



JRC REFERENCE MATERIALS REPORT

Certification of the mass fraction of NK603 in maize seed powder: ERM[®]-BF415g

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Abstract

ERM®-BF415g is a matrix reference material produced within the scope of ISO 17034 accreditation [1]. It is certified for the mass fraction of the genetically modified (GM) maize event NK603 (unique identifier MON-ØØ6Ø3-6).

NK603 maize seeds were milled, sieved and dried to obtain pure GM seed powder. The certified reference material (CRM) is available in glass vials containing at least 1 g of dried powder, which were sealed under an argon atmosphere.

Between-unit homogeneity and stability during transport and storage were assessed in accordance with ISO 33405:2024 [2]. The minimum sample size is 200 mg.

The certified value is based on the genetic purity of the base material (NK603). The purity was confirmed by event-specific quantitative polymerase chain reaction (qPCR). The measurements were performed in accordance with ISO/IEC 17025:2017 [3].

Uncertainties of the certified value include uncertainties related to possible inhomogeneity, instability and characterisation, based on the genetic purity assessment.

The material is intended for the calibration, quality control or assessment of method performance of the event-specific qPCR method for NK603 maize, validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF).

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

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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 this CRM.

Authors

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1 Introduction

1.1. Background

The European Union (EU) 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) to differentiate from products marketed as being non-GMO, conventional non-GM products or derived from organic production according to Regulation (EU) 848/2018 [4].

The products referred to as genetically modified (GM) food and feed require authorisation for marketing in the EU [5, 6]. They also require labelling if they contain more than 0.9 % of GMOs, (EC) No 1829/2003 [5]. 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, (EU) No 619/2011 [7]. The implementation of these thresholds require the availability of validated methods for GMO quantification (including DNA extraction) and of certified reference materials (CRMs) [8, 9]. Official control laboratories use the CRM for the application of food and feed law, rules on animal health and welfare, plant health and plant protection product under (EU) No 625/2017 [10].

The genetically modified *Zea mays* line NK603 with unique identifier code MON-ØØ6Ø3-6, following Commission Regulation (EC) No 65/2004 [11] was produced and commercialised by Monsanto Company (St. Louis, US), in the following referred to as Monsanto. The transgenic maize was developed to give resistance to glyphosate herbicides as a weed control option for maize crops. The genetically engineered maize contains a bacterial form of the gene *epsps*, encoding a glyphosate-tolerant form of the enzyme 5-enolpyruvylshikimate-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 70 bp DNA fragment containing two adjacent EPSPS expression cassettes, each containing a single copy of the *cp4 epsps* gene [12, 13].

ERM-BF415g was prepared using the maize seed powder of the genetically modified seeds NK603 maize (seed line 'DKC 57-40') supplied by Monsanto (St. Louis, US) to the JRC (Geel, BE) in 2003. The GM maize seed powder has been stored at -20 °C at the JRC. According to Monsanto the GM NK603 maize seeds (MON-ØØ6Ø3-6) are hybrid seeds, where the GM donor of the GM event is the female parent.

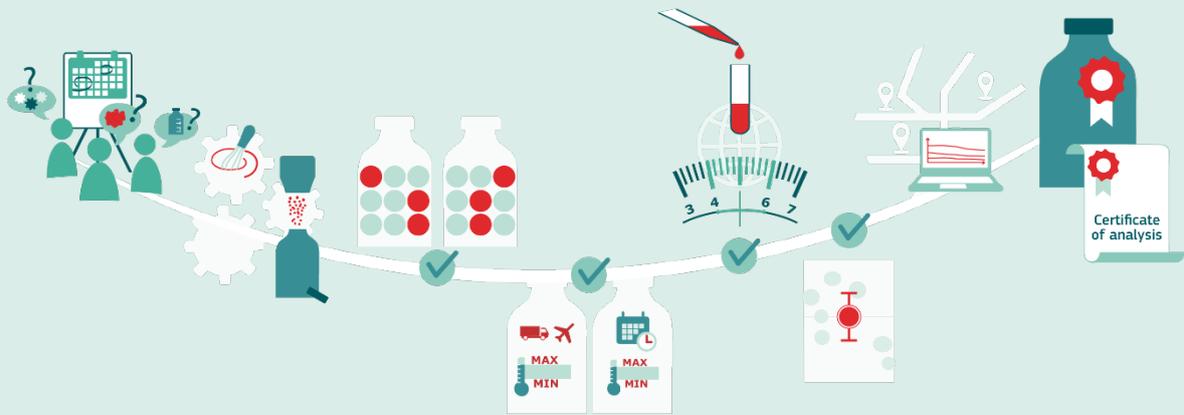
1.2. Choice of the material

This CRM, coded ERM-BF415g, consists of milled GM maize seed NK603. It belongs to the same series of the set ERM-BF415a,b,c,d,e,f materials of mixed GM concentrations closer to the EU labelling thresholds, produced in 2004 [14]. Seeds (in contrast to grains) were selected as the source of raw material because of the higher degree of purity. The seeds were milled to obtain a maize seed powder of which DNA can be extracted. ERM-BF415g is prepared to serve as calibrant, quality control or assessment of method performance of the event-specific qPCR method for NK603 maize, validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF).

The event-specific qPCR method developed by Monsanto (St Louis, US), has been validated and published in 2005 and verified in 2008 by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) (Ispra, IT) [15, 16]. The validation was performed in an international inter-laboratory collaborative study in accordance with EC Regulation No 1829/2003 [5].

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.

The genetic purity with respect to the NK603 maize event of the GM maize seeds was investigated by Monsanto (St Louis, US). For confirmation, JRC (Geel, BE) performed additional genetic purity tests on the GM maize seeds. The results obtained from Monsanto and JRC were pooled for the calculation of the certified value and the related purity uncertainty.

The uncertainty of certified value was estimated in compliance with ISO 17034 [1], which implements the basic principles of ISO/IEC Guide 98 (GUM) [17].

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 and data evaluation

European Commission, Joint Research Centre, Directorate F – Health and Food, 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

Monsanto Company (St. Louis, US)

2.3 Processing

European Commission, Joint Research Centre, Directorate F – Health and Food, Geel, BE

2.4 Stability and homogeneity measurements

European Commission, Joint Research Centre, Directorate F – Health and Food, Geel, BE
(measurements performed under the scope of ISO/IEC 17025:2017 accreditation BELAC No. 268-TEST)

2.5 Confirmation measurements

European Commission, Joint Research Centre, Directorate F – Health and Food, Geel, BE
(measurements performed under the scope of accreditation ISO/IEC 17025:2017, BELAC No. 268-TEST)

2.6 Characterisation measurements (purity)

European Commission, Joint Research Centre, Directorate F – Health and Food, Geel, BE
Monsanto Company (St. Louis, 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 Origin and purity of the starting material

The NK603 maize seeds were supplied by Monsanto (St. Louis, US) to the JRC (Geel, BE) in 2003 for the preparation of a set of CRMs coded ERM-BF415. According to the information provided by Monsanto the NK603 maize seeds (MON-00603-6) are hybrid seeds, where the GM donor of the GM event is the female parent (verified by the conversion factor of mass fraction to copy number by the EURL GMFF) [18]. Two hundred individual seeds were qualitatively tested by Monsanto for the presence of the CP4EPSPS protein by lateral flow immunoassay (LFI). All 200 seeds tested positive for the CP4EPSPS protein. Four pooled samples (75 seeds each) were tested for *GA21*, *MON863*, and *MON810* using event-specific PCR, all pools were negative for the events listed. Given the test results, it can be assumed with 95 % confidence that >98.5 % of the seeds of the lot of DKC57-40 received contain the NK603 transformation event. Upon arrival, the seeds were stored at $(4 \pm 3) ^\circ\text{C}$ in the dark prior to processing (in 2003). Once processed (Section 3.2), the maize seed powder was stored at $-20 ^\circ\text{C}$.

The genetic purity of the maize seeds with respect to the event NK603 has been tested at JRC (Geel, BE) by analysing leaf tissue from the maize plants grown from 50 individual GMO seeds on moisture paper. The genomic DNA of leaf tissue was extracted by using the commercial DNA extraction kit DNeasy® Plant minikit (Qiagen, Antwerp, BE) from 50 individual plants. The DNA extracts were diluted to $2 \text{ ng}/\mu\text{L}$, 20 ng of each sample that was analysed in triplicate by qPCR TaqMan® assay using an ABI PRISM 7700 instrument (Applied Biosystems, Lennik, BE). The genes tested were *NK603* and *Adh1* according to the method developed by Monsanto (St Louis, US) validated in-house in 2004 and by the EURL GMFF [15], and used as qualitative assessment for the purity. All the 50 plants presented positive results with respect to the transgene *NK603* and endogen *Adh1* maize, which confirm the data provided by Monsanto.

For statistical analysis, the results of the 50 plants grown from the seeds tested at the JRC (Geel, BE) and the 200 seeds tested at Monsanto were pooled. Consequently, the GM maize batch had a genetic purity above 98.8 % (based on Poisson distribution for rare events, at 95 % level of confidence).

Samples were additionally tested in 2024 on 22 targets by JRC using a pre-spotted plate PSP (EURL-SP-08/16 – PCS maize): *hmg*, *3272*, *5307*, *98140*, *BT11*, *Bt176*, *DAS40278*, *DAS59122*, *GA21*, *LY038*, *MIR162*, *MIR604*, *MON810*, *MON863*, *MON87427*, *MON87460*, *MON88017*, *MON89034*, *NK603*, *T25*, *TC1507*, *VCO 01981-5*. Among those targets, non-quantifiable traces of *MON810* and *GA21* were detected in the NK603 seed powder.

3.2 Processing

After receipt of the NK603 maize (line DKC 57-40) seeds in 2003 from Monsanto, the seeds were thoroughly cleaned; first rinsed with demineralised water and then drained and dried at $30 ^\circ\text{C}$ for 20 hours in a freeze-dryer (Martin Christ, Osterode, DE). After a two-step grinding process using a high impact mill with a triangular ribbed open grinding track under argon flushing, an additional vacuum drying at $30 ^\circ\text{C}$ was carried out to reduce the water content of the ground powder. For the second grinding step a sieve insert was used with 0.5 mm mesh width. Caution was taken to avoid increase of the grinding temperatures above $40 ^\circ\text{C}$. The sieved pure material was then homogenised by turbula-mixing for 30 minutes to ensure that the different parts of the maize seeds were equally distributed in the powder. The final powder had a water content of about 2.5 % and was stored at $-20 ^\circ\text{C}$ until 2023.

Two kilogram of the ground NK603 maize seed powder was taken for further processing in April 2023. The powder was once more turbula-mixed for 1 hour using a DynaMIX CM200 (WAB, Muttentz, CH) to ensure the homogenisation of the different parts of the maize seed tissues after storage.

Contamination with foreign DNA was avoided by using a glove box systems and separated clean areas, by treating prior to exposure all the contact surfaces and equipment with a DNA-erase solution (MP Biochemicals, Irvine, US) and by using clean and cotton free laboratory clothing.

Amber glass vials of 10 mL were filled with at least 1 g of the prepared seed maize powder NK603 using a MCPI Vibrating filling machine with feeder (FD SPac 4A MCPI, Meythet, FR). To avoid contamination, the feeder was thoroughly cleaned prior to use and the first 30 glass vials filled were discarded as a precautionary measure. Lyophilisation rubber inserts were placed in the vials' necks, leaving the vial half open. The units were then moved inside an Epsilon DF 2-100D freeze dryer (Martin Christ, Osterode, DE) where they were closed under an argon atmosphere. The capping and labelling of the vials was done using an HV 100 B 10 semi-automatic capping machine (Bausch & Ströbel, Ilshofen, DE) and a labelling machine (BBK, Beerfelden, DE).

Black coloured caps were used for the easy identification of the pure NK603 GM mass fraction level, consistent with the cap colours of previously produced GMO CRMs at JRC. Each unit is identified by a label indicating the CRM code and the unique individual unit number according to the order of filling. Some units were randomly selected for analysis. The ERM-BF415g units were stored in the dark at +4 °C.

For the purpose of this report, the term 'unit' refers to one glass vial of ERM-BF415g.

One unit of ERM-BF415g is shown in Figure 1.

Figure 1. ERM-BF415g, pure GM maize powder.



Source: JRC

3.3 Processing control

The water mass fraction of the GM powder material before and after filling ERM-BF415g units was measured with volumetric Karl Fisher titration (V-KFT). Five randomly selected units were analysed. The results passed the specification criterion (< 3 g/kg) for the material before storage, summarised in Table 1.

Particle size distribution measurements based on laser diffraction (Helos KR, Sympatec GmbH, Clausthal-Zellerfeld, DE) took place during and after final processing of ERM-BF415g. 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 GM powder bulk material before filling the units was $176 \mu\text{m} \pm 8 \mu\text{m}$ ($U, k = 2$) ($N = 1, n = 5$). Since the presence of coarse particles may affect the homogeneity of the measurands, the maximum particle size X_{99} was checked against specification before the filling of the material.

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 3-point specification of the particle size distribution ($N = 1, n = 5$) was calculated, consisting of the equivalent sphere diameters at 10 %, 50 % and 90 % of the cumulative volume distribution. These size classes were denoted X_{10} , X_{50} and X_{90} , respectively.

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 material and the processed ERM-BF415a (non-GM) and ERM-BF415g materials by laser diffraction.

	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
GM base material	21.7 ¹⁾	2.7	176 ²⁾	8	14 ³⁾	2.9	152 ³⁾	25	381 ³⁾	78
ERM-BF415g	24.3 ⁴⁾	3.5	171 ⁶⁾	8	14.5 ⁷⁾	3.2	146.8 ⁷⁾	28	368.4 ⁷⁾	79
ERM-BF415a	n.a. ⁵⁾	n.a. ⁵⁾	151 ⁶⁾	10	13.2 ⁷⁾	2.9	119.3 ⁷⁾	23	344.3 ⁷⁾	74

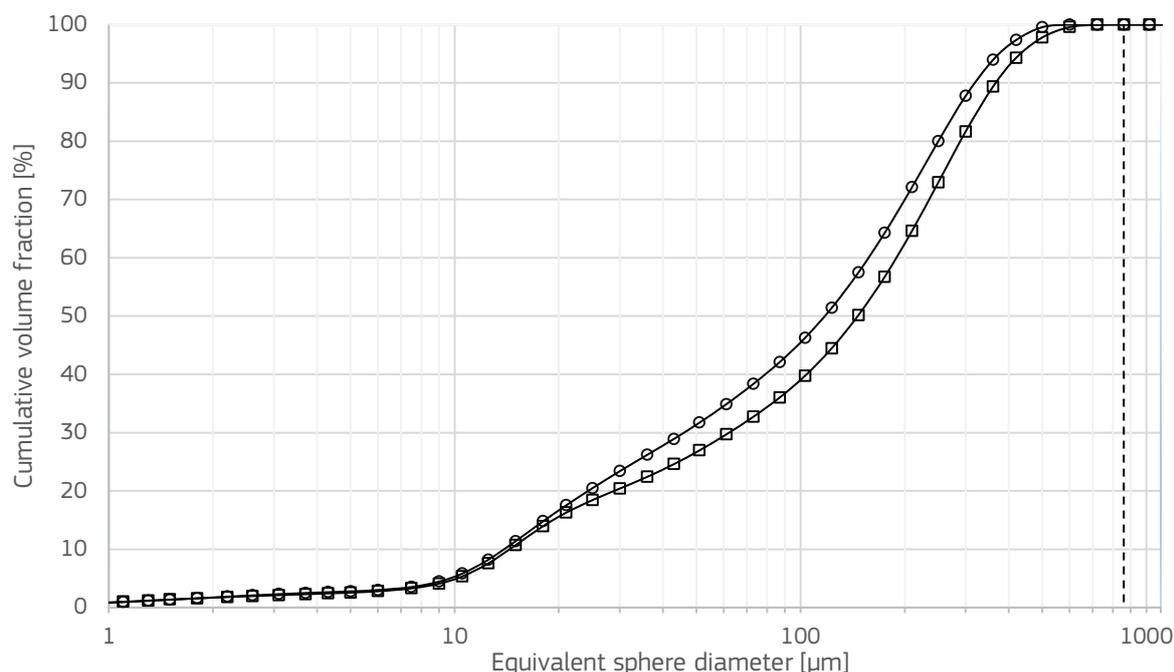
Source: JRC

- ¹⁾ Mean of one sample ($N = 1, n = 3$). 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 sample ($N = 1, n = 5$). The associated expanded uncertainty (U) with a coverage factor of $k = 2$ has been estimated based on the standard error of the mean of measurements.
- ³⁾ Mean of one sample ($N = 1, n = 5$). The associated expanded uncertainty (U) with a coverage factor of $k = 2$ has been estimated during validation of the laser diffraction method.
- ⁴⁾ Mean of five units ($N = 5, n = 2$). The associated expanded uncertainty (U) with a coverage factor $k = 2$ has been estimated during validation of the V-KFT method.
- ⁵⁾ Not determined
- ⁶⁾ Mean of five units ($N = 5, n = 2$). The associated expanded uncertainty (U) with a coverage factor of $k = 2$ has been estimated based on the standard error of the mean of measurements.
- ⁷⁾ Mean of five units ($N = 5, n = 2$). The associated expanded uncertainty (U) with a coverage factor of $k = 2$ has been estimated during validation of the laser diffraction method.

The particle size distribution of ERM-BF415g (GM material) was determined additionally after filling by laser diffraction. The results were compared with ERM-BF415a (non-GM material) produced in 2004 (Table 1). Five randomly selected units from each of the CRMs were analysed in duplicate ($N = 5, n = 2$). A Student's t -test showed with 95 % confidence that there were no significant differences between the X_{10} , X_{50} , X_{90} particle size percentiles of the volume-weighted cumulative (Q_3) distributions of the two materials. Despite a difference between the volume mean diameters, the particle size distributions of the two materials (GM and blank non-GM) were found to be sufficiently similar not to introduce a significant bias that may contribute to different DNA extraction efficiencies.

Volume-weighted cumulative distributions of ERM-BF415a and ERM-BF415g are depicted in Figure 2. The equivalent diameters of all particles were below 860 μm , fulfilling the acceptance criterion (below 1000 μm).

Figure 2. Volume-based cumulative distribution of particle size in ERM-BF415a (□) and ERM-BF415g (○) analysed by laser diffraction ($N = 5$, $n = 2$). The dashed line represents 860 μm , the highest volume mean diameter measured.



Source: JRC

3.4 Confirmation measurements

Confirmation measurements by qPCR analysis were performed on five units of ERM-BF415g. The CRMs ERM-BF415a, b, c, d and e were measured in parallel to verify the consistency of the other CRMs of the set, when being calibrated with DNA extracted from ERM-BF415g. The measurements were executed using a validated method and under the scope of ISO/IEC 17025:2017.

Five units of each GMO CRM were randomly selected. From each unit, 200 mg of powder was taken and its DNA was extracted using the in-house validated CTAB (cetyl trimethylammonium bromide) method (Annex 1). The extracted DNA quantity and quality was verified by UV photometry using the instrument NanoDrop One (Isogen Life Science/Thermo Fisher Scientific, De Meern, NL) at an absorbance wavelength of 260 nm and the absorbance ratios of 260/280 and 260/230 used to assess the purity. All samples tested showed high DNA mass concentration (above 100 ng/ μL) and good quality ratios corresponding to highly pure DNA. The gel electrophoresis showed no smear bands typical for degraded DNA, (using a DNA ladder of 100 bp to 15000 bp) which indicated that most of the extracted DNA had a size above 15000 bp.

The concentration of the extracted DNA in each sample was measured with a fluorescence intensity method using the commercial kit Quant-iT PicoGreen dsDNA (Invitrogen/Life Science Technologies Europe BV, Merelbeke, BE) and the multimode reader Synergy MX (Biotek/BRS, Beerse, BE). The DNA samples were then diluted in TE low buffer (1 mM TRIS-HCl, 0.01 mM EDTA, pH 8) to a concentration of 20 ng/ μL for normalization of the qPCR assay at 100 ng (corresponding to 5 μL DNA sample per 25 μL PCR reaction mix). The method for the quantification of the NK603 content is a real-time quantitative PCR event specific method, that amplifies a 108-bp fragment of the region that spans the 3' insert-to-plant junction in corn event NK603, validated by the EURL GMFF (JRC, Ispra, IT) using the set of primers and probes indicated by Monsanto and based on the standard curve approach on TaqMan chemistry. The relative quantification is performed with a set of primers and probes fit for the analysis of *hmg* endogenous maize gene (instead of *Adh1* original method published by EURL GMFF) [15], validated for other maize GMO events. Broothaerts et al. (2008) [19] suggested that the primers used for *Adh1* (endogenous maize gene) detection in the original qPCR method, could amplify a region which is subjected to allelic polymorphism in maize. Consequently, the relative quantification with respect to maize endogenous gene is done using a pair of specific primers and a probe for *hmg* detection.

The DNA extract obtained from the pure NK603 GMO material ERM-BF415g was diluted into nine concentration levels to build two calibration curves for the analysis of the GM and reference genes by qPCR. The analysis of the endogenous maize species *hmg* (reference gene) was performed at a concentration range of (1 to 200) ng/25 μ L reaction mix and the analysis of the GM event specific *NK603* target gene covered the concentration range of (0.05 to 20) ng/25 μ L adapted to the GM levels between 0.25 g/kg to 100 g/kg.

The amplification of the target DNA and detection was performed on a real-time quantitative PCR instrument (QuantStudio Flex System, Life Technologies Europe, Gent, BE). The parameters obtained through the analysis (i.e. efficiency of the amplification of each assay, coefficient of determination) are both registered in a control chart. The PCR efficiency control limits are arbitrarily established between 90 % and 110 %, corresponding to slope values of -3.6 and -3.1, respectively [21, 22]. For the analysis of NK603 event, the PCR efficiency was mostly just below the limit, also observed during the validation of the method in 2004, which can be due to the broad GM working concentration range (0.5 to 100) g/kg for the verification of the GM mixtures or (5 to 1000) g/kg for the verification of the produced pure material, which is far above the range normally applied for measuring the relevant GM content in real samples. The coefficient of determination for both targets complies with the acceptance criterion ($r^2 > 0.985$) [22].

The LOD of the qPCR method was determined during the validation of the method as 0.78 g/kg GM NK603 maize; it was calculated as 3.3 times the standard deviation of the results of the lowest calibration point at which the relative standard deviation S_{rel} was below 25 %.

The qPCR verification results of all the ERM-BF415 mass fractions are presented in Table 2. It has to be noted that no independent calibration has been carried out as the presented qPCR results were calibrated with DNA extracted from ERM-BF415g. Therefore the data in Table 2 can only be used for confirmation of the consistency of the GM mixtures during processing.

Table 2. NK603 maize mass fractions in the CRMs measured by qPCR.

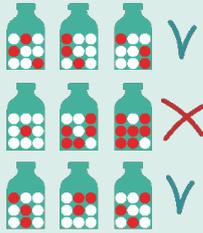
CRM code	Certified NK603 maize mass fraction, $U (k = 2)$ [g/kg]	Measured NK603 maize mass fraction, $U (k = 2)$ [g/kg]
ERM-BF415a	< 0.4 ¹⁾	< LOD ²⁾
ERM-BF415b	1 \pm 0.4	1.29 \pm 0.26 ^{3), 5)}
ERM-BF415c	4.9 \pm 0.5	5.9 \pm 1.4 ^{4), 5)}
ERM-BF415d	9.8 \pm 0.7	12.5 \pm 2.8 ^{4), 5)}
ERM-BF415e	19.6 \pm 0.9	23.0 \pm 5.2 ^{4), 5)}
ERM-BF415f	49.1 \pm 1.3	55.5 \pm 8.9 ^{4), 5)}
ERM-BF415g	-	1028 \pm 16 ^{4), 6)}

Source: JRC

- 1) Standard uncertainty of the purity estimation of the non-GM base material (LOD = 0.78 g/kg), is based on the half-width of the interval between 0 g/kg and 0.78 g/kg, divided by the square root of 3 (rectangular distribution).
- 2) Not detected, below LOD (0.78 g/kg) established during method validation
- 3) Mean of five units three replicates ($N = 5, n = 3$).
- 4) Mean of five units, two replicates ($N = 5, n = 2$)
- 5) The associated expanded uncertainty (U) with a coverage factor $k = 2$ has been estimated during validation of the qPCR method.
- 6) The associated expanded uncertainty (U) with a coverage factor of $k = 2$ has been estimated based on the standard error of the mean of measurements.

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 the pure material could be demonstrated by testing the purity of the GMO seeds. The measurements results were obtained under repeatability conditions on five units taken over the whole batch of ERM-BF415g and analysed in a random order they can be suited to investigate the CRM homogeneity (Sections 3.1 and 3.4).

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified value of the CRM is valid for all units of the material, within the stated uncertainty.

The homogeneity of ERM-BF415g can be related to the purity measurements from Monsanto and the JRC (Section 3.1). All seeds tested positive for the NK603 event. In the pure material the uncertainty contribution of the possible inhomogeneity (u_{bb}) is negligible.

4.2 Within-unit homogeneity and minimum sample size

ERM-BF415g is not expected to be inhomogeneous since it is a pure GM material. A sample size of 200 mg per analysis, taken from previous experience of maize seed powder GM CRM production, yielded a suitable amount of DNA to perform qPCR. Hence, the minimum sample size is set at 200 mg.

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 has to 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 parameters on the stability of the material. The influence of ultraviolet and visible light was minimised by storing the material in containers, which reduce light exposure. In addition, the material is 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 time and temperature parameters needed to be investigated.

The stability of ERM-BF415g was assessed using data obtained on similarly processed GMO CRMs, confirmed by short-term stability studies using an isochronous design [25] on ERM-BF415g and ERM-BF415f. For the isochronous approach, some randomly selected units are stored for a certain time at different temperature conditions. Afterwards, the units were moved to conditions where further degradation can be assumed negligible (reference temperature). At the end of the isochronous storage, samples taken from twelve units (three per time and temperature) were analysed simultaneously under repeatability conditions.

ERM-BF415g was analysed qualitatively by gel electrophoresis and ERM-BF415f (GMO nominal level 50 g/kg) was tested by qPCR. The analysis of ERM-BF415f provides the best method repeatability by qPCR analysis to see any possible trend in degradation.

5.1 Transport stability

The conditions for the transport of the material to the customers were established in two short-term stability studies on ERM-BF415g and ERM-BF415f using an isochronous scheme. To this end, 12 randomly selected units were stored at 60 °C for 0, 1, 2 and 4 weeks. The reference temperature was set to 4 °C and in addition two units stored at -70 °C were selected to check any possible difference from 4 °C. Three units per storage time were selected using a random stratified sampling scheme. From each unit, two samples were measured by gel electrophoresis on material ERM-BF415g and by event-specific qPCR on material ERM-BF415f.

Gel electrophoresis on DNA extracted from ERM-BF415g units and event-specific qPCR on DNA extracted from ERM-BF415f were performed under repeatability conditions. For the qPCR analysis all the units were measured on the same day, and all the replicates samples over two days with no analytical trend. 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 individually for each temperature. The size and integrity of the extracted DNA was evaluated visually by gel electrophoresis.

The qPCR results were screened for outliers using the single and double Grubbs test on a confidence level of 99 %.

All ERM-BF415g units tested showed clear intense bands with high relative molecular mass corresponding to fragment sizes above 15000 bp (compared to a reference ladder) with a little smear in some bands. These artefacts, which are possibly due to the conditions of the extraction method or to matrix effects, were not in correlation with the storage conditions of -70 °C, 4 °C, or 60 °C. It can be assumed that there is no significant degradation of the DNA extracted from units stored at 60 °C (Annex 2).

The data obtained on DNA extracted from ERM-BF415f with event-specific qPCR additionally were evaluated against storage time, and regression lines of mass fraction versus time were calculated to test for potential changes of the NK603 GMO event mass fraction. The slopes of the regression lines were tested for statistical significance. The graph depicting the results are shown in Annex 3.

No statistical outlier was detected for the analysis of the mass fraction of NK603 using a Grubbs test at a confidence interval of 99 %. There was no trend statistically significant at a 95 % confidence level for the material ERM-BF415g at 60 °C.

No systematic trends in the transport stability study had been observed for ERM-BF415 and similarly produced maize GMO CRMs. Therefore this material can be dispatched under ambient conditions.

5.2 Storage stability

Storage conditions and shelf-life guaranteeing the stability of the material and the certified value were established in a long-term stability study.

Data from the JRC's stability monitoring programme for GMO CRMs are available and can give an indication whether ERM-BF415g can be expected to be stable as it is produced and stored in the same way.

Previously released GMO maize seed powder CRMs were analysed for their GM mass fraction on 147 occasions over a period of 250 months. At each time point measurements were performed on two units (three replicates) stored at normal storage temperature (4 °C) and two units (three replicates) 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 qPCR measurements results from samples stored at 4 °C and -70 °C.

The data obtained from the stability monitoring of similar maize seed GMO CRMs are plotted against their storage time and linear regression lines of GM mass fractions ratio versus time are calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). No statistical outliers were detected on a 99 % confidence level. Also, no trends were found at a 95 % confidence level. Therefore, it is concluded that also ERM-BF415g is stable. The results of the long-term stability measurements are presented in Annex 4. ERM-BF415g will be 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 an RM in various ways, quality management procedures of the JRC are more stringent and allow characterisation only by either interlaboratory comparison of expert laboratories or the use of a primary method confirmed by independent analysis.

The material characterisation of ERM-BF415g was based on the purity results obtained by LFI and by PCR for 200 and 50 seeds, respectively, and using a statistical approach based on Poisson distribution. The material is certified for its NK603 maize mass fraction.

The verification of the value given by purity determination was confirmed by analysis by quantitative qPCR on the DNA extracted samples from the produced ERM-BF415g pure GM maize NK603 seed powder.

6.1 Purity of the base materials

The purity of the GM batch used for the processing of ERM-BF415g was investigated and used to calculate the certified value and its uncertainty.

The NK603 GM maize material received was tested for the presence of NK603 event on 250 seeds, all seeds analysed tested positive which resulted in a statistical purity of 98.8 % (Section 3.1). Since no evidence of quantifiable contamination was found, 100 % purity was used for the calculation of the certified NK603 maize mass fraction. The difference between the measured purity of 98.8 % and the 100 % purity was taken into account in the uncertainty estimation.

The potential presence of traces of other GM events in the material ERM-BF415g processed was verified by PCR. The analysis was performed at JRC (Geel, BE) by a qualitative PCR based, ready-to-use multi-target analytical system for GMOs using a pre-spotted 96-well plate (PSP, EURL-SP-08/16 – PCS maize) developed by the EURL GMFF (JRC, Ispra) [28, 29]. The PSP plate contains primers and probes specific for simultaneous detection of 22 targets, 1 target maize species-specific (*hmg*) and 21 targets specific GM maize events: 3272, 5307, 98140, BT11, Bt176, DAS40278, DAS59122, GA21, LY038, MIR162, MIR604, MON810, MON863, MON87427, MON87460, MON88017, MON89034, NK603, T25, TC1507, VCO 01981-5. The results indicate the presence of *hmg* (maize species-specific) and NK603 (GM target event) on the two units selected for this test. Among those GM targets, not quantifiable traces of MON810 and GA21 were detected. Any stacked event derived from any of the single inserts GMO included in the system would be detected. The seed powder used for the production of ERM-BF415g did not contain traces of other maize GM event above the LOD of the qPCR method used (Section 3.4).

Since no evidence of contamination was found in the base material (i.e. above the LOD of the method), 100 % purity was used for the calculation of the certified mass fraction of NK603.

6.2 Verification measurements

Quantitative PCR measurements demonstrated that there were no processing errors made (Section 3.4). Gel electrophoresis proved that the DNA was not significantly degraded during processing of the CRM (Annex 2).

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 33405:2024 [2] are required. Certified values of a CRM can be used for calibration and trueness controls as well as for quality control measures.

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 have 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 have usually not been assessed and only 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.

7.1 Certified value and its uncertainty

The certified value for ERM-BF415g is based on the purity assessment of 250 seeds. As no impurity was found in the NK603 maize seeds tested (Section 3.1), the certified value for ERM-BF415g is set to 1000 g/kg. For this pure GM material, the difference between the minimum statistical purity (988 g/kg) of the GM seed batch and the certified value (1000 g/kg) was used to describe the asymmetrical distribution (one side) of 95 % confidence interval of the certified mass fraction of NK603 in the maize powder.

The uncertainty of the certified GM mass fraction in ERM-BF415g is based on the uncertainty of the purity of the seeds (u_{char}).

The asymmetric expanded uncertainty was based on the rectangular distribution with 95 % confidence interval of the statistical genetic purity of the seeds (988 g/kg) and calculated as the interval between 988 g/kg and the certified value 1000 g/kg = 12 g/kg as upper limit, with a corresponding rounded lower bound of the standard uncertainty interval $u = 12 \text{ g/kg} / \sqrt{3} = 7.0 \text{ g/kg}$.

Table 3. Certified value and its calculated uncertainty for the NK603 maize mass fraction in ERM-BF415g.

	Certified value [g/kg]	Standard uncertainty contribution u_{char} [g/kg]	U_{CRM} [g/kg]
ERM-BF415g	1000 ¹⁾	7.0	+0 -12 ²⁾

Source: JRC

¹⁾ The certified value was based on the genetic purity of the maize seeds with regard to NK603 maize. All 250 seeds tested individually, were positive for the presence of the NK603 maize event.

²⁾ The asymmetric expanded uncertainty calculated with a confidence interval of 95 %, rectangular distribution with one side calculated as 98.8%, corresponding to a lower limit of 988 g/kg. The corresponding rounded lower bound of the standard uncertainty interval is calculated $12 \text{ g/kg} / \sqrt{3} = 7.0 \text{ g/kg}$.

The user is reminded that JRC only certifies this material for the NK603 maize mass fraction, and not for its transgenic and maize-specific DNA copy number ratio. Additionally, one must be careful to draw quantitative conclusions (in gene copy numbers, for instance) from measurements of 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 to a common reference, metrological traceability of its certified values to a stated reference is essential.

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

This requires that these measurements

- refer to the same property (e.g. Pb content) and the same (kind of) quantity (e.g. Pb mass fraction),
- result in a number and its uncertainty (e.g. 6 ± 2) expressed in the same measurement unit (e.g. $\mu\text{g}/\text{kg}$).

The concept of metrological traceability rests in chemistry on several anchor points, namely identity, quantity value and measurement unit. The identity of a measurand can be defined by its chemical 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 genetically modified NK603 maize event (Biosafety Clearing House, record 14776-17 [13]. The measurand is structurally defined via its DNA sequence and independent of the measurement method.

8.1.2 Quantity value

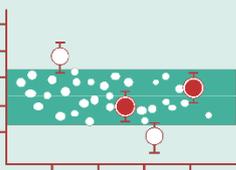
The certified value for ERM-BF415g is traceable to the International System of Units (SI). The traceability is based on the genetic purity assessment using validated quantitative and qualitative event-specific PCR methods, protein lateral flow immunoassay (LFI) and qualified analytical equipment.

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 [12] 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 this CRM ERM-BF415g is structurally defined via its DNA sequence. The CRM was prepared from pure GM seeds with the aim to use the material as calibrant and quality control to implement the mass fraction based thresholds set in the corresponding EU legislation for food and feed.

The CRM is intended for calibration, quality control or assessment of method performance of the event-specific qPCR method for NK603 maize, validated by the EURL GMFF. Consequently, in combination with the

validated measurement method [15], this CRM is establishing the arbitrary reference system required for quantification of NK603 maize seed powder.

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 $(4 \pm 3)^\circ\text{C}$ in the dark. Care should be taken to avoid any change of the moisture content once the units are open, as the material is hygroscopic. The user should close any unit immediately after taking a sample.

For more information regarding the shelf-life of reference materials please consult ERM Application Note 7 [30].

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

For general information on handling of reference materials, please consult ERM Application Note 6 [31].

9.4 Minimum sample size

The minimum sample size for DNA extraction is 200 mg in order to obtain a suitable DNA amount for subsequent analysis by qPCR.

9.5 Use of the certified value

The intended use of these materials is for calibration, quality control or assessment of method performance of the event-specific qPCR method for NK603 maize, validated by the EURL GMFF. As with any reference material, it can be used for establishing control charts or validation studies.

The user is reminded that this reference material is certified for its NK603 maize mass fraction and it should be used for measurement results expressed in mass fractions, see Application Note 4 [20]. The exact relationship between the certified GM powder mass fraction 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, for instance, requires the use of a conversion factor that is only an approximate value, thereby adding additional uncertainty to the measurement result [18].

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. It is recommended to use different GM concentration levels of the set ERM-BF415 for calibration and quality control.

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 [23]).

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}):

$$u_{\Delta} = \sqrt{u_{\text{meas}}^2 + u_{\text{CRM}}^2}$$

- 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 $\Delta_{\text{meas}} \leq U_{\Delta}$ 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 material(s) 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.

10 Conclusions

ERM-BF415g is a matrix reference material certified for the mass fraction of NK603 maize. ERM-BF415g was produced within the scope of ISO 17034 accreditation.

ERM-BF415g, together with the already certified reference materials ERM-BF415a, ERM-BF415b, ERM-BF415c, ERM-BF415d, ERM-BF415e and ERM-BF415f, is intended to support the implementation of the labelling requirements that are set in the EU legislation for food and feed, Regulation (EC) No 1829/2003 [5] and Regulation (EC) NO 619/2011 [7]. In combination with the event-specific qPCR method validated by the EURL GMFF [15], this CRM is establishing the arbitrary reference system required for the quantification of NK603 maize.

The following certified value was assigned:

Table 4. Certified value assigned to ERM-BF415g.

ERM [®] - BF415g MAIZE SEED POWDER		
	Certified value ²⁾ [g/kg]	Uncertainty ³⁾ [g/kg]
NK603 ¹⁾	1000	+0 -12

¹⁾ Genetically modified maize with the unique identifier MON-00603-6.

²⁾ Certified values are values that fulfil the highest standards of accuracy. The certified value is based on the genetic purity of the maize seeds, > 98.8 % with regard to the NK603 maize event. The certified value and its uncertainty are traceable to the International System of Units (SI).

³⁾ The asymmetric expanded uncertainty is based on the 95 % confidence interval of the purity of the seeds. The rounded lower bound of the standard uncertainty interval corresponds to 7 g/kg. It is estimated in accordance with ISO 17034:2016 and ISO 33405:2024 and assuming a rectangular distribution.

Source: JRC

ERM-BF415g is certified for its NK603 maize mass fraction. The material is intended for the calibration, quality control or assessment of method performance of the event-specific qPCR method for NK603 maize, validated by EURM-GMFF [15, 16].

References

- [1] ISO 17034:2016, General requirements for the competence of reference material producers, International Organization for Standardization, Geneva, Switzerland
- [2] ISO 33405:2024, Reference materials — Approaches 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 (EU) 848/2018 of the European Parliament and of the Council of 30 May 2018 on Organic Production and Labelling of Organic Products and Repealing Council Regulation (EC) No 834/2007. Off. J. Eur. Union, L 150:1-92
- [5] Commission 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
- [6] Commission implementing Decision (EU) No 684/2015 of 24 April 2015 authorising the placing on the market of genetically modified maize NK603 (MON-00603-6) and renewing the existing maize NK603 (MON-00603-6) products, pursuant to Regulation (EC) No 1829/2003 of the European Parliament and of the Council. Off. J. Union, L112:6-10.
- [7] 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
- [8] Commission implementing regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council, Off. J. Union, L157:1-48
- [9] ISO 21571:2005, Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Nucleic acid extraction
- [10] Commission Regulation (EU) No 625/2017 of the European Parliament and the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products. Off. J. Eur. Union, L 95:1-142
- [11] 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, L 10:5-10
- [12] Euginius, (EUropean GMO INitiative for a Unified Database System) https://euginius.eu/euginius/pages/gmo_detail.jsf?qmoname=NK603 (last accessed on 30/01/2025)
- [13] Biosafety Clearing House (BCH), <https://bch.cbd.int/en/database/LMO/BCH-LMO-SCBD-14776-17> (last accessed on 30/01/2025)
- [14] S. Trapmann, P. Conneely, P. Corbisier, D. Gancberg, S. Gioria, M. Van Nyen, H. Schimmel, H. Emons; The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of NK603 Maize Certified Reference Material ERM®-BF415 (ERM®-BF415a/ERM®-BF415b/ERM®-BF415c/ERM®-BF415d, ERM®-BF415e, ERM®-BF415f); EUR Report 21270 Luxembourg: Office for Official Publications of the European Communities, 2005, ISBN 92-894-8063-7
- [15] M. Mazzara, C. Paoletti C., J. Puimalaainen, D. Rasulo, G. Van Den Eede, Event Specific Method for the Quantitation of Maize Line NK603 Using Real-Time PCR - Validation Report CRLVL27/04VP (2005) EU Database of Reference Methods for GMO (Analysis: entry name: QT-EVE-ZM-008), European Union Reference Laboratory for GM food and feed, <https://gmo-crl.jrc.ec.europa.eu/method-validation/details/all/3/NK603> (last accessed on 30/01/2025)

-
- [16] Verification of the Performance of a NK603 Event-specific Method on Maize Line NK603 Using Real-time PCR (2008), European Union Reference Laboratory for GM food and feed <https://gmo-crl.jrc.ec.europa.eu/method-validation/details/all/3/NK603> (last accessed 30/01/2025)
- [17] 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.
- [18] Guidance documents, Table conversion factors (CF) experimentally determined on certified reference materials (CRM), European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF), <https://gmo-crl.jrc.ec.europa.eu/doc/CF-CRM-values.pdf> (last accessed on 30/01/2025)
- [19] W. Broothaerts, P. Corbisier P, H. Schimmel, S. Trapmann, S. Vincent, H. Emons, A single nucleotide polymorphism in the adh1 reference gene affects the quantification of genetically modified maize (*Zea mays* L.), *Journal of Agricultural and Food Chemistry* 56 (2008) 8825-883
- [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/e/132/User-support-Application-Notes> (last accessed on 30/01/2025)
- [21] 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
- [22] 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
- [23] 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 30/01/2025)
- [24] T.P.J. Linsinger, J. Pauwels, , A.M.H. Van der Veen, , H.G. Schimmel A. Lamberty, Homogeneity and stability of reference materials, *Accred. Qual. Assur.* 6 (2001) 20-25.
- [25] 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
- [26] T.P.J. Linsinger, J. Pauwels, A. Lamberty, H.G. Schimmel, A.M.H. Van der Veen, L. Siekmann, Estimating the uncertainty of stability for matrix CRMs, *Fres. J. Anal. Chem.* 370 (2001) 183-188
- [27] DIN 1333:1992-02, Presentation of numerical data, Zahlenangaben, Deutsches Institut für Normung e.V., Berlin, Germany
- [28] M. Querci, N Foti, A. Bogni, L. Kluga, H. Broll, G. van de Eede, Real-Time PCR Based Ready-to-Use MultiTarget Analytical System for GMO Detection, *Food Anal. Methods* (2009), DOI 10.1007/s12161-009-9093-0.
- [29] S.F. Rosa, F. Gatto, A. Angers-Loustau, M. Petrillo, J. Kreysa, M. Querci, Development and applicability of a ready-to-use PCR system for GMO screening. *Food Chemistry* (2016) 201: 110-119.
- [30] 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 30/01/2025)
- [31] ERM Application Note 6: Use of ERM certificates and materials, <https://crm.jrc.ec.europa.eu/e/132/Usersupport-Application-Notes> (last accessed on 30/01/2025)
- [32] ISO/IEC Guide 99:2007, International vocabulary of metrology – Basic and general concepts and associated terms (VIM), International Organization for Standardization, Geneva, Switzerland

List of abbreviations

<i>A</i>	Absorbance
<i>Adh1</i>	Alcohol dehydrogenase 1 enzyme encoded gene
ANOVA	Analysis of variance
<i>b</i>	Slope in the equation of linear regression $y = a + bx$
bp	Base pair of DNA
<i>c</i>	Mass concentration $c = m/V$ (mass/volume)
CTAB	Cethyl trimethyl ammonium bromide
CI	Confidence interval
Cq	Quantification cycle (also referred to as threshold cycle, Ct)
CRM	Certified reference material
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
ERM [®]	Trademark owned by the European Commission; used by the JRC for reference materials
EU	European Union
EURM [®]	Trademark owned by the European Commission; used by the JRC for reference materials
EURL GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed
g	The relative centrifugal force or g force
GE	Gel electrophoresis
GM	Genetically modified
GMO	Genetically modified organism
GUM	Guide to the Expression of Uncertainty in Measurement
H	Hour
<i>hmg</i>	High mobility group proteins encoded gene
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
<i>k</i>	Coverage factor
LFI	Lateral flow immunoassay
LOD	Limit of detection
LOQ	Limit of quantification
<i>M</i>	Molar mass
min	Minute

n	Number of replicate analysis per unit
N	Number of units analysed
n.a.	Not applicable
p	Number of technically valid datasets
PCR	Polymerase chain reaction
PSA	Particle size analysis
qPCR	Quantitative PCR
RM	Reference material
RMP	Reference material producer
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSD	Relative standard deviation
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®	<i>Thermus aquaticus</i> (Taq) DNA polymerase-based technology for fluorescent signal generation in real-time PCR
TBE	Tris borate EDTA buffer
TE	Buffer containing TRIS and EDTA
T	Temperature
t	Time
TRIS	Tris(hydroxymethyl)aminomethane
u	Standard uncertainty
U	Expanded uncertainty
u_{bb}^*	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability/intermediate precision; an additional index "rel" is added as appropriate
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_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
U_{meas}	Standard measurement uncertainty
U_{meas}	Expanded measurement uncertainty
U_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
UV	Ultraviolet
V	Volume
VIM	International Vocabulary of Metrology – Basic and General Concepts and Associated Terms
V-KFT	Volumetric Karl Fischer titration
w	week
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 50 % 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 used.

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 Na₂EDTA pH 8.0
- 2) CTAB buffer B
 - 1 % (w/v) CTAB
 - 0.1 M Tris-HCl, pH 8.0
 - 15 mM Na₂EDTA, 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 Na₂EDTA, pH 8.0
- 8) Proteinase K, 20 mg/mL
- 9) RNase A, 100 mg/mL
- 10) QIAGEN G2 buffer
- 11) QIAGEN Genomic-tip 20/G columns
- 12) QIAGEN QBT buffer
- 13) QIAGEN QC buffer
- 14) QIAGEN QF buffer
- 15) Isopropanol

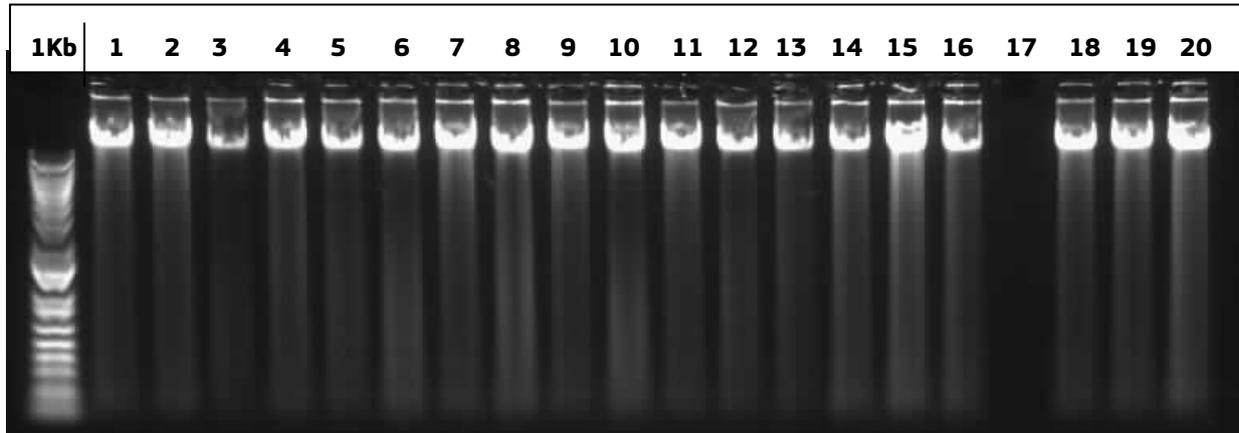
Protocol CTAB-based DNAextraction method

- a) Weigh 200 mg powder into a 2 mL microcentrifuge tube.
- b) Add 1.3 mL of CTAB buffer A and mix thoroughly by vortexing.
- c) Add 5 µL RNase A and 10 µL Proteinase K and mix by briefly vortexing.
- d) Incubate 45 min at 60 °C, mixing by shaking at 1400 rpm in a thermomixer
- e) Centrifuge 10 min at 16000 x g at RT.
- f) Transfer 750 µL supernatant to a 2 mL micro-centrifuge tube containing 1 mL of chloroform/octanol (volume fraction: 24:1).
- g) Mix thoroughly by inverting, incubate 5 min at RT.
- h) Centrifuge 10 min at 16000 x g at RT.
- i) Transfer the 600 µL upper phase to a new 2 mL micro-centrifuge tube containing 1400 µL of CTAB B.

- j) Mix thoroughly by inverting, incubate 20 min at RT.
- k) Centrifuge 15 min at 16000 x g at RT.
- l) Carefully discard the supernatant by pipetting and conserve the pellet.
- m) Add 200 μ L of 1.2 M NaCl.
- n) Incubate 5 min at 50 °C shaking at 1400 rpm in a thermomixer.
- o) Add 1.6 mL G2 buffer, add 2.5 μ L RNase A, add 20 μ L Proteinase K.
- p) Incubate 1 h at 50 °C, shaking at 650 rpm in a thermomixer.
- q) Centrifuge 5 min at 16000 x g at RT.
- r) Continue with the purification with QIAGEN genomic tip 20 columns.
- s) Store at RT overnight, at +4 °C (short term) or -20 °C (long term).

Annex 2. Results of the short-term stability measurements

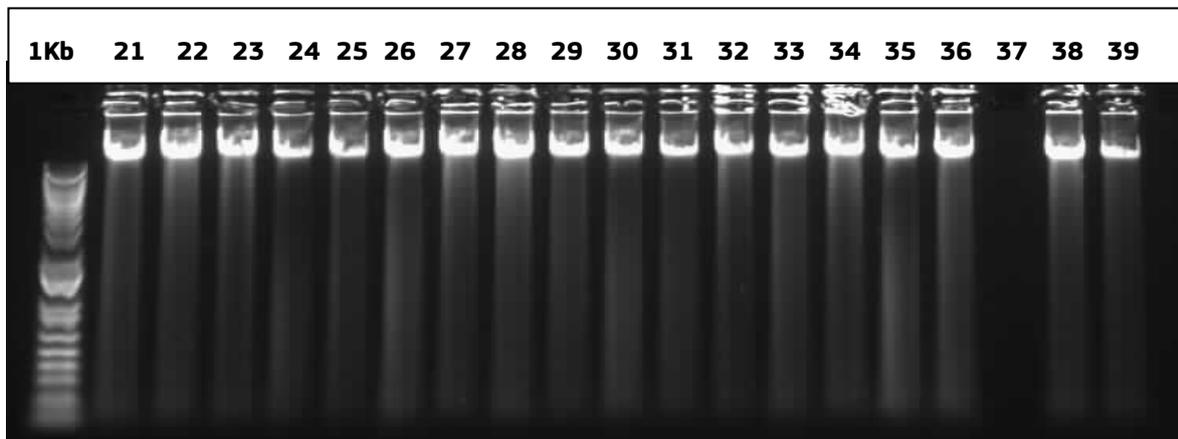
Figure 3. Gel electrophoresis of samples ERM-BF415g submitted to isochronous study at three temperatures (-70, +4, +60) °C (5 units/ 2 replicates) analysed in random order.



Day 1 DNA extraction

1. ERM-BF415g 0w/4°C	6. ERM-BF415g 4w/60°C	11. ERM-BF415g 0w/-70 °C	16. ERM-BF415a (neg control qPCR)
2. ERM-BF415g 0w/-70°C	7. ERM-BF415g 0w/-70°C	12. ERM-BF415g 2w/60°C	17. DNA blank CTAB (02/10/23)
3. ERM-BF415g 4w/60°C	8. ERM-BF415g 2w/60°C	13. ERM-BF415g 1w/60°C	18. ERM-BF415g calibrant 2023
4. ERM-BF415g 1w/60°C	9. ERM-BF415g 0w/60°C	14. ERM-BF415g 1w/60°C	19. ERM-BF415g calibrant 2023
5. BF415g 2w/60°C	10. ERM-BF415g 4w/60°C	15. ERM-BF415g 0w/4°C	20. BF415 calibrant 2004

Agarose electrophoresis 0.75 % in TBE buffer (Tris Borate, EDTA) with GelRed as reagent dye for DNA. Run 100 V / 60 min. 10 µL **1 Kb ladder plus** (Invitrogen cat 10448-085, Lot #0067388) with fragments from 0.1–15 Kb
5 µL DNA **Samples DNA extracts** – CTAB extraction method (02-03/10/2023). Buffer TE low (1 mM TRIS HCl; 0, 01 mM EDTA) prepared 20/03/2023.



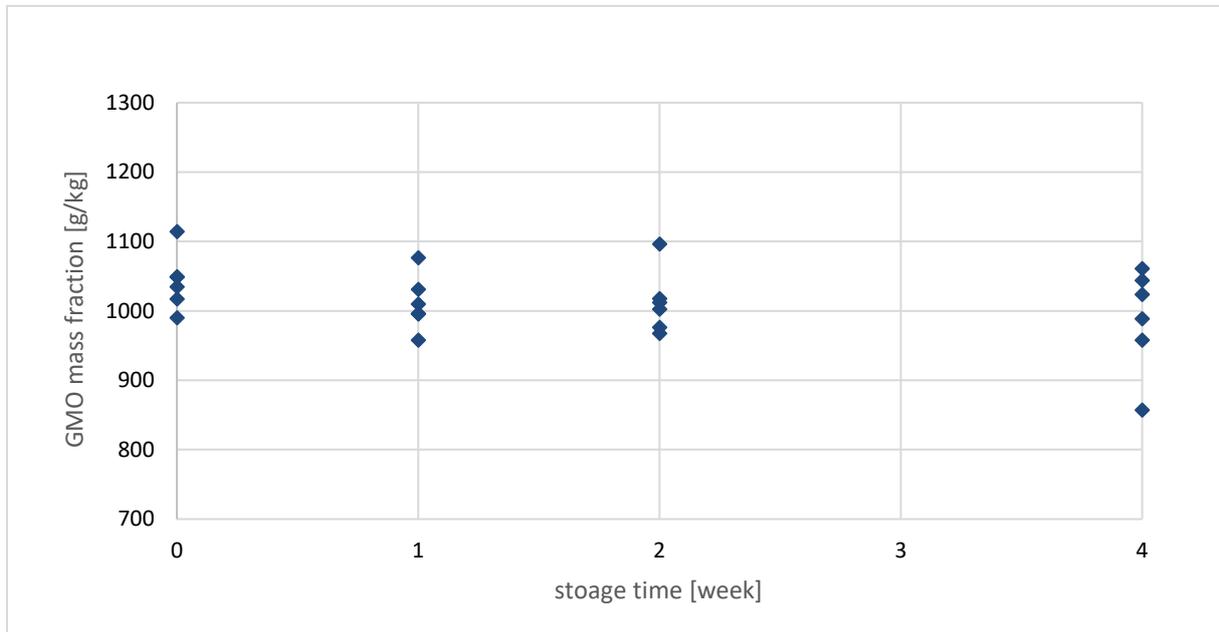
Day 2 DNA extraction

21. ERM-BF415g 1w/60°C	26. ERM-BF415g 2w/60°C	31. ERM-BF415g 2w/60°C	36. ERM-BF415a (neg control qPCR)
22. ERM-BF415g 0w/4°C	27. ERM-BF415g 0w/-70 °C	32. ERM-BF415g 0w/-70°C	37. B2 blank CTAB
23. ERM-BF415g 0w/-70°C	28. ERM-BF415g 0w/4w	33. ERM-BF415g 1w/60°C	38. ERM-BF415g (calibrant 2023)
24. ERM-BF415g 4w/60°C	29. ERM-BF415g 1w/60°C	34. ERM-BF415g 0w/4°C	39. BF415 (calibrant 2004)
25. ERM-BF415g 2w/60°C	30. ERM-BF415g 4w/60°C	35. ERM-BF415g 4w/60°C	

Source: JRC

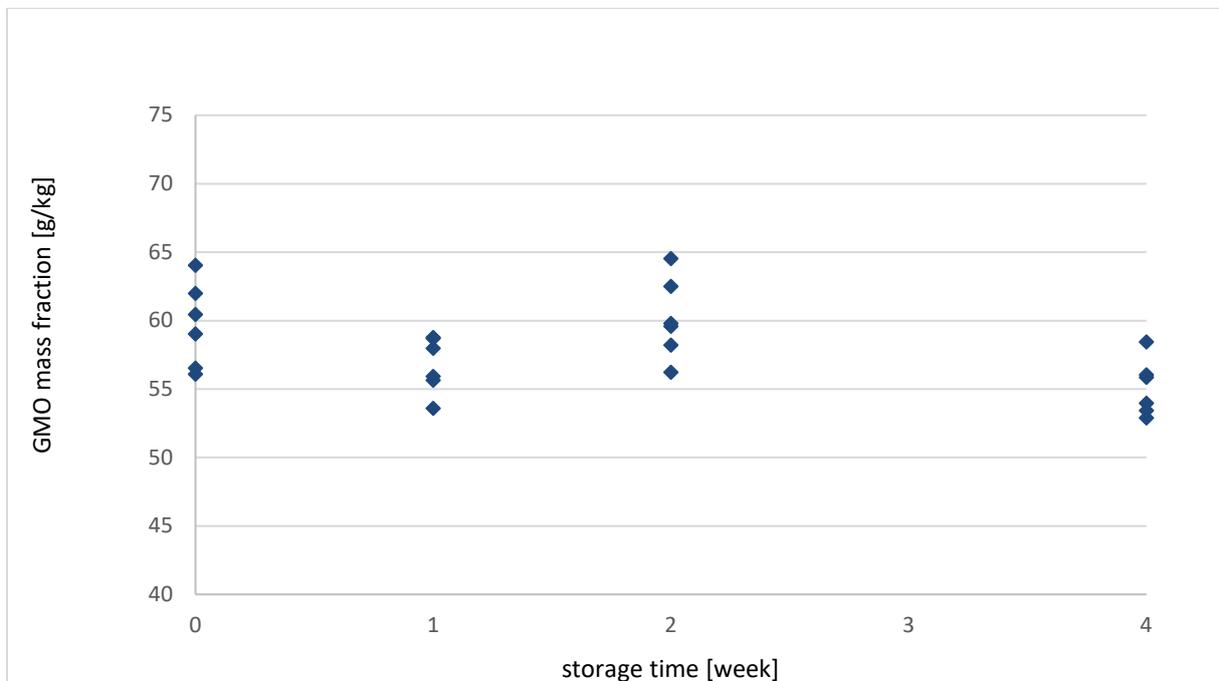
Annex 3. Results of the short-term stability (4 weeks / 60 °C) measurements of materials ERM-BF415g and ERM-BF415f

Figure 4A. ERM-BF415g – Transport stability assessed at 60 °C during 4 weeks. Measurement results GM mass fraction of three units (two replicates) per time point measured by qPCR. The graph represents GMO mass fraction versus storage time, given in weeks.



Source: JRC

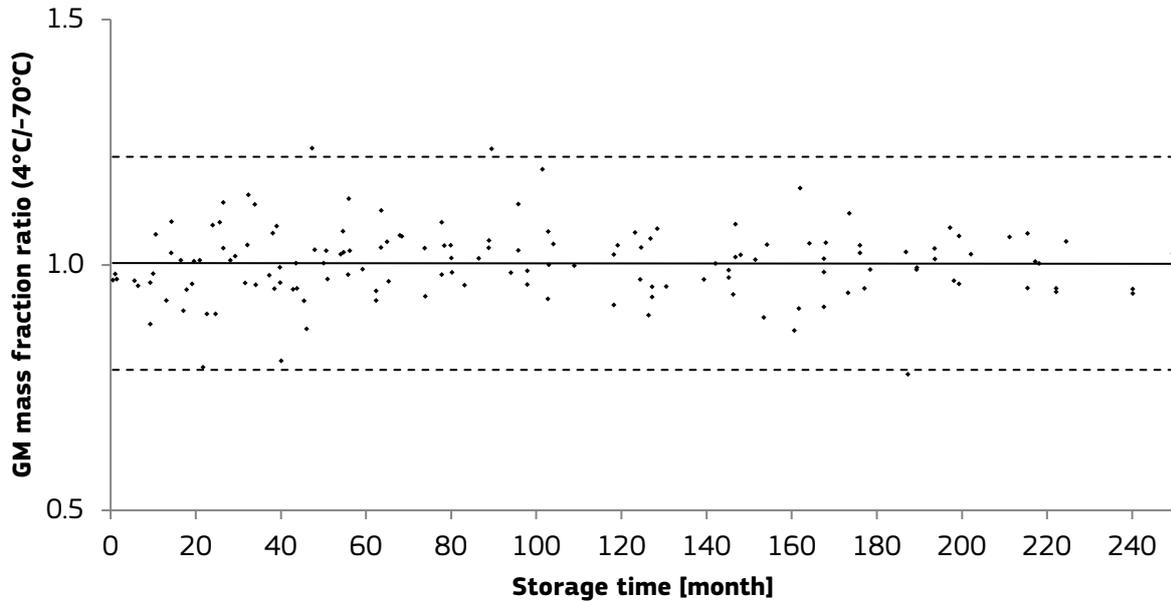
Figure 4B. ERM-BF415f – Transport stability assessed at 60 °C during 4 weeks. GM mass fraction measured by quantitative PCR measurements. The graph represent GMO mass fraction versus storage time.



Source: JRC

Annex 4. Results of the stability monitoring measurements

Figure 4. Storage stability of maize GMO CRMs assessed at 4 °C over 250 months. GM mass fraction ratio measured by qPCR ; results of 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, ERM-BF439, and ERM-BF447. The dash lines give the limits of 3s (standard deviation) of the measurement results. The straight line is the least-squares linear regression for all data points.



Source: JRC

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