3), with the expanded uncertainty calculated using a coverage factor k = 2 (Table 1).

The milled raw materials were used to prepare the blank material (non-GM maize seed powder) and the pure GM DP23211 maize material. Both materials were treated according to the same procedure and strict measures were taken to avoid cross-contamination.

A feeder, FD SPAc 4A (MCPI, Meythet, FR), was used to fill 10 mL amber glass vials with at least 1 g of powder. To avoid cross-contamination, the equipment was cleaned between two batches and the first 30 units of each batch were discarded as an additional precaution. Lyophilisation inserts were placed in the vials' necks. The units were then placed in a freeze-dryer (Epsilon DF 2-100D, Martin Christ, Osterode, DE) to provide an argon atmosphere, and were closed inside the freeze-dryer using a hydraulic device. Capping and labelling was executed using an HV 100 B 10 semi-automatic capping machine (Bausch & Ströbel, Ilshofen, DE) and a labelling machine (BBK, Beerfelden, DE).

Colour coded caps were used to facilitate the identification of the two mass fraction levels of the DP23211 maize event: nominal 0 g/kg = silver (BF445a), nominal 1000 g/kg = black (BF445b), consistent with the cap colours of previously produced JRC GMO CRMs. Each of the units was identified by a label indicating the ERM batch code and an individual unit number according to the filling order. After the inventory and the selection of units for future analysis according to a random stratified sampling scheme, the remaining units were stored in the dark at (4 ± 3) °C.

For the purpose of this report, the term 'unit' refers to one vial of ERM-BF445a or ERM-BF445b.

One unit of each ERM-BF445a (blank) and ERM-BF445b are shown in Figure 1.

Figure 1. Set of ERM-BF445

3.3 Processing control

The sample size per unit of ERM-BF445 is 1 g.

Particle size distribution measurements based on laser diffraction (Helos KR, Sympatec GmbH, Clausthal-Zellerfeld, DE) took place during and after final processing of ERM-BF445.

The particle size distributions of the non-GM and GM powders were compared. The cumulative volume distribution of the particles derived from laser diffraction data is based on their equivalent spherical diameters, i.e. the diameter of the particles derived from the volume occupied upon their rotation. Based on that, the volume mean diameter and associated expanded uncertainty of the non-GM and GM base powders was 110 µm ± 8 µm and 166 µm ± 14 µm, respectively (Table 1).

However, since most particles are not spherical, the calculated volumes of the particles based on their equivalent diameters will overestimate the mean particle size. Therefore, a three-point specification of the particle size distribution (N = 1, n = 3) was calculated, consisting of the equivalent sphere diameters at 10 %, 50 % and 90 % of the cumulative volume distribution (Table 1). These size classes were denoted X_{10}, X_{50} and X_{90}, respectively. A t-test showed with 95 % confidence that there were significant differences between the X_{10}, X_{50}, X_{90} particle sizes and between the volume mean diameters of the non-GM and GM maize powders.

Based on the significant different particle size distributions there could be a DNA extraction bias between the non-GM and GM base powders. It is therefore essential to prepare mixtures of non-GM and GM maize on the basis of extracted DNA of each of the powders separately, see Section 9.3.

Table 1. The water mass fraction determined by V-KFT and the volume mean diameter and percentiles of cumulative particle size distributions of the base materials by laser diffraction.

Base material	Water mass fraction [g/kg]		Volume mean diameter [µm]		X_{10} [µm]		X_{50} [µm]		X_{90} [µm]	
	$\bar{x}$	U	$\bar{x}$	U	$\bar{x}$	U	$\bar{x}$	U	$\bar{x}$	U
Non-GM powder	31.1 [1)]	3.9	110 [2)]	8	12 [3)]	3	96 [3)]	16	231 [3)]	48
GM powder	17.5 [1)]	2.2	166 [2)]	14	19 [3)]	4	138 [3)]	23	355 [3)]	73

1) Mean of one unit (N = 1, n = 3). The associated expanded uncertainty (U) with a coverage factor k = 2 has been estimated during validation of the V-KFT method.

2) Mean of one unit (N = 1, n = 5). The associated expanded uncertainty (U) with a coverage factor of k = 2 is based on the standard deviation of measurements.

3) Mean of one unit (N = 1, n = 3). The associated expanded uncertainty (U) with a coverage factor of k = 2 has been estimated during validation of the laser diffraction method.

For each of the CRMs produced, five randomly selected units were analysed by V-KFT to determine the residual water mass fraction in the powder. The results are summarised in Table 2.

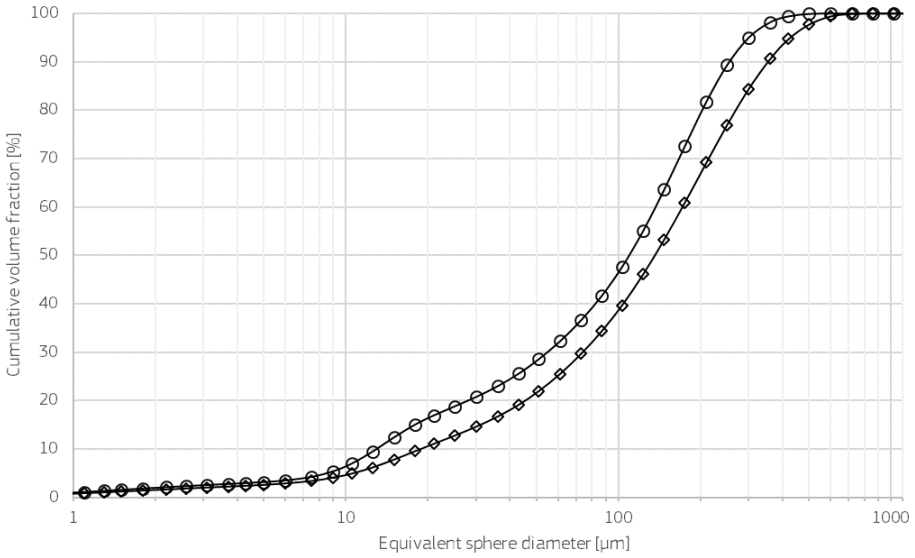
Table 2. Water mass fractions of ERM-BF445 CRMs determined by V-KFT (N = 5, n = 1).

CRM code	Water mass fraction [g/kg]	
	$\bar{x}$	U (k = 2) [1)]
ERM-BF445a	37	4
ERM-BF445b	18.8	2.1

1) The associated expanded uncertainty (U) has been estimated during validation of the V-KFT method on maize powder

The particle size distribution of the CRMs was determined by laser diffraction. Five randomly selected units from each of the CRMs were analysed in duplicate (N = 5, n = 2). The equivalent diameters of all particles were below 860 µm (Figure 2). The volume mean diameters measured by laser diffraction were 124 µm ± 5 µm (U) and 166 µm ± 11 µm (U) for ERM-BF445a and b, respectively.

Figure 2. Volume-based cumulative distribution of particle size in ERM-BF445a (O), ERM-BF445b (◇) analysed by laser diffraction (N = 5, n = 2).



3.4 Confirmation measurements

For confirmation, samples from ERM-BF445a and ERM-BF445b were analysed using the event-specific qPCR method, delivered by Pioneer Hi-Bred International, Inc. (Johnston, IA, US). The method was first validated in-house and the measurements were executed under the scope of ISO/IEC 17025:2017 [3].

DNA was extracted from 200 mg samples taken from ERM-BF445a and ERM-BF445b, based on a DNA extraction method with Cetyltrimethylammonium bromide (CTAB) and followed by a purification over Genomic-tip 20/G columns (QIAGEN Benelux BV, Antwerp, BE) according to manufacturer's instructions (Annex 1). Gel-electrophoresis and spectrophotometry (NanoDrop One, Isogen Life Science BV, De Meern, NL) was used to determine the size, integrity and quality of the extracted DNA samples. All samples showed very high molecular weight and intact DNA, with some smear of minimally degraded DNA (data not shown).

After extraction, the DNA quantity in the extracts was measured with a fluorescence based method using Quant-iT™ PicoGreen® dsDNA (Invitrogen/Life Technologies Europe BV, Merelbeke, BE) and a Biotek Synergy MX spectrofluorometer (BRS, Drogenbos, BE). The DNA extracts were diluted in a TE-low buffer solution (pH 8.0, 1 mmol/L Tris and 0.01 mmol/L EDTA) to a concentration of 50 ng/µL (approximately 250 ng DNA per 25 µL PCR reaction mix).

For each reaction mix, 5 µL of the diluted DNA extracts of the samples, calibrant or control sample was added to a 20 µL mixture of event-specific or species specific primers and probes and master mix (SsoAdvanced Univ Probes Supermix from Bio-Rad Laboratories NV, Temse, BE).

The quantitative PCR for the ERM-BF445b samples was calibrated with genomic DNA extracted from pure DP23211 maize powder. For the calibration curve of the maize species-specific and the event-specific gene, the DNA was used from approximately 500 ng to 5 ng DNA per 25 µL PCR reaction mix. Amplification and detection was performed on a quantitative PCR system (QuantStudio 7 Flex System, Life technologies Europe, Gent, BE). ERM-BF445a samples were analysed using a qualitative PCR set-up.

The efficiency of the amplification of the PCR assays was assessed from the slope of the regression lines of the Cq values as a function of the calibrants' DNA quantity (DP23211 or endogene) in the different DNA solutions used. The PCR efficiency should be between 90 % and 110 %, corresponding to the slope values of -3.6 and -3.1 [11, 12]. For both the maize species-specific and the event-specific assays the PCR

amplification efficiencies were within the limits. The coefficient of determination (r^2) for both the endogene and transgene plates was above the acceptance criterion (≥ 0.98) [12].

The LOD of the qPCR method was established during in-house validation and calculated as 3.3-fold the standard deviation (s) (in % GM) of the lowest calibration point at which s_{rel} was below 25 %. The results of the quantification of the DP23211 maize event are shown in Table 3.

The PCR measurement results confirmed that the DP23211 maize event could not be detected in the non-GM material, ERM-BF445a. No independent calibration was carried out and therefore the data in Table 3 can only be used for confirmation of the consistency of the powders during processing. However, a significant bias was found between the qPCR results for ERM-BF445b and the respective certified values (Table 5).

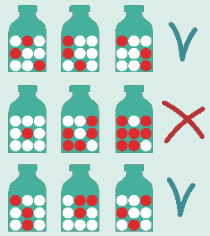
Table 3. The DP23211 maize mass fraction in the CRMs measured by event-specific PCR.

CRM code	DP23211 maize mass fraction [g/kg]	U_{meas} (k = 2) [g/kg]
ERM-BF445a	Not detected [1)]	-
ERM-BF445b	1038 [2)]	± 46 [3)]

[1)] Samples were measured qualitatively, no DP23211 maize event was detected in 45 PCR cycles. Two samples (extraction replicates) from each of ten randomly selected units (N = 10, n = 2), with each sample measured in three qualitative PCR replicates.
[2)] Mean of two samples (extraction replicates) from each of ten randomly selected units (N = 10, n = 2), with each sample measured in three quantitative PCR replicates, using genomic DNA from pure DP23211 maize powder for calibration.
[3)] Expanded uncertainty with a coverage factor k = 2 based on the standard deviation of measurements performed under repeatability conditions.

4 Homogeneity

Box 3. Homogeneity assessment



A key requirement for any RM produced as a batch of units is equivalence between those units. It is important to know how much the variation between units contributes to the uncertainty of the certified value. Consequently, ISO 17034 [1] requires RMPs to quantify the between-unit variation in homogeneity studies.

The within-unit homogeneity is correlated to the minimum sample size, which is the minimum amount of sample that is, for a given measurand, representative of the whole unit and that should be used in an analysis. Using sample intakes equal to or above the minimum sample size guarantees the assigned value within its stated uncertainty.

The homogeneity of ERM-BF445a was demonstrated by testing the purity of the non-GM seeds. The DP23211 maize event was not detected in the milled seed lots tested by Pioneer Hi-Bred International, Inc. (Johnston, IA, US) and also not in the 10 units of ERM-BF445a as tested for the confirmation measurements. The powder used for the production of ERM-BF445a did not contain traces of DP23211 maize (Section 3.1 and 3.4, Table 3).

The homogeneity of ERM-BF445b is related to the purity study based on results from Pioneer Hi-Bred International, Inc. (Johnston, IA, US) and the JRC (Geel, BE) (Section 3.1).

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample size is respected, but determines the minimum size of sample that is representative for the whole unit.

4.1 Between-unit homogeneity

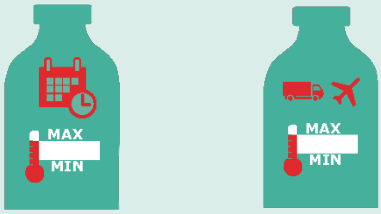
The standard uncertainties of characterisation for ERM-BF445a and ERM-BF445b are 0.6 g/kg and 3 g/kg respectively (Table 4). Based on the purity data (Section 3.1 and 3.4), it is highly unlikely that between-bottle variation (caused by undetected impurity or de-mixing) would be higher than 1/3 of these uncertainty values, thus making the uncertainty contribution for standard uncertainty related to a possible between-unit inhomogeneity (u_{bb}) negligible.

4.2 Within-unit homogeneity and minimum sample size

ERM-BF445a and ERM-BF445b are pure non-GM and GM materials, respectively. Therefore, the minimum sample intake for these materials is not linked to the within-unit homogeneity. However, based on the PCR measurements carried out, it was concluded that also for these two pure materials the suitable minimum sample intake for PCR is 200 mg in order to extract a significant amount of DNA.

5 Stability

Box 4. Stability assessment



Stability testing is necessary to establish the conditions for storage as well as the transport conditions of the RMs to the customers. During transport, especially in summer, temperatures up to 60 °C can be reached, and stability under these conditions must be demonstrated if the RMs are to be transported without any additional cooling.

Time, temperature, light (including ultraviolet radiation) and water content were regarded as the most relevant factors influencing the stability of the materials. The influence of ultraviolet and visible light was minimised by storing the material in containers, which reduce light exposure. In addition, materials are stored in the dark and transported in boxes, thus removing any possibility of degradation by light. The water content was adjusted to an optimum during processing. Therefore, only the influences of time and temperature needed to be investigated.

The stability of the ERM-BF445a and ERM-BF445b materials was assessed, based on experience with similar produced maize GMO CRMs and confirmed by a short-term stability study that was carried out using an isochronous design [13]. In this approach, units of ERM-BF445a and b were stored for a certain time at different temperature conditions. Afterwards, the units were moved to conditions where further degradation can be assumed negligible (reference conditions). At the end of the isochronous storage, samples taken from the units were analysed simultaneously under repeatability conditions.

The ERM-BF445a and ERM-BF445b materials are pure non-GM and GM maize powders and general degradation of the matrix implies also general degradation of the DNA in the matrix. In the case of the pure GM maize powder, DNA degradation would affect equally the transgene (DP23211) and the endogene (*hmg*), the target genes for the event-specific PCR method and the ratio transgene/endogene would stay equal. Consequently, the PCR method cannot be used to detect the degradation of the matrix and DNA in the pure GM material. For that reason, gel-electrophoresis was selected as detection method for the stability where degraded DNA that is fragmented in different lengths (with different molecular weight), would be visible on gel. Although gel-electrophoresis is a qualitative method and cannot be used to quantify the uncertainty contribution relating to the transport and storage stability of these CRMs, it can be used to test and compare the DNA integrity of the different samples of the short-term stability study.

5.1 Transport stability

The conditions for the transport of the ERM-BF445 materials to the customers were confirmed in a short-term stability study. To this end, units were stored at 60 °C and 4 °C for 3 weeks and the reference temperature was set to -70 °C. Three units per storage temperature were selected using a random stratified sampling scheme. From each unit, two samples were measured by gel-electrophoresis (data not shown).

The measurements were performed under repeatability conditions, and a randomised sequence was used to differentiate any potential drift in the measurement results from a potential trend over storage time. The data were evaluated visually based on the gel-electrophoresis images.

Some of the 60 °C samples for ERM-BF445a showed a slightly more intense smear around 600 base pairs (bp) compared to the 4 °C and -70 °C samples. The 60 °C samples for ERM-BF445b show a slight smear around 1500 bp, while the 4 °C and -70 °C samples express a slight smear at the high molecular weight region. There is no difference between -70 °C and 4 °C within the ERM-BF445a samples or within ERM-BF445b samples. The cause of the smears, could be related to the many manipulations of the DNA during the extraction protocol. However, all tested samples of ERM-BF445a and ERM-BF445b show a broad and intense high molecular weight band on gel which shows that high molecular weight DNA can be extracted that is suitable for PCR.

From similarly produced maize GMO CRMs, data are available from short-term stability studies at conditions of 60 °C, 18 °C and 4 °C for 0, 1, 2 and 4 weeks (for example the maize GMO CRMs: ERM-BF433, ERM-BF438, ERM BF439, ERM-BF412k and ERM BF446). For these studies, 10 % GM powders were used and were

measured by quantitative PCR. This allowed assessment of the stability of each base material in the 10 % GM mixtures. The data were evaluated against storage time, and regression lines of mass fraction versus time were calculated, to test for potential increases or decreases of GM maize mass fraction due to shipping conditions. These results showed that all these materials are stable at the tested temperatures proving that not only the GM maize powders but also the non-GM maize powders were stable at storage and dispatch conditions. Based on these data, there is no reason to doubt about the stability of ERM-BF445a and ERM-BF445b at the selected dispatch conditions. The material can be dispatched without further precautions under ambient conditions.

5.2 Storage stability

Storage conditions and shelf life guaranteeing the stability of the materials and the certified values were established.

Data from JRC's stability monitoring programme for GMO CRMs can give an indication whether ERM-BF445 can be expected to be stable as they are all produced and stored in the same way.

Previously released GM maize powder CRMs were analysed for their GM mass fraction on 134 occasions over a period of 19 years. At each time point measurements were performed on units stored at assigned storage temperature (4 °C) and at a reference temperature (-70 °C). Each of these studies can be viewed as a two-point isochronous study. The evaluation was based on the ratio of quantitative PCR measurement results from samples stored at 4 °C and -70 °C.

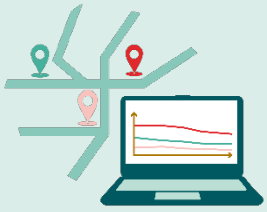
The stability monitoring data were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. In addition, the data were plotted against storage time and linear regression lines of GM mass fraction versus time were calculated. The slope of the regression line was tested for statistical significance (loss/increase due to storage). No statistical outliers were detected for the previous and similar produced maize CRMs. No statistically significant trend was detected on a 95 % confidence level for the tested temperature.

The integrity of the DNA extracted from the samples of the 4 °C short-term stability study (Section 5.1) was checked by gel-electrophoresis, which is a qualitative method. Consequently, a ratio of the short-term stability samples at 4 °C and -70 °C could not be quantified and these data cannot be directly compared with the stability monitoring data and cannot be used to estimate a quantifiable uncertainty contribution relating to the storage of the CRM.

The stability monitoring data of similar produced maize CRMs show clearly that maize CRMs are stable at 4 °C. The data of the short-term stability study, based on images from gel-electrophoresis, show no visible difference between -70 °C and 4 °C samples and therefore confirm that also ERM-BF445 can be stored at 4 °C.

The results of the stability monitoring measurements are shown in Annex 2. The materials are included in the JRC's regular stability monitoring programme to control its further stability.

Box 5. Stability monitoring



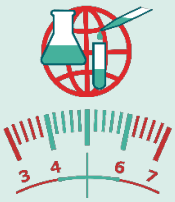
RMs are produced as batches that should last for ten years or longer. This long lifetime means that a storage stability study of limited duration cannot provide a definite "use by" date for the material. It therefore needs to be complemented by stability monitoring throughout the lifetime of the RM.

Therefore, the stability of RMs whose assigned values might change is regularly monitored. The monitoring frequency depends on the outcome of the storage stability assessment.

If the tests confirm the stability of the assigned values, the material remains on sale. If not, possible actions include the retraction of the value in question, retraction of the complete material or a change of the certified value. Customers are notified if the change is larger than the uncertainty of the assigned value.

6 Characterisation

Box 6. Reference material characterisation



Material characterisation is the process of determining the property value(s) of a RM. While ISO 17034 [1] allows to characterise a RM in various ways. Quality management procedures of the JRC define only a limited number of characterisation approaches such as e.g. inter-laboratory comparison or the use of a primary method confirmed by independent analysis.

The two CRMs ERM-BF445a and b are maize powder materials processed from non-GM and GM seeds. ERM-BF445a is prepared from the pure blank material and ERM-BF445b from the pure GM material. ERM-BF445 is certified for the mass fraction of DP23211 maize event and the certification is based on their purity.

6.1 Purity of the base materials

The purity of the GM and non-GM batches used for the processing of these powders was investigated to calculate the certified value and their uncertainties.

The purity of the GM DP23211 maize material was based on the pooled measurements results from JRC (Geel, BE) and Pioneer Hi-Bred International, Inc. (Johnston, IA, US). In total 379 seeds were tested and one of the seedlings tested at JRC (Geel, BE) was found negative for the DP23211 maize event (Section 3.1).

The non-GM maize powder used for the production of ERM-BF445a did not contain traces of the DP23211 maize event assessed by the qualitative real-time PCR event-specific method used (Section 3.1 and 3.4).

The potential presence of certain GM events in both the GM and non-GM maize powders was verified by using a qualitative PCR-based ready-to-use multi-target analytical system for GM detection developed by the EURL-GMFF (Ispra, IT) [14]. This test was performed at JRC (Geel, BE) by using a pre-spotted 96-well plate containing primers and probes for simultaneous detection of 21 individual specific GM maize events (3272, 5307, 98140, BT11, Bt176, DAS-40278, DAS-59122, GA21, LY038, MIR162, MIR604, MON810, MON863, MON87427, MON87460, MON88017, MON89034, NK603, T25, TC1507 and VCO-01981-5) and the primers and probes for the taxon-specific assay for maize, the *high mobility group* gene (*hmg*). Any stacked events derived from the single-insert GMOs included in the system would also be detected. The results indicated that the *hmg* was detected but none of the GM events tested were detected in the non-GM and GM maize powders used for the production of ERM-BF445.

6.2 Verification measurements

Real-time PCR measurements demonstrated that no production errors were made (Section 3.4). Gel-electrophoresis proved that the DNA was not degraded during processing of the CRM (data not shown).

7 Value Assignment

Box 7. Assignment of values to a reference material

Based on the outcome of characterisation measurements three types of values can be assigned, namely certified, indicative or additional material information values.

Certified values are values that fulfil the highest standards of accuracy. Procedures at JRC Directorate F require a sufficient number of datasets to assign certified values. Full uncertainty budgets in accordance with ISO 17034 [1] and ISO Guide 35 [2] are required. Certified values of a CRM can be used for calibration and trueness controls.

Indicative values are values where either the uncertainty is deemed too large or too few independent datasets are available to allow certification. Indicative values of an RM can be used for statistical quality control (homogeneity and stability has been assessed) but not for calibration, demonstration of method or laboratory proficiency or method trueness.

Additional material information values are values for which homogeneity and stability has usually not been assessed and insufficient data for characterisation is available. Consequently, an estimate of the reliability of the values is not possible and no uncertainty is given. Additional material information values cannot be used for calibration, demonstration of method or laboratory proficiency or method trueness. They can be used to e.g. anticipate possible interferences in measurement processes.

Certified values and their uncertainties were assigned.

The certified values and the related uncertainties are based on the purity values of the non-GM seeds and GM seeds used.

For the blank material, the half-width of the rectangular 95 % confidence interval between the purity of the seeds (> 999 g/kg) and 1000 g/kg was included in the calculation of the standard uncertainty related to the characterisation ($u_{char,1}$) ,Table 4. Calculating the U_{CRM} for the blank material on the basis of this standard uncertainty ($u_{char,1}$) gives a value of U = 0.6 g/kg (assuming k = 2).

Based on the high purity, the certified value for ERM-BF446a is artificially set at 0.0 g/kg. The assigned asymmetric expanded uncertainty, 1.0 g/kg, is based on the difference between the certified value (0.0 g/kg) and the difference (1.0 g/kg) between the purity 999 g/kg and 1000 g/kg (Table 4). This difference is, therefore, a conservative estimate of the uncertainty of the certified value.

For the pure GM material, the purity (987 g/kg) of the GM seed batch was used to describe the triangular 95 % confidence interval of the certified mass fraction of the DP23211 maize powder. As an impurity was found in the DP23211 maize seeds tested (Section 3.1), the certified value for ERM-BF445b is set at the level of the purity found, 987 g/kg.

The half-width of the triangular 95 % confidence interval between the measured purity of the GM maize, 987 g/kg (Section 3.1) and 1000 g/kg was taken into account in the standard uncertainty calculation of $u_{char,2}$. Calculating U_{CRM} for the pure GM material on the basis of the only quantifiable standard uncertainty ($u_{char,2}$) gives a value of U = 6 g/kg (assuming k = 2) (Table 4).

The certified values and their uncertainties are summarised in Table 4.

Table 4. Certified values and their uncertainties for the DP23211 mass fraction in ERM-BF445.

CRM code	Certified value [g/kg]	$u_{char,\ 1}$ [g/kg]	$u_{char,\ 2}$ [g/kg]	U_{char} [3] [g/kg]	U_{CRM} [4] [g/kg]
ERM-BF445a	0.0 [1]	0.2702	n.a. [7]	0.5405	+ 1.0 [5] - 0.0
ERM-BF445b	987 [2]	n.a. [7]	2.5231	5.0461	± 6 [6]

1) The certified value was based on the high purity (> 99.9 %) of the conventional, non-genetically modified maize seeds. No contamination was detected in this material when using an event-specific quantitative polymerase chain reaction assay targeting the DP23211 maize event.
2) The certified value was based on the genetic purity of the maize seeds, 98.7 % with regard to DP23211 maize. In total 379 seeds were tested individually for the presence of the DP23211 maize event of which 378 tested positive.
3) The estimate of the expanded uncertainty U_{char} with coverage factor k = 2
4) Uncertainties are always rounded up [15] and in a way that the rounding error corresponds to 3 % to 30 % of the uncertainty.
5) The asymmetric uncertainty is based on the rectangular 95 % confidence interval of the purity of the seeds (> 99.9 %). The upper bound of the expanded uncertainty interval is 1.0 g/kg and the lower bound is 0.0 g/kg. The corresponding rounded upper bound of the standard uncertainty interval is 1.0 g/kg / √3 = 0.6 g/kg.
6) The expanded (k = 2) uncertainty is based on the half width of the triangular 95 % confidence interval of the purity of the seeds (98.7 %).
7) Not applicable

As no proof could be delivered regarding how the certified GM powder mass fractions are related to the corresponding transgenic and species name-specific DNA copy number ratios, the user is reminded that JRC only certifies these materials for their mass fraction of event DP23211 maize. Additionally, one must be careful to draw quantitative conclusions (in gene copy numbers, for instance) from measurements on unknown samples, as DNA- and/or protein-based quantification of GMOs may vary with the specific matrix and the species variety tested.

8 Metrological traceability and commutability

8.1 Metrological traceability

Box 8. Metrological traceability

Metrological traceability of measurement results is a key requirement for ensuring the comparability of data. As CRMs are used to make measurement results traceable, metrological traceability of its certified values to a stated reference is essential.

The certified value of a CRM is metrologically traceable if the measurements used for establishing it can be related to a reference through an unbroken chain of calibrations.

This requires that these measurements

- refer to the same property (e.g. Pb) and the same (kind of) quantity (e.g. Pb content),
- result in a number and its uncertainty (e.g. 6 ± 2) expressed in the same measurement unit (e.g. µg/kg).

The concept of traceability rests on several anchor points, namely identity, quantity value and measurement unit. The identity of a measurand can be defined by its structure alone or can be operationally defined, the quantity value of the measurand can refer to the SI, or to other appropriate references.

8.1.1 Identity

The identity of the measurand is based on the documentary traceability to the DP23211 maize event (Biosafety Clearing House, record ID 116060) [7].

8.1.2 Quantity value

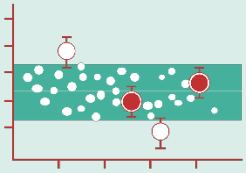
The traceability of the certified values of ERM-BF445a and ERM BF445b is based on the genetic purity assessment of the pure non-GM and GM powders, respectively. JRC (Geel, BE) used an in-house validated event specific DP23211 maize quantitative and/or qualitative PCR method and verified equipment. The measurements were performed under the scope of ISO/IEC 17025:2017 accreditation. The event-specific PCR method for DP23211 maize will be published by the (EURL-GMFF) (Ispra, IT) after validation in an international inter-laboratory collaborative study. The purity assessments by Pioneer Hi-Bred International, Inc. (Johnston, IA, US) were based on qualitative DP23211 insert specific and construct specific methods, conducted according to Good Laboratory Practices Standards (40 Code of Federal Regulation (CFR) 160, Environmental Protection Agency, US). The certified values are therefore retraceable to the International System of Units (SI).

8.2 Commutability

Box 9. Commutability

Commutability is a prerequisite for RMs intended to be used for calibration or quality control of different measurement procedures targeting the same measurand. The concept of commutability of a RM is defined by the VIM [16] as:

"*property of a reference material, demonstrated by the closeness of agreement between the relation among the measurement results for a stated quantity in this material, obtained according to two given measurement procedures, and the relation obtained among the measurement results for other specified materials*"



Commutability is a property of an RM indicating how well an RM mimics the characteristics of a typical routine sample in various measurement procedures for a stated measurand.

The same RM may be commutable for some measurement procedures but non-commutable for others. A commutability statement is therefore only valid for the mentioned measurement procedure(s).

The certified value of the CRMs ERM-BF445a and ERM-BF445b is structurally defined via its DNA sequence. The CRMs were prepared from pure non-GM and DP23211 GM maize seed powder with the aim to implement the mass fraction based thresholds set in the corresponding EU legislation for food and feed.

The CRMs are to be used for quality control or calibration of real-time PCR measurements of the maize GM event DP23211 in food and feed. Consequently, in combination with the measurement method validated by the EURL-GMFF [9], this set of CRMs is establishing the arbitrary reference system required for quantification of DP23211 maize. The differences in the particle size distribution observed between the ERM-BF445a powder and ERM-BF445b powder, prohibits that powder mixtures should be made. Instead, to ensure a common reference system, mixtures should only be made at DNA level, after extraction, purification and quantification of the DNA.

Commutability of this reference material does not need to be assessed.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

The material is for in-vitro use only; it does not contain any viable seeds.

9.2 Storage conditions

The materials should be stored at (4 ± 3) °C in the dark. Care should be taken to avoid moisture uptake once the units are opened, as the material is hygroscopic. The user should close the unit immediately after taking a sample.

For more information regarding the shelf life of reference materials please consult ERM Application Note 7 [17].

Note that the European Commission cannot be held responsible for changes that may happen to samples after opening or when the material is stored differently from the stated storage conditions at the customer's premises.

9.3 Use of the material

The particle size distributions of the non-GM and GM DP23211 maize powders in ERM-BF445a and ERM-BF445b are significantly different. Consequently, there could be a DNA extraction bias between the non-GM and GM powders. If mixtures are prepared, they need to be prepared after DNA extraction and based on the DNA quantity (Section 3.3). This procedure is described in the European technical guidance document for the flexible scope accreditation of laboratories quantifying GMOs [18].

For general information on handling of reference materials, please consult ERM Application Note 6 [19].

9.4 Minimum sample size

The minimum sample size for DNA extraction is 200 mg.

ERM-BF445a and ERM-BF445b are pure non-GM and GM materials, respectively. Therefore, the minimum sample size for these materials is not linked to the within-unit homogeneity. Nevertheless, to obtain a significant amount of DNA, the minimum sample size for DNA extraction is 200 mg.

9.5 Use of the certified values

The intended use of these materials is for calibration or quality control of DP23211 maize detection methods. As with any reference material, they can be used for establishing control charts or validation studies.

The user is reminded that this reference material is certified for its DP23211 maize mass fraction and should be used for measurements expressed in mass fractions, see ERM-Application Note 4 [20]. The exact relationship between the certified GM powder mass fractions and the corresponding DNA copy number ratio is not provided in this report. Changing the measurement unit from mass fraction to copy number per haploid genome equivalent, requires the use of an established conversion factor with an established uncertainty, thereby adding additional uncertainty to the measurement result [21].

Use as a calibrant

If this matrix material is used as calibrant, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty.

Comparing a measurement result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1 [22]).

When assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is summarised here:

— Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).

- Combine the measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_{Δ}) from the combined uncertainty (u_{Δ}) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %.
- If $\Delta_{meas} \leq U_{\Delta}$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

Use in quality control charts

The materials can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart. In the case of ERM-BF445, mixtures with target concentrations of DP23211 maize should only be prepared based on extracted DNA of ERM-BF445a and ERM-BF445b (Section 3.3 and 9.3). An explanation how to prepare these mixtures is given in the European technical guidance document for the flexible scope accreditation of laboratories quantifying GMOs [18].

10 Conclusions

ERM-BF445a and ERM-BF445b are matrix reference materials certified for the mass fraction of DP23211 maize. These materials were produced and certified in accordance with ISO 17034:2016 [1] and ISO Guide 35:2017 [2]. ERM-BF445 was produced within the scope of ISO 17034 accreditation.

ERM-BF445a and ERM-BF445b are produced with the aim to support the implementation of the mass fraction based thresholds and labelling requirements that are set in the EU legislation for food and feed, Regulation (EC) No 1829/2003 [4] and Commission Regulation (EU) No 619/2011 of 24 June 2011 [5]. In combination with the real-time PCR measurement method validated by the EURL-GMFF [9], this set of CRMs is establishing the arbitrary reference system required for quantification of DP23211 maize.

The following certified values were assigned:

Table 5. Certified values and their uncertainties for ERM-BF445.

DP23211 maize [1] mass fraction		
	Certified value [2] [g/kg]	Uncertainty [7] [g/kg]
ERM-BF445a	0.0 [3]	+ 1.0 [4] - 0.0
ERM-BF445b	987 [5]	± 6 [6]

[1] Genetically modified maize with the unique identifier DP-Ø23211-2

[2] Certified values are values that fulfil the highest standards of accuracy. The certified value and its uncertainty are traceable to the International System of Units (SI).

[3] The certified value was based on the purity of the conventional, non-genetically modified maize seeds. No contamination was detected when using an event-specific quantitative polymerase chain reaction assay targeting the DP23211 maize event.

[4] The asymmetric uncertainty is based on the rectangular 95 % confidence interval of the purity of the seeds (> 99.9 %). The upper bound of the expanded uncertainty interval is 1.0 g/kg and the lower bound is 0.0 g/kg. The corresponding rounded upper bound of the standard uncertainty interval is 1.0 g/kg / √3 = 0.6 g/kg.

[5] The certified value is based on the genetic purity of the maize seeds, 98.7 % with regard to DP23211 maize. In total 379 seeds were tested individually for the presence of the DP23211 maize event, of which 378 tested positive.

[6] The expanded (k = 2) uncertainty is based on the half width of the triangular 95 % confidence interval of the purity of the seeds (98.7 %).

[7] Uncertainties are always rounded up [15] and in a way that the rounding error corresponds to 3 % to 30 % of the uncertainty.

The materials are intended for the calibration of methods, quality control and assessment of method performance.

References

[1] ISO 17034:2016, General requirements for the competence of reference material producers, International Organization for Standardization, Geneva, Switzerland

[2] ISO Guide 35:2017, Reference materials – Guidance for characterization and assessment of homogeneity and stability, International Organization for Standardization, Geneva, Switzerland

[3] ISO/IEC 17025:2017, General requirements for the competence of testing and calibration laboratories, International Organization for Standardization, Geneva, Switzerland

[4] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. Off. J. Eur. Union, L 268:1-28

[5] Commission Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired. Off. J. Eur. Union, L 166:9-15

[6] Commission Regulation (EC) No 65/2004 of 14 January 2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms. Off. J. Eur. Union, L10:5-10

[7] Biosafety Clearing House: https://bch.cbd.int/en/database/LMO/BCH-LMO-SCBD-116060-2, (last accessed on 22/11/2022)

[8] EUginius (EUropean GMO INItiative for a Unified Database System): https://www.euginius.eu/euginius/pages/gmo_detail.jsf?gmoname=DP23211, (last accessed on 14/11/2022)

[9] European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), https://gmo-crl.jrc.ec.europa.eu/method-validations (last accessed on 11/11/2022)

[10] ISO/IEC Guide 98-3:2008, Uncertainty of measurement – Part 3: Guide to the Expression of Uncertainty in Measurement (GUM 1995), International Organization for Standardization, Geneva, Switzerland

[11] ISO 20395:2019(E), Biotechnology — Requirements for evaluating the performance of quantification methods for nucleic acid target sequences — qPCR and dPCR, International Organization for Standardization, Geneva, Switzerland

[12] European Network of GMO Laboratories (ENGL) working group on "Method Performance Requirements" (WG-MPR), European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing, (2015) JRC95544

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

[14] M. Querci, N Foti, A. Bogni, L. Kluga, H. Broll, G. van de Eede, Real-Time PCR Based Ready-to-Use Multi-Target Analytical System for GMO Detection, Food Anal. Methods (2009), DOI 10.1007/s12161-009-9093-0

[15] DIN 1333:1992-02, Zahlenangaben, Deutsches Institut für Normung e.V., Berlin, Germany

[16] ISO/IEC Guide 99:2007, International vocabulary of metrology – Basic and general concepts and associated terms (VIM), International Organization for Standardization, Geneva, Switzerland

[17] ERM Application Note 7: Prolonging the validity of reference material certificates, https://crm.jrc.ec.europa.eu/e/132/User-support-Application-Notes (last accessed on 21/11/2022)

[18] S. Trapmann, C. Charles Delobel, P. Corbisier, H. Emons, L. Hougs, P. Philipp, M. Sandberg, M. Schulze, European technical guidance document for the flexible scope accreditation of laboratories quantifying GMOs, https://crm.jrc.ec.europa.eu/graphics/cms_docs/accreditation_scope_gmo.pdf (last accessed on 21/11/2022)

[19] ERM Application Note 6: Use of ERM certificates and materials, https://crm.jrc.ec.europa.eu/e/132/User-support-Application-Notes (last accessed on 21/11/2022)

[20] S. Trapmann, ERM-Application Note 4: Use of Certified Reference Materials for the Quantification of GMO in Food and Feed, https://crm.jrc.ec.europa.eu/graphics/cms_docs/erm4_english.pdf (last accessed on 21/11/2022)

[21] Guidance documents page of the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), Table conversion factors (CF) experimentally determined on certified reference materials (CRM), https://gmo-crl.jrc.ec.europa.eu/doc/CF-CRM-values.pdf (last accessed on 21/11/2022)

[22] T.P.J. Linsinger, ERM Application Note 1: Comparison of a measurement result with the certified value, https://crm.jrc.ec.europa.eu/e/132/User-support-Application-Notes (last accessed on 21/11/2022)

List of abbreviations

bp	Base pairs
Cq	Quantification cycle (also referred to as threshold cycle, Ct)
CRM	Certified reference material
DNA	Deoxyribonucleic acid
DvSSJ1	*Diabrotica virgifera* smooth septate junction protein 1
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
ERM®	Trademark owned by the European Commission; used by the JRC for reference materials
EU	European Union
EURL-GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed
g	The relative centrifugal force or the g force
GM	Genetically modified
GMO	Genetically modified organism
GUM	Guide to the Expression of Uncertainty in Measurement
IPD072Aa	Insecticidal protein derived from *Pseudomonas chlororaphis*
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
k	Coverage factor
KFT	Karl Fischer titration
LOD	Limit of detection
LOQ	Limit of quantification
M	Molar mass
min	Minute
n	Number of replicate analysis per unit
N	Number of units analysed
n.a.	Not applicable
PAT	Phosphinothricin acetyltransferase
PCR	Polymerase chain reaction
PMI	Phosphomannose isomerase
PSA	Particle size analysis
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
RMP	Reference material producer
RNA	Ribonucleic acid

rpm	Revolutions per minute
RT	Room temperature
r^2	Coefficient of determination of the linear regression
s	Second
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added when appropriate; this parameter is linked to the homogeneity of the material
SI	International System of Units
T	Temperature
t	Time
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation in real-time PCR
TE	Buffer containing TRIS and EDTA
TRIS	Tris(hydroxymethyl)aminomethane
u	Standard uncertainty
U	Expanded uncertainty
u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{Δ}	Combined standard uncertainty of measurement result and certified value
u_{meas}	Standard measurement uncertainty
U_{meas}	Expanded measurement uncertainty
V	Volume
VIM	International Vocabulary of Metrology – Basic and General Concepts and Associated Terms
V-KFT	Volumetric Karl Fischer titration
Δ_{meas}	Absolute difference between mean measured value and the certified value
X_{10}	Particle diameter corresponding to 10 % of the cumulative undersize distribution (here by volume)
X_{50}	Median particle diameter corresponding to 50th percentile of the cumulative undersize distribution (here by volume)
X_{90}	Particle diameter corresponding to 90 % of the cumulative undersize distribution (here by volume)

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Annexes

Annex 1. DNA extraction method as modified in-house

Solutions and reagents

1) CTAB buffer A
   2 % (w/v) CTAB
   1.4 M NaCl
   0.1 M Tris-HCl, pH 8.0
   15 mM Na_2EDTA pH 8.0
2) CTAB buffer B
   1 % (w/v) CTAB
   0.1 M Tris-HCl, pH 8.0
   15 mM Na_2EDTA, pH 8.0
3) Chloroform/Octanol (volume fraction: 24:1)
4) 1.2 M NaCl
5) Ethanol
6) 70 % Ethanol/Nuclease free water ((volume fraction: 70:30)
7) TE low buffer
   1 mM Tris, pH 8.0
   0.01 mM Na_2EDTA, pH 8.0
8) Proteinase K, 20 mg/mL
9) RNase A, 100 mg/mL
10) QIAGEN Genomic-tip 20/G columns
11) QIAGEN QBT buffer
12) QC buffer
13) QF buffer
14) Isopropanol

Protocol CTAB extraction

a) Weigh 200 mg powder into a 2 mL microcentrifuge tube.
b) Add 1 mL of CTAB buffer A and mix thoroughly by vortexing.
c) Add 10 µL RNase A and mix by briefly vortexing.
d) Incubate 15 min at 65 °C, mix a few times by shaking
e) Add 20 µL Proteinase K and mix by briefly vortexing
f) Incubate 15 min at 65 °C, mix a few times by shaking
g) Centrifuge 10 min at 13000 x *g* at RT.
h) Transfer the supernatant to a 2 mL microcentrifuge tube containing 500 µL of chloroform/octanol (volume fraction: 24:1).
i) Vortex 10 s and centrifuge 10 min at 13000 x *g* at RT.
j) Transfer the upper phase to a new 2 mL microcentrifuge tube containing 700 µL of chloroform/octanol (volume fraction: 24:1).

k) Vortex 10 s and centrifuge 5 min at 13000 x *g* at RT.

l) Transfer upper phase to a new 2 mL microcentrifuge tube, carefully determining the volume transferred.

m) Add 2 volumes of buffer B and mix by pipetting up and down.

n) Incubate for 60 min at RT.

o) Centrifuge for 10 min at 13000 x *g* at RT, discard the supernatant by pipetting and conserve the pellet.

p) Add 400 µL of 1.2 M NaCl and vortex gently.

q) Add 400 µL of chloroform/octanol (volume fraction: 24:1), vortex 10 s and centrifuge 5 min at 13000 x *g* at RT.

r) Transfer upper phase to a new 1.5 mL microcentrifuge tube, carefully determining the volume transferred.

s) Add 2 volumes of cold (-20 °C) ethanol to each tube and mix by inverting 10 times.

t) Centrifuge for 10 min at 20000 x *g* at 4 °C.

u) Carefully discard the supernatant by pipetting and wash the pellet with 500 µL of cold (-20 °C) 70 % ethanol.

v) Vortex briefly and centrifuge for 5 min at 13000 x *g* at 4 °C.

w) Discard the supernatant by pipetting and air-dry the pellet for about 5 min.

x) Dissolve the DNA pellet in 80 µL of TE low buffer preheated to 50 °C.

y) Incubate for 10 min at 50 °C, shaking at 350 rpm.

z) Continue with the purification, or store at RT overnight, at +4 °C (short term) or -20 °C (long term).

Protocol purification

1) Equilibrate a QIAGEN Genomic-tip 20/G column with 900 µL of QBT buffer

2) Apply the sample to the equilibrated Genomic-tip 20/G

3) Wash the genomic-tip 20/G with 3x1 mL of QC buffer

4) Place a 2 mL eppendorf tube under the column and elute the genomic DNA with 1 mL of QF buffer

5) Add 700 µL of isopropanol, invert 10 times

6) Centrifuge 30 min at 13,000 x *g* at 4 °C, discard the supernatant

7) Wash the pellet with 1 mL 70 % ethanol

8) Centrifuge 10 min at 13,000 x *g* at 4 °C

9) Discard the supernatant and air-dry the pellet for 10 min

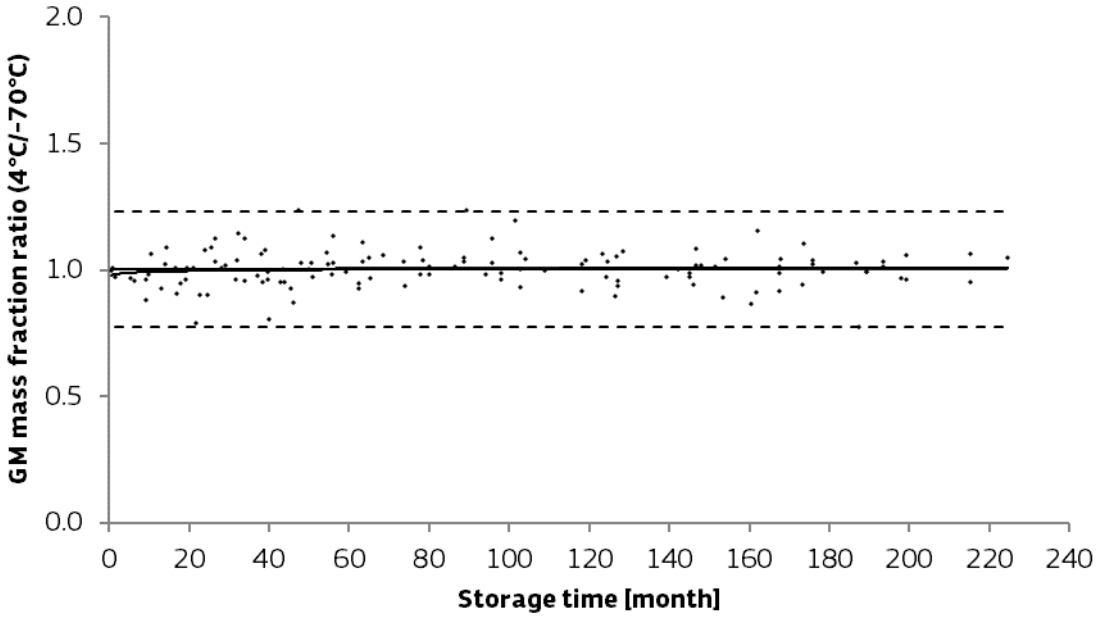
10) Dissolve DNA pellet in 100 µL of TE Low preheated at 50 °C

11) Incubate 10 min at 50 °C

12) Let the pellet dissolve completely overnight at RT and store at +4 °C (short term) or -20 °C (long term).

Annex 2. Results of the stability monitoring measurements

Figure 2.1. Storage stability of maize GMO CRMs assessed at 4 °C over 224 months. Quantitative PCR measurement results of ERM-BF446, ERM BF411, ERM-BF412, ERM-BF412k, ERM-BF413, ERM-BF413k, ERM-BF414, ERM-BF415, ERM-BF416, ERM-BF417, ERM-BF418, ERM-BF420, ERM-BF423, ERM-BF424, ERM-BF427, ERM-BF433, ERM-BF438, and ERM-BF439 (data from the stability monitoring program, last update 12/2022). The dashed lines give the limits of 3s obtained for the measurement results. The straight line is a least-squares linear regression for all data points.



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