



Institute for Reference  
Materials and Measurements



## **CERTIFICATION REPORT**

### **The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of NK603 Maize**

### **Certified Reference Materials ERM<sup>®</sup>-BF415**

**(ERM<sup>®</sup>-BF415-a/ERM<sup>®</sup>-BF415-b/  
ERM<sup>®</sup>-BF415-c/ ERM<sup>®</sup>-BF415-d/  
ERM<sup>®</sup>-BF415-e/ERM<sup>®</sup>-BF415-f)**



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**(ERM<sup>®</sup>-BF415a/ERM<sup>®</sup>-BF415b/ERM<sup>®</sup>-BF415c/  
ERM<sup>®</sup>-BF415d, ERM<sup>®</sup>-BF415e, ERM<sup>®</sup>-BF415f)**

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# **ADDENDUM**

## **Certification report ERM-BF415**

### **EUR 21270 EN**

#### **Additional information**

#### **ANNEX**

#### **Use of ERM-BF415 for measurement results based on copy number ratios**

For the preparation of the CRMs ERM-BF415, earlier certified as IRMM-415, Monsanto (St. Louis, MO, USA) supplied seeds of non-modified maize (seed variety 'RX670') and GM NK603 maize (seed line 'DKC 57-40') to the IRMM. According to Monsanto the GM NK603 maize seeds (unique identifier MON-ØØ6Ø3-6) are hybrid seeds, where the GM donor of the GM event is the female parent.

ERM-BF415 has been certified for its GM mass fraction and not for its GM copy number ratio. If users intend to use ERM-BF415 for GM measurement results expressed in copy number ratios, the estimation of the copy number ratio and the related uncertainty needs to be carried out according to the principles explained in ERM Application note 4 [2].

For the estimation of the copy number ratio the measurement unit of the certified value given in g/kg to the measurement unit '1' for ratios, expressed in percent, is required. Furthermore, the hybrid status of the seeds used for the production of the CRMs needs to be considered as well as the DNA extractability of the non GM and GM seed powder.

This estimation is linked to additional standard uncertainties, which need to be taken into consideration in the combined expanded uncertainty. These additional standard uncertainties refer to:

- (1) Measurement uncertainty of the quantification method
- (2) Possible variation of the maize genome size [3]
- (3) Possible effect caused by endoreduplication
- (4) Possible effect caused by the GM trait introduction of the maize event.

As the GM trait introduction via the female parent could not be verified by IRMM it is strongly recommended to take both possibilities of the GM trait introduction (introduction via the female or male parent) into consideration when calculating the combined expanded uncertainty. On the other hand it is reasonable to consider the maize seeds used for the production of the matrix to be hybrids into which the GM trait has been introduced by one parent.

The principles of the approach to estimate the copy number ratio value and its expanded combined uncertainty of a mass fraction certified maize CRM are outlined in ERM Application note 4 [2].

#### **References**

- [1] Trapmann S, Conneely P, Contreras M, Corbisier P, Gancberg D, Hannes E, Gioria S, Muñoz-Pineiro A, Van Nyen M, Schimmel H, Szilágyi S, Emons E (2005) The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of 1507 Maize - Certified Reference Materials ERM®-BF418, EC certification report EUR 21689 EN, ISBN 92-894-9748-3
- [2] Application note 4: European Reference Materials - Use of certified reference materials for the quantification of GMO in food and feed.  
[http://www.erm-crm.org/html/ERM\\_products/application\\_notes/application\\_note\\_4/index.htm](http://www.erm-crm.org/html/ERM_products/application_notes/application_note_4/index.htm)
- [3] Poggio L, Rosato M, Chiavarino AM, Naranjo CA 1998: Genome Size and Environmental Correlations in Maize (*Zea mays* ssp. *mays*, Poaceae), *Annals of Botany* 82, 107-115

## SUMMARY

This report describes the preparation and certification of dry-mixed maize powder CRMs with different mass fractions of genetically modified NK603 maize powder (Certified Reference Materials ERM-BF415a, ERM-BF415b, ERM-BF415c, ERM-BF415d, ERM-BF415e and ERM-BF415f). Reference Material ERM-BF415 was originally certified as IRMM-415. The CRMs were processed in 2003 and certified in 2004 by the European Commission, Directorate General Joint Research Centre, the Institute for Reference Materials and Measurements (IRMM) in Geel, Belgium. The CRMs are intended for the quality control and calibration of methods for the detection of genetically modified food. The NK603 concentration of ERM-BF415 was verified with the help of DNA-based detection methods. The CRMs are available in the form of glass bottles containing 1 g of maize powder packed under argon atmosphere. Seeds of non-modified maize (seed variety RX670) and NK603 maize (line DKC 57-40) both produced by Monsanto (St. Louis, MO, USA) were rinsed with demineralised water, additionally drained and dried at 30 °C in order to minimise dust contamination from other crops. After a two step grinding process, the materials were prepared by turbula-mixing and dry-mixing of non-modified maize powder and NK603 maize powder. ERM-BF415 was certified to contain the following NK603 concentrations: ERM-BF415a certified value  $< 0.4$  g GM / kg maize; ERM-BF415b certified value  $1.0 \pm 0.4$  g GM / kg maize; ERM-BF415c certified value  $4.9 \pm 0.5$  g GM / kg maize; ERM-BF415d certified value  $9.8 \pm 0.7$  g GM / kg maize; ERM-BF415e certified value  $19.6 \pm 0.9$  g GM / kg maize; ERM-BF415f certified value  $49.1 \pm 1.3$  g GM / kg maize. The minimum sample intake recommended is 100 mg.



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## GLOSSARY

<i>adh1</i>	<i>alcohol dehydrogenase</i> (endogenous maize) gene
avr.	average
CRM	certified reference material
CTAB	cetyltrimethylammonium bromide
Ct-value	concentration threshold value
DNA	deoxyribonucleic acid
CP4EPSPS	5-enolpyruvylshikimate-3-phosphate synthase derived from <i>Agrobacterium</i> <i>sp.</i> strain CP4
ERM <sup>®</sup>	trademark European Reference Material
FAM	6-FAM <sup>™</sup> fluorescent dye
GA21	GM maize event GA21
GM	genetically modified
GMO	genetically modified organism
IRMM	Institute for Reference Materials and Measurements
KFT	Karl Fischer Titration
LOD	limit of detection
LOQ	limit of quantification
m/m	mass per mass
MON 810	GM maize event MON 810
MON 863	GM maize event MON 863
NK603	GM maize event NK603
n	number of samples analysed
NAA	neutron activation analysis
PCR	polymerase chain reaction
PSA	particle size analysis
RSD	relative standard deviation
rt-PCR	real-time PCR
std dev	standard deviation
UV	ultra violet



## 1 Introduction

Legislation in the European Union demands the labelling of food products consisting of, or containing more than 0.9 % genetically modified organisms (GMOs), provided the GMO has been placed on the market in accordance with Community legislation [1]. This enforces the necessity on the one hand to develop and validate reliable quantitative detection methods and on the other hand to develop and produce certified reference materials (CRM) to calibrate and control the correct application of detection methods. Therefore, mixtures of genetically modified (GM) and non-GM powders have been prepared and certified as CRMs.

A set of CRMs of maize powder with different mass fractions (< 0.4, 1.0, 4.9, 9.8, 19.6, 49.1 g GM / kg maize) of dried powder of GM maize carrying the transformation event NK603 (line DKC 57-40) was processed and certified by IRMM. The six CRMs (ERM-BF415a, ERM-BF415b, ERM-BF415c, ERM-BF415d, ERM-BF415e and ERM-BF415f) are available from IRMM and its authorised distributors [2].

ERM-BF415 has been produced with the help of dry-mixing techniques in order to minimise DNA and protein degradation during the processing.

## 2 CRM Preparation

### 2.1 Characterisation of the starting materials

For the preparation of the CRMs, 140 kg of non-modified maize (hybrid seed line RX670) and 50 kg GM NK603 maize (hybrid seed line DKC 57-40) both of seed quality were supplied to IRMM by Monsanto (St. Louis, MO, USA).

The delivered non-GM and GM seed batches have been tested by Monsanto laboratories (St. Louis, MO, USA) for their purity by lateral flow immunoassay. Two hundred individual GM seeds randomly chosen from the GM seed batch tested positive for the presence of the CP4EPSPS protein. Additionally four pooled samples (75 seeds each) were tested for GA21, MON 863 and MON 810 by event specific PCR and all pools tested negative for these events. For the non-GMO seed material four pooled samples (75 seeds each) were tested for GA21, MON 863, MON 810 and NK603 by event specific PCR. The pools were negative for the events listed. With a 95 % confidence it was concluded that 98.5 % of the GM batch contain the NK603 transformation event and with 99 % confidence that the non-GM batch is free of GA21, MON 863, MON 810 and NK603.

Sub-batches of around 70 kg of non-modified maize and 10 kg of NK603 maize were used for the processing of ERM-BF415.

The purity and genetic composition of the sub-batches have been controlled at IRMM with the help of DNA extracted from leaves of seedlings. Seeds of each batch were randomly chosen and grown on moistened paper. 50 non-GM and 50 GM seeds were germinated and DNA was extracted from 110 mg pieces of the young leaves. The DNA was extracted using the DNeasy® Plant Mini kit (Qiagen, Hilden, DE), analysed on a 1 % agarose gel and quantified using the PicoGreen® dsDNA quantification kit (Molecular Probes Europe, Leiden, NL). The average DNA yield was  $4.0 \pm 1.7 \mu\text{g} / 110 \text{ mg GM tissue}$  and  $4.4 \pm 1.7 \mu\text{g} / 110 \text{ mg non-GM tissue}$ .

Real time-PCR (rt-PCR) detection was performed at IRMM following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA). Primer pairs specific for the event NK603 and the *adh* endogenous maize gene have been used together with a reporter-dye labelled with FAM. The threshold cycle values (Ct-value) obtained after the PCR on the 50 GM plants were compared to a calibration curve obtained with 100 % NK603 powder. The calculated average percentage of NK603 maize was for  $n = 50$ :  $111.5 \pm 23.7 \%$ . It was concluded, that all GM positive plants were therefore heterozygous (table 1).

**Table 1: Purity test and genetic composition of the GM and non-GM sub-batches used for the production of ERM-BF415**

Sub- batch	PCR method performed and primers used <sup>1</sup>	Number of plants tested	Number of positives	Number of negatives
Non-GM	event specific real-time PCR	50	0	50
GM	event specific real-time PCR	50	50	0

<sup>1</sup> primer sequences of the event specific NK603 method are confidential and have been provided by Monsanto

Additionally the purity of the ground non-GM base material has been tested at IRMM. No GM contamination in the non-GM material was found and it could be concluded that contamination is below the detection limit (LOD) of the rt-PCR method applied (table 2). The LOD was calculated as  $(3.3 \cdot \sigma) / b$ , with  $\sigma$  representing the standard deviation of a defined GM percentage and 'b' the slope of the calibration curve. The defined GM percentage was the lowest GM percentage for which the amplification efficiency was optimal. The efficiency of the amplification was determined based on the slope of the regression line between the GM percentage and the Ct-values, which should not be lower than the theoretical value of 3.322. The LOD has been established on NK603 powder and was 0.030 % (table 11).

**Table 2: Quantification of GM contamination in the non-GM base material by event specific rt-PCR, each determination in triplicate using 100 mg / extraction**

Non-GM raw material	Number of measurements  n	Concentration of GM contamination <sup>2</sup>	
		[copy number %]	std dev
Event specific rt-PCR <sup>1</sup>	5	< 0.030 %	-

<sup>1</sup> Primer sequences of the event specific NK603 method are confidential and have been provided by Monsanto

<sup>2</sup> Percentage is given as an indicative value since it is lower than the LOD (see table 11)

In order to verify that the extractable DNA content of GM and non-GM starting material is the same, the DNA was extracted from the twice ground powders (as described in chapter 2.2) using a CTAB method [3]. The DNA was afterwards quantified with the help of PicoGreen (Molecular Probe, Leiden, NL) in a spectrofluorometer (Fluostar Galaxy from BMG Labtechnologies GmbH, Offenburg, DE) and by UV with a Biophotometer (Eppendorf, Hamburg, DE). No significant differences in DNA extractability in the base material have been observed when applying the CTAB method (table 3). The ratio between the extractable DNA content of the two materials was calculated with the following formula:

Extractable DNA in 100 mg NK603 maize powder  
 Extractable DNA in 100 mg non - GM maize powder

Different extraction efficiencies on the GM and non-GM powder would influence the GM concentration measured and one should only use extraction methods validated for that purpose.

**Table 3: Ratio of extractable DNA of GM and non-GM powdery base material.**

<b>Extraction method</b>	<b>n</b>	<b>Spectrofluorimeter (PicoGreen) [ratio, std dev]</b>	<b>Biophotometer (UV) [ratio, std dev]</b>
CTAB [4]	10	0.90 ± 0.42	1.08 ± 0.40

## 2.2 Processing of powdery base materials

During the processing of ERM-BF415 the GM and non-GM powdery base materials were treated separately. Cross contamination and contamination with foreign DNA were avoided with the help of glove box systems, clean cells, disposable lab clothing and treatment of all contact surfaces prior to exposure to the base materials with a DNA destroying solution.

The kernels used for processing were rinsed in demineralised water and after draining dried under vacuum at 30 °C for around 20 hours. This treatment led to a water loss of between 1.5 and 2.5 mass %. The dried starting materials were then ground using a high impact mill with a triangular ribbed open grinding track under Argon flushing. An additional vacuum drying at 30 °C has been carried out to reduce the water content of the once ground powder further. For the second grinding step a sieve insert has been used with 0.5 mm mesh width. Slow feeding of the mill ensured that the whole powder passed the sieve and that no selective grinding occurred. Caution was taken to avoid increase of the grinding temperatures above 40 °C.

The ground powder was mixed with the help of a turbula mixer for 30 minutes to improve the distribution of the different parts of the maize kernels separated by the milling process. Particle size analysis proved that both base materials had similar particle size distributions. Prior to dry-mixing both powders had a water content around 2.5 %.

### **2.3 Quantitative preparation of GM / non-GM mixtures**

Mixtures containing nominal 0, 0.1, 0.5, 1, 2 and 5 % (m/m) NK603 maize powder and non-genetically modified maize powder were prepared quantitatively using a dry-mixing method. Water contents of the ground GM and non-GM powders, used as base materials, were determined in duplicate by volumetric Karl Fischer titration (KFT, Metrohm, Berchem, BE) in order to correct for the water content of the material. A 10 % GM mix was produced first using 100 % GM and non-GM base material. All lower concentrations were achieved by further dilution with non-GM maize powder. Powders were weighed using a calibrated balance. The calculated mass fractions were in a first step manually pre-mixed in container and afterwards turbula mixed. The whole material was then transferred into a dry-mixing device and mixed for 2 min.

### **2.4 Bottling**

The dry-mixed products were bottled in thoroughly cleaned 10-mL brown glass vials using an automatic sampling device. The first 30 filled bottles of each batch were discarded as an additional measure against carry over contamination. Rubber stoppers were automatically placed on the bottle opening. Before final closure of the vials the air was evacuated in a freeze-drier and replaced with argon. With the help of the hydraulically moveable device of the freeze drier, the vials were closed. All vials were sealed with aluminium caps to prevent opening of rubber stoppers during storage and transport. Colour coded caps have been used to easier distinguish between the different GM concentrations: nominal 0 % (certified value < 0.4 g GM / kg maize) - silver, nominal 0.1 % (certified value 1.0 g GM / kg maize) - yellow, nominal 0.5 % (certified value 4.9 g GM / kg maize) - blue, nominal 1 % (certified value 9.8 g GM / kg maize) - red, nominal 2 % (certified value 19.6 g GM / kg maize) - green and nominal 5 % (certified value 49.1 g GM / kg maize) - pink.

### **2.5 Processing control**

The water content of the dried powders was determined by volumetric KFT and amounted typically to values in the range of 1.2 to 1.9 g/100 g (table 4). Particle size measurements of the various powders were carried out using a particle size analyser (PSA, Sympatec, Clausthal-Zellerfeld, DE). The powders had a maximum particle size below 750 µm an average particle size around 110 µm (table 5).

Additionally a sieving test following ISO 3310-1 using sieves with meshes of 90, 125, 180, 250, 355, 500 and 710 µm has been carried out. The results confirmed that the maximum particle size of the final product is < 710 µm. The average particle size analysed with the sieving test is slightly higher than measured before and was between 125 and 180 µm (table 6).

**Table 4: Water content of ERM-BF415**

CRM	Certified mass fraction NK603 [g /kg]	Water content [g/100 g]		
		n	Mean	std dev
ERM-BF415a	< 0.4	5	1.60	0.15
ERM-BF415b	1.0 ± 0.4	5	1.36	0.16
ERM-BF415c	4.9 ± 0.5	5	1.17	0.16
ERM-BF415d	9.8 ± 0.7	5	1.89	0.33
ERM-BF415e	19.6 ± 0.9	5	1.31	0.11
ERM-BF415f	49.1 ± 1.3	5	1.8	0.11

**Table 5: Particle size distribution of ERM-BF415**

CRM	Certified mass fraction NK603 [g /kg]	Average particle size (n = 5)		Maximum particle size* (n = 5)	
		avr. [µm]	std dev	avr. [µm]	std dev
ERM-BF415a	< 0.4	111.2	3.8	≤ 595	44.7
ERM-BF415b	1.0 ± 0.4	108.6	6.6	≤ 603	120.5
ERM-BF415c	4.9 ± 0.5	111.6	8.1	≤ 483	43.8
ERM-BF415d	9.8 ± 0.7	114.9	2.9	≤ 735	0
ERM-BF415e	19.6 ± 0.9	110.7	12.0	≤ 599	91.0
ERM-BF415f	49.1 ± 1.3	109.5	6.5	≤ 563	115.4

\* measuring categories were 435-515, 515-615 and 615-735 µm

**Table 6: Particle size distribution of ERM-BF415, determined by sieving test (n = 1)**

CRM	Sample intake	Particle size distribution [accumulated mass %]						
		Fraction < 90 µm	Fraction < 125 µm	Fraction < 180 µm	Fraction < 250 µm	Fraction < 355 µm	Fraction < 500 µm	Fraction < 710 µm
ERM-BF415a	10 g	27.1	42.1	56.2	73.0	92.8	99.7	100
ERM-BF415b	10 g	29.0	43.1	57.2	73.4	91.8	99.7	100
ERM-BF415c	10 g	27.6	40.7	54.7	71.9	91.7	99.8	100
ERM-BF415d	10 g	26.5	40.3	55.0	73.4	91.9	99.8	100
ERM-BF415e	10 g	24.6	39.7	54.0	72.0	91.5	99.7	100
ERM-BF415f	10 g	24.2	40.4	55.5	71.8	91.7	99.7	100

The contribution of the particle size to the uncertainty of the certified value for the maize powder certified reference material has been estimated for the DNA based methods with the help of a programme and DNA extractability data obtained for the various particle size fractions [4]. The estimates for the influence of the particle size distribution on the uncertainty of the certified value with a given sample intake of 100 mg and an average particle size of 140  $\mu\text{m}$  can be found in table 7.

**Table 7: Uncertainty contribution of the particle size calculated for a sample intake of 100 mg (according to [4], average particle size 140  $\mu\text{m}$ )**

<b>GM content of the CRM [g/kg]</b>	<b>CRM std dev [g/kg] ERM-BF412 [5]</b>	<b>CRM std dev [g/kg] ERM-BF411 [6]</b>
1.0	0.29	0.35
5.0	0.71	0.69
10.0	0.91	1.07
20.0	1.26	1.51
50.0	1.81	2.63

### 3 Homogeneity

#### 3.1 Homogeneity study for dry-mixed maize powder

Prior to the production a homogeneity study for dry-mixed maize powders has been carried out by mixing Au-spiked maize powder with non-spiked, non-GM maize powder. All powders used in the homogeneity study have been processed in the same way as described for the base materials in chapter 2.2. A 10 % dilution was produced first and afterwards diluted two times further to reach concentrations of 1 and 0.1 % Au spiked in non-spiked maize. During the analysis the Au concentration of the three mixtures was determined with the help of neutron activation analysis (NAA). NAA proved the homogeneity of the dry-mixed maize powder at a sample intake level of 50 mg (table 8) and the adequacy of the dry-mixing technology for the preparation of the non-GM / GM maize mixtures.

**Table 8: Homogeneity study on dry-mixed Au-spiked maize powder with non-spiked maize powder, results of Au determination by neutron activation analysis (NAA) with a sample intake of 50 mg (n = 6)**

Material	Mixture parts [%]		Results NAA	
	Au-spiked	Non-spiked	Au content [µg /g]	RSD [%]
Au-spiked maize	100	0	1300	3.5
Non-spiked maize	0	100	0.005	10.0
10 % mixture	10	90	132	5.5
1 % mixture	1	99	12.5	4.8
0.1 % mixture	0.1	99.9	1.24	14.6

#### 3.2 Minimum sample intake for analysis

For the recommended sample intake of 100 mg per analysis, one may estimate on the basis of the particle size distribution (average particle size 111 µm) and the density of maize (0.94 g/mL) that the number of particles in a 100 mg sample is larger than  $10^5$ . Consequently 100 mg of ERM-BF415b (0.1 % NK603 maize) contains at least 100 GM particles. On this basis uncertainties due to sample inhomogeneity were estimated (chapter 5). Referring to the particle size distribution it is advised to use sample intakes not smaller than 100 mg.



## **4 Stability**

Earlier productions of wet-mixed maize GMO CRMs proved to have a long-term stability of at least 2.5 - 4 years provided the dried powder was stored at +4 °C in the dark and under argon. It can be assumed that the stability of dry-mixed powders is even higher. An intensive post-certification monitoring is carried out in half-year intervals in order to monitor the stability of ERM-BF415.

## 5 Certified mass fractions and uncertainty budgets

The materials ERM-BF415a, ERM-BF415b, ERM-BF415c, ERM-BF415d, ERM-BF415e and ERM-BF415f form a set of 6 reference materials certified for the mass fraction of NK603 maize powder. The certified mass fractions are based on quantitative dry-mixing of non-modified maize powder with NK603 maize powder. Taking into account the uncertainties of the weighing and of the water contents of the starting materials, uncertainties for the certified mass fractions at 100 mg level were estimated (table 9).

It must, however, be emphasised that due to the relatively large uncertainty inherent to quantification of the total DNA content, the DNA / dry powder mass fraction of different lots of maize kernels cannot be determined with the highest precision. Therefore, the ratio GM-DNA / non-GM-DNA in the reference materials may significantly deviate from the certified powder mass ratio values. Precise production techniques in combination with sound purity controls of the GM and non-GM raw materials allow improving the GM quantification.

The certified value is based on the mass fraction of dried non-genetically modified powder and dried genetically modified powder mixed and corrected for the water content. The combined uncertainty of the certified value comprises of the uncertainty introduced due the mass determination procedure, the humidity determination, the inhomogeneity at 100 mg level, the purity of non-GM and GM base material.

**Table 9: Uncertainty estimation (in g GM dry powder per kg maize dry powder)**

CRM, certified content [g /kg]		<u>Standard Uncertainty</u>					<u>Combined uncertainty</u>	<u>Expanded uncertainty</u>
		(u <sub>1</sub> ) <sup>1</sup>	(u <sub>2</sub> ) <sup>2</sup>	(u <sub>3</sub> ) <sup>3</sup>	(u <sub>4</sub> ) <sup>4</sup>	(u <sub>5</sub> ) <sup>5</sup>	(u <sub>c</sub> )	(U = 2 * u <sub>c</sub> )
ERM-BF415a	< 0.4	-	-	-	-	-	-	-
ERM-BF415b	1.0	0.0029	0.0014	0.0814	0.1732	0.0040	0.1914	0.3829
ERM-BF415c	4.9	0.0129	0.0068	0.1817	0.1732	0.0200	0.2522	0.5045
ERM-BF415d	9.8	0.0238	0.0111	0.2575	0.1732	0.0402	0.3140	0.6280
ERM-BF415e	19.6	0.0413	0.0222	0.3635	0.1732	0.0801	0.4132	0.8265
ERM-BF415f	49.1	0.0987	0.0555	0.5748	0.1732	0.2004	0.6429	1.2858

<sup>1</sup> mass determination uncertainty introduced, mainly based on the uncertainty of the balance

<sup>2</sup> water content average std dev 0.08 %, for ERM-BF415b and ERM-BF415c three dilution steps taken into consideration, for ERM-BF415d, ERM-BF415e and ERM-BF415f two dilution steps taken into consideration.

<sup>3</sup> inhomogeneity at 100 mg level with an average particle size of 111 µm and a density of 0.94 g/mL

<sup>4</sup> purity of non-GM base material

<sup>5</sup> purity of GM base material

## 6 Verification of NK603 maize mixtures

All six materials were analysed for consistency and not with the aim to quantify the gravimetrically prepared materials of verified purity.

For the verification study an event specific rt-PCR method has been used. The amplified PCR products are measured cycle-by-cycle with target specific reporter and quencher dyes, which lead to an increased fluorescence. The number of cycles (Ct-value) which are required to pass a fluorescence threshold correlates with the amount of target DNA in the starting sample. Results obtained can be found in table 10. The detection and quantification limits of the event specific NK603 quantification method have been established by dilution of DNA extracted from the 100 % powder in nuclease free water (table 11).

**Table 10: Quantification of NK603 DNA with event specific real-time PCR (confidential method provided by Monsanto), DNA extracted using the CTAB method, determination in triplicate using 100 mg / extraction.**

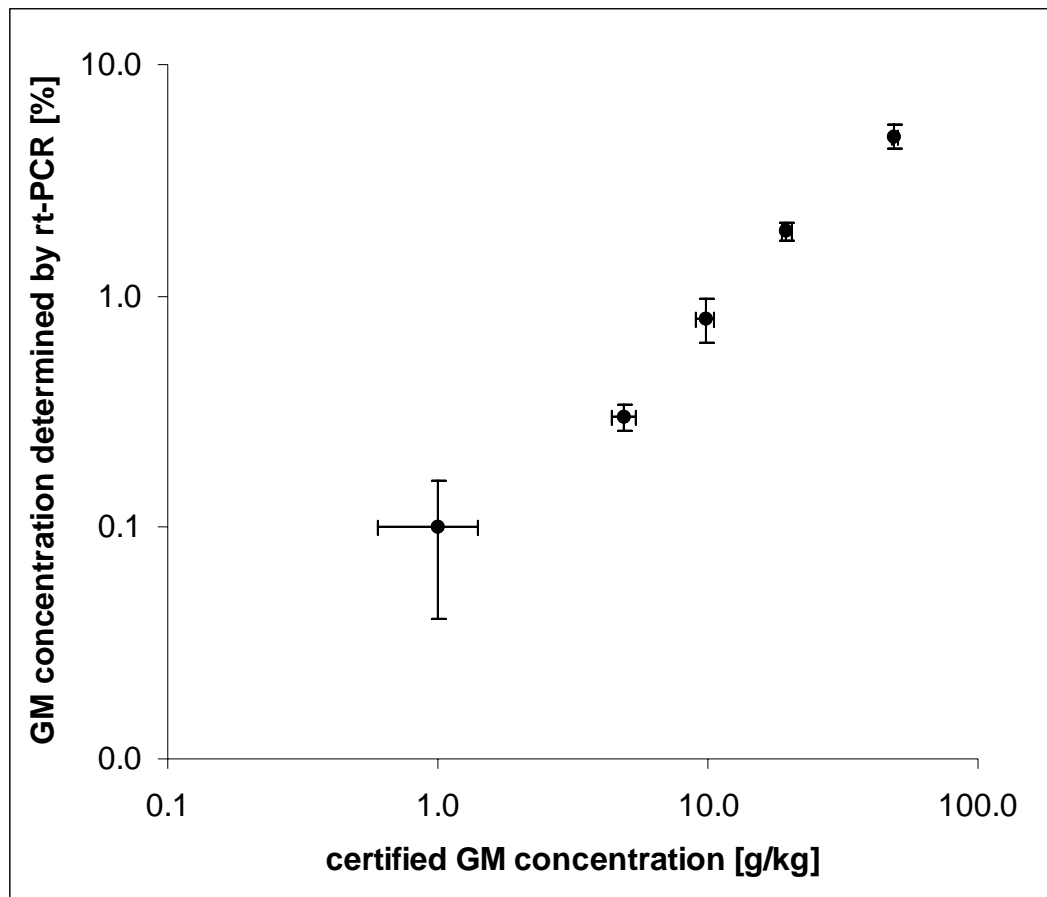
CRM	Certified GM Concentration [g /kg]	Number of measurements  n	Event specific real-time PCR	
			[copy number %]	std dev
ERM-BF415a	< 0.4	5	< 0.030 <sup>1</sup>	-
ERM-BF415b	1.0 ± 0.4	5	0.10	0.06
ERM-BF415c	4.9 ± 0.5	5	0.3	0.04
ERM-BF415d	9.8 ± 0.7	5	0.8	0.17
ERM-BF415e	19.6 ± 0.9	5	1.9	0.17
ERM-BF415f	49.1 ± 1.3	5	4.9	0.57

<sup>1</sup> value below LOD (see table 11)

**Table 11: Limit of detection (LOD) and limit of quantification (LOQ) of the real-time PCR methods used for the verification, established by dilution of DNA extracted from 100 % powder in non GMO DNA extracted from verified non-GM plants**

Real-time PCR method	LOD [copy number %]	LOQ [copy number %]
NK603 event specific	0.030	0.095

Results above the LOD obtained with the event specific PCR screening method (table 10) are compared to the certified values in figure 1. Quantification of the GM content of six mixtures of NK603 powder by real-time PCR proved the consistency of CRM ERM-BF415. However, one has to be careful to draw quantitative conclusions from measurements of unknown samples as DNA and/or protein based GM quantification may depend on maize varieties.



**Figure 1: Quantification of NK603 GM CRMs by event-specific real-time PCR**

## **7 References and acknowledgements**

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The Certification of Reference Materials of Dry Mixed Maize Powder with different Mass Fractions of NK603 Maize

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**Abstract**

This report describes the preparation and certification of dry-mixed maize powder CRMs with different mass fractions of genetically modified NK603 maize powder (Certified Reference Materials ERM<sup>®</sup>-BF415-0, ERM<sup>®</sup>-BF415-1, ERM<sup>®</sup>-BF415-2, ERM<sup>®</sup>-BF415-3, ERM<sup>®</sup>-BF415-4 and ERM<sup>®</sup>-BF415-5). European Reference Material ERM-BF415 was originally certified as IRMM 415. The CRMs were processed in 2003 and certified in 2004 by the European Commission, Directorate General Joint Research Centre, the Institute for Reference Materials and Measurements (IRMM) in Geel, Belgium. The CRMs are intended for the quality control and calibration of methods for the detection of genetically modified food. The NK603 concentration of IRMM-415 was verified with the help of DNA-based detection methods. The CRMs are available in the form of glass bottles containing 1 g of maize powder packed under argon atmosphere. Seeds of non-modified maize (seed variety RX670) and NK603 maize (line DKC 57-40) both produced by Monsanto (St. Louis, MO, USA) were rinsed with demineralised water, additionally drained and dried at 30 °C in order to minimise dust contamination from other crops. After a two step grinding process, the materials were prepared by turbula-mixing and dry-mixing of non-modified maize powder and NK603 maize powder. IRMM-415 was certified to contain the following NK603 concentrations: ERM<sup>®</sup>-BF415-0 certified value < 0.4 g GM / kg maize; ERM<sup>®</sup>-BF415-1 certified value  $1.0 \pm 0.4$  g GM / kg maize; ERM<sup>®</sup>-BF415-2 certified value  $4.9 \pm 0.5$  g GM / kg maize; ERM<sup>®</sup>-BF415-3 certified value  $9.8 \pm 0.7$  g GM / kg maize; ERM<sup>®</sup>-BF415-4 certified value  $19.6 \pm 0.9$  g GM / kg maize; ERM<sup>®</sup>-BF415-5 certified value  $49.1 \pm 1.3$  g GM / kg maize. The minimum sample intake recommended is 100 mg.

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