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CERTIFICATION REPORT

Certification of Plasmid DNA containing NK603 Maize DNA Fragments

Certified Reference Material ERM®-AD415

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

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

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CERTIFICATION REPORT

Certification of Plasmid DNA containing NK603 Maize DNA Fragments

Certified Reference Material ERM®-AD415

B. Jeynov, E. de Andrade, W. Broothaerts, P. Corbisier, S. Mazoua, A. Merveillie, S. Trapmann, H. Emons

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

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ABSTRACT

This report describes the preparation, characterisation, stability and suitability studies of the certified reference material (CRM) ERM®-AD415 which contains a plasmid (pIRMM-0086) carrying a defined deoxyribonucleic acid (DNA) fragment specific for a genetic modification present in *Zea mays* L. line NK603 as well as defined DNA fragments specific for the *Zea mays* taxon, of which one has been certified. The maize NK603 event is registered in the EU register of GM Food and Feed according to Regulation (EC) No 65/2004 and has received the unique identifier maize MON-ØØ6Ø3-6.

The CRM was processed in 2009 and certified in 2010 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE. The CRM is available in plastic tubes containing 500 µL of the plasmid DNA in 1 mmol/L Tris, 0.01 mmol/L EDTA pH 8.0 buffer ($T_1E_{0.01}$) and 1 ng/µl ColE1 plasmid used as background DNA. Each tube contains approximately 10^9 copies of the ERM-AD415 plasmid which correspond approximately to 4.1 ng of DNA.

The plasmid contains a 108 bp fragment of the region that spans the 3' insertion locus of the event NK603 and four fragments specific for the maize taxon: a 79 bp fragment of the maize endogenous *high mobility group* gene (*hmg*), a 151 bp fragment of the maize endogenous *starch synthase IIb* gene (*zssIIb*), a 135 bp fragment which targets to the 5'-untranslated region of the maize endogenous *alcohol dehydrogenase 1* gene (Zm*adh1* target) and a 70 bp fragment which targets to the junction between the second intron and the third exon of the maize endogenous *alcohol dehydrogenase 1* gene (*adh1* target).

The certified values are the numbers of cloned DNA fragments for the NK603 and *hmg* PCR targets per plasmid.

The minimum sample intake is 50 µL to perform the dilution series. A minimum sample intake of 5 µL is used per real-time PCR assay.

PLASMID DNA CONTAINING NK603 MAIZE DNA FRAGMENTS

	Number of DNA fragments per plasmid	
	Certified value [2]	Uncertainty [3]
Fragment of 3' insertion-specific DNA / pIRMM-0086 [1]	1	negligible
Fragment of *hmg* DNA / pIRMM-0086 [1]	1	negligible

[1] The sequence identity was confirmed by dye terminator cycle sequencing of the 3' insert-plant junction and *hmg* fragments present in *Zea mays* L. line NK603. The estimated error probability of the sequence identification of each fragment is lower than 0.0002 %.

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

[3] The uncertainty of the certified value refers to a standard uncertainty. It is estimated by a type B evaluation based on information provided in Section 8.1 of the present certification report.

The intended use of this CRM is for calibration of the event-specific method for the quantification of the NK603 event validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), "Event-specific method for the quantitation of maize line NK603 using real-time PCR", available on the homepage of the European Union Reference Laboratory for Genetically Modified Food and Feed (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) published on 10/01/2005. With respect to the findings reported by Broothaerts et al. (2008) J Agric Food Chem 56(19): 8825-31, the method for the quantification of the maize NK603 event described here targets a 79 bp fragment from the endogenous maize gene *hmg* instead of the 70 bp fragment of the maize endogenous gene *adh1* used in the method validated by the EURL-GMFF.

GLOSSARY

6-FAM	6-carboxylfluorescein dye
α	error probability
A	absorbance
Ap(R)	ampicillin resistance
adh1[1]	*alcohol dehydrogenase 1* gene
ANOVA	analysis of variance
BLASTN	Basic Local Alignment Search Tool for Nucleotides
bp	base pair
cp	copy number
CRM	Certified Reference Material
Ct	number of PCR cycles to pass a set threshold
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
EPSPS[1]	5-enolpyruvylshikimate-3-phosphate synthase
ERM	European Reference Material
EURL-GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed, formerly named Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF)
gDNA	genomic DNA
gDNA$_\ell$	genomic DNA extracted from NK603 maize leaves
gDNA$_s$	genomic DNA extracted from the NK603 seed powder CRM ERM-BF415e
GM	genetically modified
GMO	genetically modified organism
hmg[1]	*high mobility group* gene
IRMM	Institute for Reference Materials and Measurements
LB	Luria-Bertani
M	molar mass
n	number of replicates
N	number of tubes analysed
N_A	Avogadro constant
N_d	number of data sets
N_{ds}	number of data sub-sets
NCBI	National Centre for Biotechnology Information
NIH	National Institutes of Health
OD	optical density
ORF	open reading frame
oriV	plasmid origin of replication
p	probability
PCR	polymerase chain reaction
pDNA	plasmid DNA
q	quality value assigned to a base in DNA sequencing
R^2	coefficient of determination
rel	relative (subscript indicating the relative nature of the value)
RNA	ribonucleic acid
RSD	relative standard deviation
S	size of a DNA fragment expressed in number of bp
s	standard deviation
SI	International System of Units
TAMRA	6-carboxytetramethylrhodamine
$T_1E_{0.01}$	1 mmol/L Tris, 0.01 mmol/L EDTA buffer, pH 8.0
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
TBE	Tris-borate-EDTA buffer
U_{CRM}	expanded uncertainty of the certified value
u	standard uncertainty
u_{lts}	standard uncertainty contribution of the long-term stability of the material
UNG	uracil N-glycosylase
UV	ultra violet
$\overline{x}$	mean
z	estimated error probability per base in DNA sequencing
zssIIb[1]	*starch synthase IIb* gene

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

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1 INTRODUCTION

Legislation in the European Union demands the 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 measurement methods, and on the other hand to develop and produce reference materials to calibrate and control the correct application of detection methods. This threshold (0.9 %) is commonly understood as a mass fraction. In October 2004 the European Commission recommended expressing the content of GM food and feed as the percentage of GM DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes (EC) No 787/2004 [2].

Calibrants are required to determine the ratio of the number of copies of transgenic and taxon-specific genes. Here we describe the development of a new pure DNA calibrant containing a DNA sequence specific for the NK603 event and four different DNA fragments each containing a different *Zea mays* taxon-specific sequence. As for any plasmid harbouring a single copy of a number of unique sequences, the ratio between the sequences is equal to 1.

With respect to the findings of Broothaerts et al. [3] the reference gene target *adh1* as described in the event-specific NK603 quantification method [4] is not suitable for the quantification of the maize NK603 event. Therefore the plasmid calibrant was designed to contain one copy of each of the maize taxon-specific targets *adh1*, *hmg*, Zm*adh1* and *zssIIb*. To offer a reliable reference system ERM-AD415 is certified for the number of NK603 event and *hmg* targets present per pIRMM-0086 plasmid.

The identity of the cloned sequences has been confirmed by the dye terminator cycle sequencing method. The nucleotide sequence of the 3' insert-plant junction (maize NK603 event) cloned into the plasmid calibrant was identical to the nucleotide sequence provided by Monsanto Company (St. Louis, MO, US).

Likewise, the nucleotide sequences of the *zssIIb*, *adh1*, Zm*adh1* and *hmg* fragments cloned into the plasmid calibrant were identical to the nucleotide sequences referred to as AF019297, AY691949 (for both *adh1*-specific fragments) and AJ131373, respectively, in the National Institutes of Health (NIH, Bethesda, MD, US) genetic sequence database (GenBank®) and correspond to the *adh1*, *hmg1* and *zssIIb* genes present in *Z. mays* species [5-7].

The maize NK603 event is registered in the Organisation for Economic Co-operation and Development Unique Identification Registry (available through the Biosafety Clearing-House database for living modified organisms [8]) as specified in Regulation (EC) No 65/2004 of 14/01/2004, establishing a system for the development and assignment of unique identifiers for genetically modified organisms [9]. The maize NK603 event received the unique identifier maize MON-ØØ6Ø3-6.

2 DESIGN OF THE PROJECT AND CERTIFICATION PROCEDURE

The major objective of the project was the production of a plasmid DNA reference material for the calibration of real-time PCR quantification methods.

The material is intended to be used as calibrant for the quantification of the NK603 event expressed as the ratio between the number of copies of the NK603 event and the number of copies of a taxon-specific *Z. mays hmg* gene. The plasmid calibrant is certified for containing one single DNA fragment of those two genes per plasmid. The cloned DNA fragments are identical to the sequences published in the NIH genetic sequence database (GenBank).

The relative number of copies of the NK603 event per haploid genome of maize that are present in a DNA extract can be calculated by application of the EURL-GMFF validated quantification method (modified with respect to the reference gene target, as described in this report) using the ERM-AD415 calibrant.

3 PARTICIPANTS

Processing, stability study and value assignment

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

Characterisation

Eurofins Medigenomix GmbH, Martinsried, DE
European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
QIAGEN Sequencing Services, Hilden, DE

Suitability study

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

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

4 DESCRIPTION AND PROCESSING OF THE MATERIAL

4.1 DESCRIPTION OF THE STARTING MATERIAL

4.1.1 Cloning of the transgenic target

A 167 bp fragment containing part of the maize chromosome and part of the NK603 transgenic sequence (3' insert-plant junction sequence) was amplified by the Platinum® Pfx DNA polymerase (Invitrogen, Carlsbad, CA, US) using as template genomic DNA extracted from seed powder of the NK603 event maize variety DKC 57-40. The amplicon was ligated in the plasmid vector pCR2.1 (Invitrogen, Carlsbad, CA, US) and *Escherichia coli* Top10 cells were transformed with the ligation products. Transformed cells were selected on LB plates supplemented with 50 µg/mL kanamycin and 100 µg/mL ampicillin and identified by PCR screening using primers flanking the border regions of the cloned fragment. The correct insertion was confirmed by DNA sequence analysis on plasmid DNA isolated from single colonies. One single clone bearing the plasmid named pIRMM-0082 was selected.

4.1.2 Cloning of the endogenous targets

A 351 bp fragment of the endogenous *high mobility group* (*hmg*) gene from maize was amplified by the high fidelity polymerase Platinum Pfx polymerase using genomic DNA extracted from MON 810 *Z. mays* as template. The amplicon was cloned in pCR2.1 to obtain the plasmid called pIRMM-0034. This plasmid was digested with *Eco*RI to release the fragment specific for the *hmg* gene. Subsequently, this fragment was ligated into the *Eco*RI restricted pUC18 vector (pIRMM-0012) using the rapid DNA ligation kit (Roche, Mannheim, DE) to yield the plasmid pIRMM-0067 and *E. coli* Top10 cells were transformed with the ligation products. Transformed cells were selected on 100 µg/mL ampicillin-containing LB plates and identified by PCR screening using primers flanking the border regions of the cloned fragment. The correct insertion was confirmed by restriction enzymatic digestion using *Eco*RI and *Pvu*II on plasmid DNA isolated from single colonies.

A 270 bp fragment of the endogenous *alcohol dehydrogenase 1* gene containing the 70 bp target *adh1* was amplified by the Platinum Pfx DNA polymerase using as template genomic DNA extracted from seed powder prepared from the non-GM maize variety RX670. The amplicon was ligated in the vector pCR2.1 and *E. coli* Top10 cells were transformed with the ligation products. Transformed cells were selected on LB plates supplemented with 50 µg/mL kanamycin and 100 µg/mL ampicillin and identified by PCR screening using primers flanking the border regions of the cloned fragment. The correct insertion was confirmed by DNA sequence analysis on plasmid DNA isolated from single colonies. One single clone bearing the plasmid named pIRMM-0080 was selected.

A 249 bp fragment of the endogenous *starch synthase IIb* gene containing the 151 bp target *zssIIb* was amplified by the Platinum Pfx DNA polymerase using as template genomic DNA extracted from seed powder of the NK603 event maize variety DKC 57-40. The amplicon was ligated in pCR2.1 and *E. coli* Top10 cells were transformed with the ligation products. Transformed cells were selected on LB plates supplemented with 50 µg/mL kanamycin and 100 µg/mL ampicillin and identified by PCR screening using primers flanking the border regions of the cloned fragment. The correct insertion was confirmed by DNA sequence analysis on plasmid DNA isolated from single colonies. One single clone bearing the plasmid named pIRMM-0081 was selected.

A 1266 bp fragment of the endogenous *alcohol dehydrogenase 1* gene containing the 135 bp target Zm*adh1* was amplified by the Platinum Pfx DNA polymerase using genomic DNA extracted from seed powder of the NK603 event maize variety DKC 57-40. The amplicon was ligated in pCR2.1 and *E. coli* Top10 cells were transformed with the ligation products.

Transformed cells were selected on LB agar plates supplemented with 50 µg/mL kanamycin and 100 µg/mL ampicillin and identified by PCR screening using primers flanking the border regions of the cloned fragment. The correct insertion was confirmed by DNA sequence analysis on plasmid DNA isolated from single colonies. One single clone bearing the plasmid named pIRMM-0087 was selected.

4.1.3 Construction of the multiple target plasmid

The multitarget plasmid was assembled in four consecutive DNA fragment insertions into the acceptor pUC18-based plasmid vector bearing the *hmg* maize endogenous target (pIRMM-0067). The plasmids pIRMM-0067 (containing the *hmg*-specific sequence) and pIRMM-0080 (containing the *adh1*-specific sequence) were both digested using the *Bam*HI restriction endonuclease, the relevant digestion products ligated and *E. coli* Top10 cells transformed with the ligation products. Transformed cells were plated on LB agar plates containing 100 µg/mL ampicillin and identified by restriction enzyme digestion of plasmid DNA extracts from single bacterial colonies. The corresponding plasmid was named pIRMM-0083.

The NK603 target fragment was inserted by restriction digestion of both plasmids pIRMM-0083 and pIRMM-0082 using *Xba*I, the relevant digestion products ligated and *E. coli* Top10 cells transformed with the ligation products. Transformed cells were plated on LB agar plates containing 100 µg/mL ampicillin and identified by restriction enzyme digestion of plasmid DNA extracts from single bacterial colonies. The corresponding plasmid was named pIRMM-0084.

The *zssIIb* target fragment was inserted by restriction digestion of both plasmids pIRMM-0084 and pIRMM-0081 using *Pst*I, the relevant digestion products ligated and *E. coli* Top10 cells transformed with the ligation products. Transformed cells were plated on LB agar plates containing 100 µg/mL ampicillin and identified by restriction enzyme digestion of plasmid DNA extracts from single bacterial colonies. The corresponding plasmid was named pIRMM-0085.

The Zm*adh1* target fragment was inserted by restriction digestion of pIRMM-0085 using *Sma*I resulting in a blunt-end linearised vector and amplification of a 340 bp fragment containing the 135 bp amplicon of the *alcohol dehydrogenase 1* gene (containing the Zm*adh1*-specific sequence) by the Platinum Pfx DNA polymerase using as template the plasmid pIRMM-0087. In a next step the two blunt-end products were ligated and *E. coli* Top10 cells transformed with the ligation products. Transformed cells were plated on LB agar plates containing 100 µg/mL ampicillin and identified by PCR screening using primers flanking the border regions of the cloned fragment. The correct insertions were confirmed by DNA sequence analysis on plasmid DNA isolated from single colonies. The corresponding plasmid was named pIRMM-0086 (Figure 1).

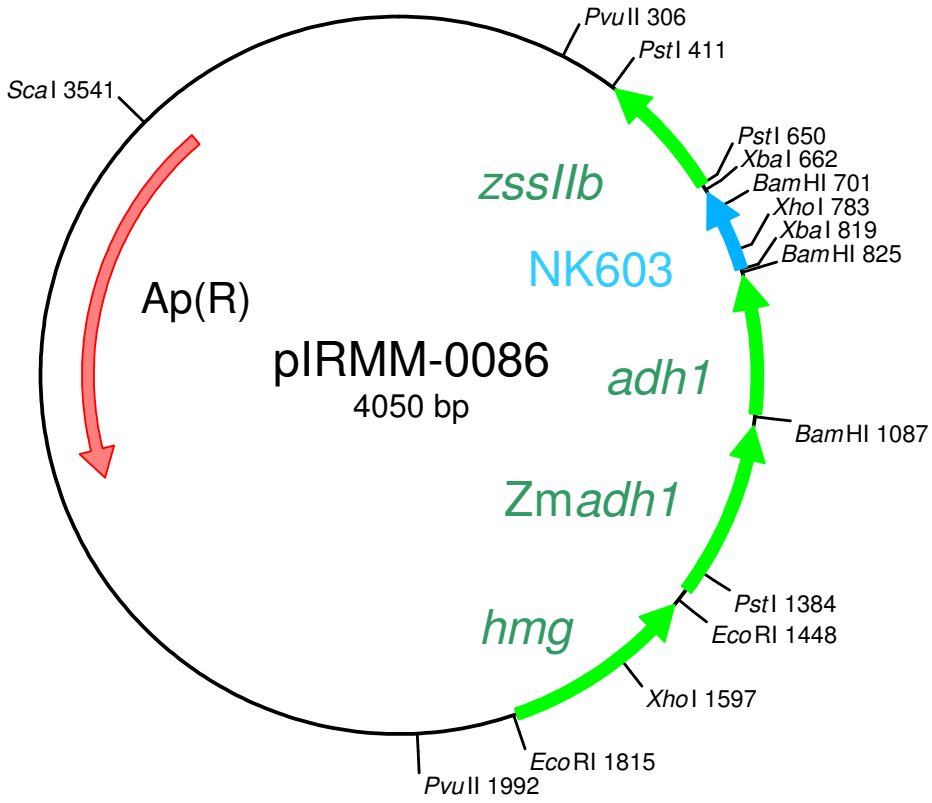

Figure 1: Circular map of pIRMM-0086 representing the 3' insertion-plant junction and the maize endogenous target inserts as well as the enzyme restriction sites used in the cloning and restriction analysis.

4.2 PURITY OF THE MATERIAL

The purity of the plasmid pIRMM-0086 was first analysed by DNA restriction analysis and agarose gel electrophoresis. No other DNA bands than those expected after restriction of the pIRMM-0086 with *Sca*I (Figure 2**,** lane 2), *Pvu*II (Figure 2, lane 3) and *Sca*I/*Xho*I (Figure 2**,** lane 4) could be seen after ethidium bromide staining. This confirmed the correct cloning of the fragments. Moreover, no smear and/or RNA bands were visible, as demonstrated by agarose gel electrophoresis (Figure 2, lanes 2-4). However, remaining traces of genomic DNA from host bacterial cells or traces of RNA molecules can not be excluded in the final plasmid preparation. Nevertheless, such traces do not affect the real-time PCR measurements or the ratio of the target sequences, as the used primers and probes are highly specific for the targeted sequences and do not hybridise to other DNA fragments that could be present in the final plasmid preparation. Such traces may introduce, however, a bias in the UV absorbance-based DNA quantification of the plasmid solution, and therefore an erroneous estimation of the number of plasmid copies in the tube. For this reason, the DNA concentration in each tube can only be given as an approximate value. A BLASTN 2.2.24+ analysis of the *hmg*- and NK603-specific detection PCR primers did not reveal more than 70 % nucleic acid sequence identity with the genomic DNA of *E. coli* from the NCBI database (data not shown). Therefore, specific PCR amplification occurring due to a potential contamination with bacterial genomic DNA is not possible. In addition, real-time PCR analyses of each of the cloned targets, performed on either genomic DNA extracted from seed powder or plasmid DNA, had comparable PCR efficiencies, demonstrating no significant effect of potentially contaminating bacterial genomic DNA on the real-time PCR measurements. Furthermore, this effect was ruled out by digital PCR [10] experiments that confirmed also the expected ratio between the two target sequences (DNA copy number

ratio of the NK603 event and *hmg* sequences in ERM-AD415 and its expanded uncertainty (k = 2), 1.03 ± 0.14). Based on all these observations, it can be reasonably concluded that no contamination with external genomic DNA or a large amount of RNA molecules occurred during the plasmid preparation.

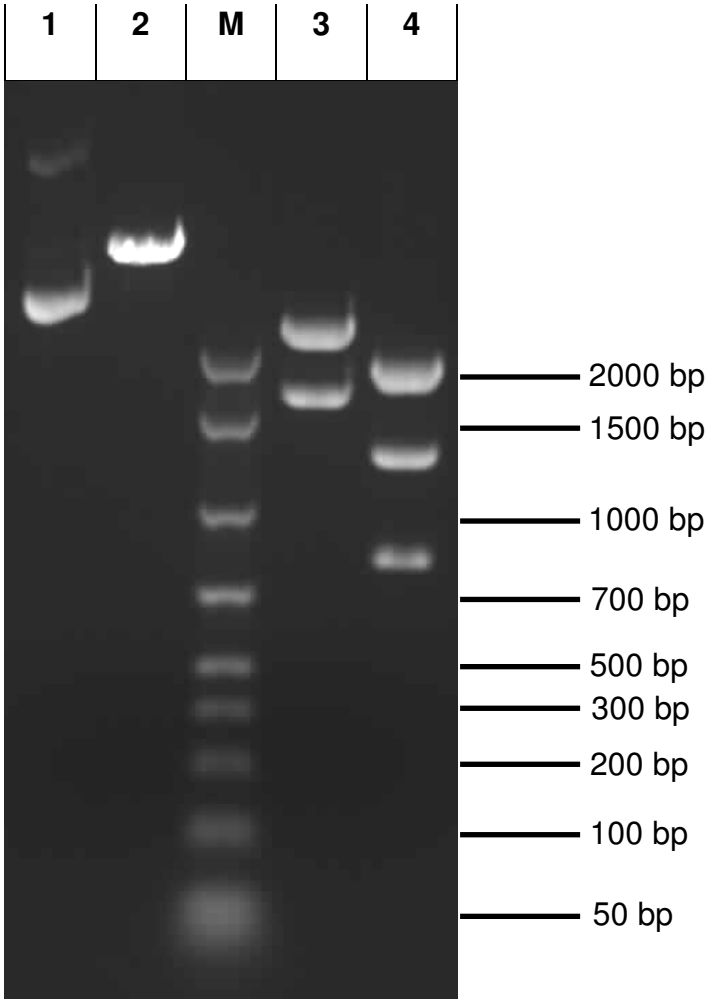

Figure 2: Restriction analysis of pIRMM-0086. Lane 1: unrestricted pIRMM-0086; Lane 2: pIRMM-0086 restricted with *Sca*I (plasmid linearisation expected); Lane 3: pIRMM-0086 restricted with *Pvu*II (expected fragments: 1686 bp, 2364 bp); Lane 4: pIRMM-0086 restricted with *Sca*I/*Xho*I (expected fragments: 814 bp, 1290 bp, 1946 bp); M: molecular DNA marker (Amplisize™ 50-2000 bp ladder, Bio-Rad Laboratories, Hercules, CA, US).

Despite the fact that the enzyme restriction conditions were chosen to allow a full digestion of the intermediate plasmids used for the assembly of pIRMM-0086, it is very difficult to prove that all plasmids were indeed fully digested, as traces of undigested plasmids will not be visible after gel electrophoresis and ethidium bromide staining.

The *E. coli* cells could be transformed with two types of plasmids: pIRMM-0086 present in large amount and traces of undigested pIRMM-0085. However, as those 2 synthetic plasmids have the same origin of replication (*ori*V from ColE1 plasmid) they belong to the same incompatibility group. As a result, the transformed bacterial clones can only bear one single plasmid. As the plasmid production was started from a separate colony, only one

type of plasmid can be present in a single colony. We can therefore conclude that each single bacterium extracted from one colony contains only one type of plasmid[2].

As additional proof of purity, plasmid DNA isolated from the transformed *E. coli* cells was sequenced completely to verify that all target DNAs were present and correctly cloned. The sequence analysis did not reveal the presence of a mixed population of plasmids.

4.3 PROCESSING OF THE MATERIAL

Fifty mL of LB media containing 100 µg/mL of ampicillin were inoculated with a single colony of *E. coli* containing the plasmid pIRMM-0086 and shaken at 200 rpm overnight at 37 °C. The bacterial pellet was lysed in the presence of RNase, following the recommended protocol and buffers of a QIAprep Spin Miniprep kit (QIAGEN Benelux B.V., Venlo, NL). The plasmid DNA was purified on a silica membrane and eluted in 10 mmol/L Tris-HCl buffer, pH 8.5. The resulting DNA solution was stored at -20 °C until further use.

The plasmid DNA concentration and purity of the preparation were measured by UV spectrophotometry and fluorometry. A DNA mass concentration and standard deviation of 195 ± 18 µg/mL were obtained using the Picogreen® dsDNA quantitation kit (Molecular Probes Inc, Eugene, OR, US). The purity of the nucleic acid preparation was assessed spectrophotometrically by comparing the UV absorbance of the sample at 260 nm to that at 280 nm. Taking into account the generally accepted mean extinction coefficient for double-stranded DNA at 260 nm and 280 nm, pure nucleic acid samples are expected to have an A_{260}/A_{280} ratio approximately 1.8 and an A_{260}/A_{230} ratio above 2.0 [11]. The A_{260}/A_{280} and A_{260}/A_{230} ratios measured for the plasmid solution were 1.86 and 2.26 respectively, indicating a sufficient DNA purity; however, such values do not exclude traces of contaminating proteins [11].

The measured DNA mass concentration was used to calculate the number of copies of plasmid that were present per volume. For this estimation, knowing the accurate size (*S*) of the plasmid and the mean molar mass (*M*) of a nucleotide the plasmid number content can be obtained using the following formula:

for 1 g of DNA containing *x* copies of a plasmid

$$x = \frac{N_A}{2 \cdot M \cdot S}$$

where,

x = number content (expressed in number of plasmids/g),
N_A = Avogadro constant (expressed in mol^{-1}),
M = mean molar mass of a nucleotide (expressed in g/mol),
S = size of the plasmid molecule (expressed in number of nucleotides).

[2] The synthetic vectors used (pUC18 and pCR®2.1) in our cloning strategy were chosen as being high copy vectors from the same incompatibility group (incQ). Those plasmids have their own origin of replication (oriV) and are able to replicate independently of the host chromosome. A bacterial host cannot however contain different plasmids that have the same mechanisms of replication, because the control of the replication (in Gram-negative bacteria) is exercised through trans-acting molecules (theta replication mode). The inevitable consequence of this is that one of the plasmids would eventually be lost from the cell simply as a result of random partitioning of plasmids into daughter cells during cell division. Thus the plasmids would appear to be incompatible. Two or several plasmids from the same incompatibility group cannot coexist in the same cell [12].

The number of plasmids per volume can be calculated by measuring the DNA concentration of the solution using the following formula:

$$\text{copy number concentration}\,[\text{cp/µL}] = \frac{\text{DNA mass concentration}\,[\text{g/µL}] \cdot N_A\,[\text{bp/mol}]}{2 \cdot M\,[\text{g/mol}] \cdot \text{plasmid size}\,[\text{bp/cp}]}.$$

A solution containing approximately $2{\cdot}10^6$ cp/µL of the plasmid pIRMM-0086, corresponding to a DNA concentration of 8.3 pg/µL was prepared by diluting the stock plasmid solution in a background of ColE1 plasmid DNA (product number D9683, Sigma-Aldrich, Bornem, BE) at a final concentration of 1 ng/µL. The buffer used for the dilution contained 1 mmol/L Tris, 0.01 mmol/L EDTA pH 8.0 ($T_1E_{0.01}$).

4.4 FILLING OF TUBES

The above plasmid solution was sterilised by filtration through a filter with a pore size of 0.2 µm and filled manually in pre-labelled high recovery polypropylene tubes with 500 µL of the plasmid solution under sterile conditions. Each tube contains approximately 10^9 copies of the ERM-AD415 plasmid, which corresponds to about 4.14 ng of DNA.

The tubes were placed in cardboard boxes each containing 100 tubes. Tubes for the short- and long-term stability studies, and for additional characterisation were selected randomly from the entire batch. Each box was then sealed under light vacuum in a plastic pouch and frozen either at (-20 ± 5) °C or at (-70 ± 10) °C.

Each tube was identified by a numbered label as shown in Figure 3.

Figure 3: Prototype label for ERM-AD415.

4.5 DISPATCHING OF TUBES

Tubes of the processed ERM-AD415 were dispatched to the participants in the interlaboratory comparison launched to evaluate the suitability of calibrants (Section 8.3). Unprocessed samples of plasmid DNA containing purified pIRMM-0086 in nuclease-free water were sent to two independent sequencing service companies to determine the complete nucleic acid sequence of the plasmid. All samples were packed in containers filled with dry ice.

5 PROCEDURES

5.1 METHODS USED FOR THE STABILITY STUDY

Simplex real-time PCR was used to identify and quantify the amount of both target DNAs present in the sample. The set up of the quantitative real-time PCR reaction was done according to the procedure described in the method validated by the EURL-GMFF for the event-pecific quantification of the maize NK603 event [4] with exception of the following changes: the reference gene target was changed from *adh1* to *hmg* to avoid erroneous quantification of the copy number of the reference target as reported by Broothaerts et al. [3]; the real-time PCR reaction volume used in this certification study was 25 µL; the baseline and threshold of the individual real-time PCR measurements were set automatically. The two tables in Annex 1 list the primers used for the quantification of the NK603 event and reference gene target (*hmg)* by real-time PCR and summarise deviations from the EURL-GMFF protocol, respectively.

Briefly, the sequence identification and quantification of the NK603 transgenic target was performed using an event-specific method targeting a 108 bp fragment of the 3' plant junction region and a 79 bp fragment of the *hmg* gene used as normaliser. The final forward and reverse primer amount of substance concentrations in the real-time PCR reaction were 300 nmol/L and 150 nmol/L for the *hmg* and NK603 targets respectively, while the amount of substance concentrations for the *hmg* and NK603 probes were 160 nmol/L and 50 nmol/L, respectively. TaqMan® Universal PCR MasterMix analyses were carried out according to the manufacturer's instructions (Applied Biosystems, Lennik, BE) with a thermal profile consisting of an initial denaturation step at 95 °C for 10 min, followed by 45 amplification cycles of 15 s at 95 °C and 1 min at 60 °C. The TaqMan® Universal PCR MasterMix contained AmpliTaq Gold® DNA polymerase for a better yield and a more robust 5' nuclease assay than AmpliTaq® DNA polymerase and AmpErase® UNG to protect against subsequent reamplifications from PCR products minimising as such the carry-over contamination. The MasterMix also contained a passive reference dye to correct for inter-well signal variation caused by slight differences in the reaction volume.

Five µL of DNA solution were added to 20 µL master mix containing the appropriate primers and probe for the simplex real-time PCR. Both the *hmg* and the NK603 probe were 5′ labelled with 6-FAM. The fluorescence was recorded on-line (in real-time) during the PCR amplification process and its intensity was proportional to the number of target DNA sequences.

5.2 METHODS USED FOR CHARACTERISATION

5.2.1 Gel electrophoresis

Plasmid DNA samples (5 µL total volume) were analysed by gel electrophoresis, after enzymatic restriction, using a Minicell System (Bio-Rad Laboratories, Hercules, CA, US) in agarose gels of a 1, 2 or 2.5 % (mass fraction) containing ethidium bromide at a mass concentration of 0.5 µg/mL. Gels were run for 60 min at 80 V in a TBE buffer (90 mmol/L Tris, 90 mmol/L boric acid, 2 mmol/L EDTA, pH 8.0). DNA was visualised by UV and photographed using GeneSnap (Syngene, Leusden, NL).

5.2.2 Fluorometry and UV spectrometry

The extracted plasmid DNA was quantified using the PicoGreen dsDNA Quantitation Kit according to the manufacturer's instructions using a lambda DNA standard solution. The purity of the DNA in the solution was analysed at 230 nm, 260 nm and 280 nm using a UV/VIS spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies Inc, Wilmington,

DE, US). The ratios of the absorbances at 260 nm and 280 nm (A_{260}/A_{280}) and of the absorbances at 260 nm and 230 nm (A_{260}/A_{230}) were calculated to provide an estimation of the purity of the extracted DNA.

5.2.3 DNA sequencing

The plasmid pIRMM-0086 was sequenced by three independent laboratories: JRC-IRMM (Geel, BE), Eurofins Medigenomix GmbH (Martinsried, DE) and QIAGEN Sequencing Services (Hilden, DE).

Sequencing at IRMM was performed on an ABI Prism® 3130*xl* Genetic Analyser (Applied Biosystems, Lennik, BE) using the BigDye® Terminator v1.1 cycle sequencing kit protocol. This sequencing analysis targeted the regions of DNA insertion in pIRMM-0086 generated during the construction of the plasmid. Cycle sequencing utilises successive rounds of denaturation, annealing and extension in a thermocycler to create a linear amplification of extension products. With dye terminator labeling, each of the four dideoxy terminators is tagged with a different fluorescent dye. The 3130*xl* RapidSeq36_POP7 run module was used, and the samples were analysed using the ABI sequencing analysis software v5.2 Patch 2.

Eurofins Medigenomix GmbH (Martinsried, DE) performed the sequencing analysis of the complete plasmid by primer walking on both DNA strands. All sequences were generated using BigDye-terminator chemistry (version 3.1) of Applied Biosystems (Foster City, CA, US) following standard protocols. For PCR reactions Primus 96 HPL Thermalcyclers (MWG AG, Ebersberg, DE) were used. Sequencing reaction cleanup was done by gel filtration through Performa DTR 96-well plates (Edge Biosystems, Gaithersburg, MD, US). Finally all reactions were run on ABI Prism® 3730*xl* capillary sequencers.

The sequence quality values which are a transformed estimate of the probability of correctness (1 - probability of error) are represented in a log scale from 0 to 50 for single strand sequencing. The use of a log-transformed error probability facilitates working with error rates in the interval of highest interest (very close to 0). The quality value q assigned to each base (i.e. base-call) is defined by the following equation:

$$q = -10 \log_{10} z$$

where z is the estimated error probability for a base.

The sequences generated by Eurofins Medigenomics had a quality value of 78 and higher, which means that the estimated probability for each base to be incorrect was less than 1/63000000.

QIAGEN Sequencing Services (Hilden, DE) also performed sequencing of the complete plasmid on both DNA strands with the BigDye® Terminator v3.1 cycle sequencing kit according to the protocol supplied by Applied Biosystems on a Model 3700 and 3730 automated DNA sequencers (PE Biosystems). Assembly of all sequencing data was carried out using the STADEN software package (version 4.5; http://www.mrc-lmb.cam.ac.uk/pubseq) and the DNASTAR package (DNASTAR Inc., Madison, WI, US). The sequences provided by QIAGEN had an estimated probability to return an incorrect base of less than 1/20000.

5.3 METHODS USED FOR THE SUITABILITY STUDY

A study designed to perform the sequence identification and quantification of the targets *hmg* and NK603 present in ERM-AD415 and for evaluation of the analytical behaviour of DNA calibrants used in the quantification of the NK603 event in maize seed powder was

included as part of the interlaboratory comparison organised to assess the copy number ratio of ERM®-BF415e [13]. The set up of the quantitative real-time PCR reaction was as described in Section 5.1.

For the purpose of the study comparing the analytical behaviour of pDNA and gDNA three types of DNA were used: plasmid DNA ERM-AD415 (pDNA), genomic DNA extracted from seed powder certified for its mass fraction content of NK603 (ERM-BF415e) (gDNA*s*) and genomic DNA extracted from NK603 plant leaves (gDNA*l*). Genomic DNA from NK603 plant leaves was obtained at IRMM by DNA extraction of maize leaves harvested from germinated NK603 maize seeds heterozygous for the GM event using the protocol for automated DNA extraction and the Chemagic DNA Plant Kit (Chemagen Biopolymer-Technologie AG, Baesweiler, DE) following the manufacturer's protocol. For the extraction of genomic DNA from seed powder, samples of ERM-BF415e were used. The plasmid DNA, genomic DNA from plant leaves extracted at IRMM and the maize powder ERM-BF415e were shipped to the participating laboratories on dry ice. For each data set, the genomic DNA from seed powder was obtained by each participant using one of the three DNA extraction methods: i) a modified cetyltrimethylammonium bromide (CTAB) method [14], ii) CTAB (modified as mentioned above) followed by additional purification (Genomic-tip 20/G column kit and procedure from QIAGEN, Hilden, DE), iii) DNA extraction method provided with the GENE*Spin* kit for DNA extraction (GeneScan Analytics GmbH, Freiburg, DE). The extraction methods used in the study are described in detail in the report describing the certification of ERM-BF415e for its DNA copy number ratio [13].

6 HOMOGENEITY

6.1 HOMOGENEITY ASSESSMENT

The certified value for ERM-AD415 is expressed as number of DNA fragments per pIRMM-0086 plasmid. This quantity defines the structure (DNA sequence) of the pure substance (pIRMM-0086), therefore the homogeneity of the material is not considered in the estimation of the uncertainty of the certified value.

6.2 MINIMUM SAMPLE INTAKE

In the frame of the short-term stability study and the interlaboratory comparison addressing the analytical behaviour of plasmid and genomic DNA with respect to the real-time PCR quantification of the NK603 event in maize (Section 7.1 and 8.3) it was shown that using 50 µL of ERM-AD415 solution for the preparation of a calibration curve by serial dilution led to reliable PCR results. The validity of the PCR measurements was, however, not tested with smaller starting volume intakes. Therefore the recommended minimum sample intake for setting up a calibration curve for real-time PCR using the described method [3-4] is 50 µL.

7 STABILITY

7.1 SHORT-TERM STABILITY STUDY

7.1.1 Design of the short-term stability study

The short term stability of ERM-AD415 was evaluated by analysing 5 tubes stored at (-20 ± 5) °C and (4 ± 3) °C for 1, 2 and 4 weeks. Three replicates from each tube were analysed (N = 5, n = 3) at several dilution levels. The same number of tubes (5) was stored at (-70 ± 10) °C as reference. Each reference tube was analysed in triplicate by simplex real-time PCR to reveal changes in the amount of the two fragments present in the plasmid due to the different test temperatures and times of storage.

7.1.2 Results of the short-term stability study

The ratio of both sequences was analysed and did not indicate any significant change at (4 ± 3) °C or (-20 ± 5) °C (Figure 4). DNA copy number ratios followed a normal distribution at all tested temperatures. When scrutinising the data, one outlier was detected by the single Grubbs test at a 95 % confidence level. As no technical reason to exclude the data of one tube was found the outlying data was retained. Regression analysis was done for each of the storage temperatures to reveal any trend in the ratio between the two targets in relation to the time of storage. A *t*-test showed no significant change over the time period of 4 weeks (95 % confidence level) for the material kept at (-20 ± 5) °C and (4 ± 3) °C when testing the plasmids at final copy number concentrations estimated to be between 400 000 cp/µL and 20 cp/µL.

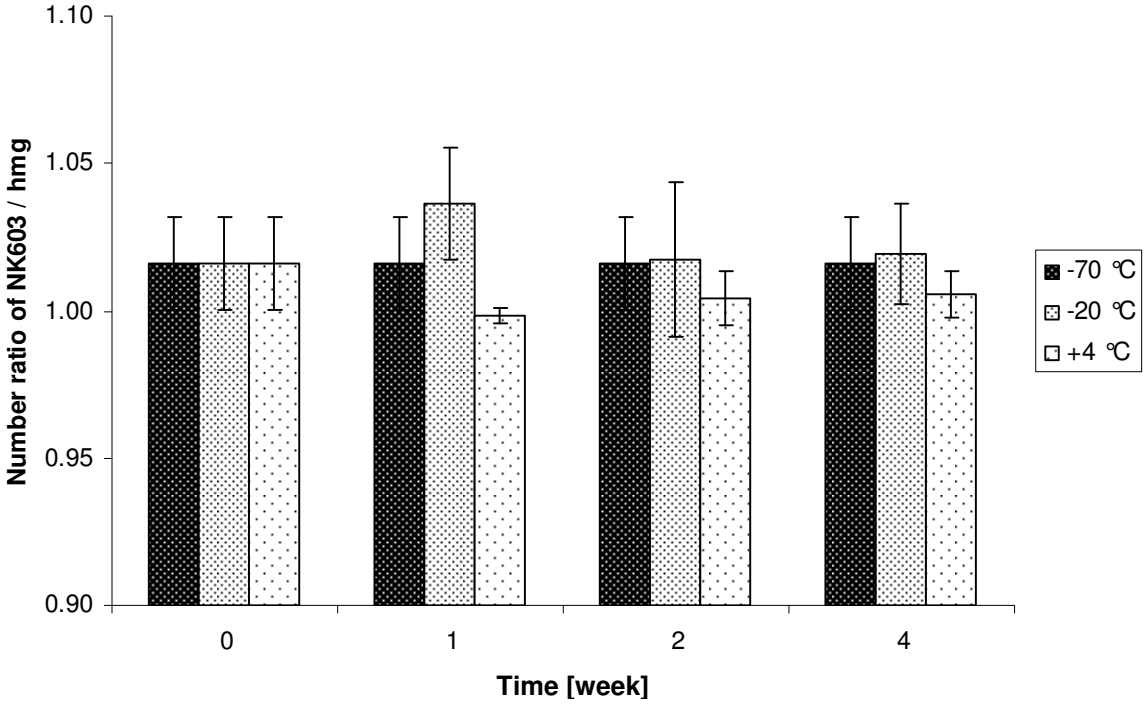

Figure 4: Short-term stability of ERM-AD415 stored at different temperatures for 0, 1, 2 and 4 weeks and analysed by NK603 and *hmg* specific real-time PCR. The bars indicate the interval $\bar{x}$ ± standard deviation s for N = 5, n = 3.

7.1.3 Conclusion from the short-term stability study

The short-term stability study performed with ERM-AD415 proved that the ERM-AD415 withstands temperatures of up to 4 °C for the tested periods of time.

More information about the stability of materials has been gained on similar plasmid DNA that was processed using the same extraction and purification protocol, mixed in the same proportion with the same batch of ColE1 background DNA, based on the same vector, stabilised in the same buffer composition and stored in the same type of tubes. Because of the similar processing and identical nature of the DNA, it is reasonable to think that both materials will have the same stability features.

Data from the isochronous long-term stability study of the ERM-AD413 stored at (18 ± 5) °C during 24 months (see details in Section 7.2) indicate that the CRM can be shipped using cooling elements.

7.2 LONG-TERM STABILITY STUDY

7.2.1 Design of the long-term stability study

As mentioned above, ERM-AD415 has been produced following the same procedure as for ERM®-AD413 [15]. Therefore, to estimate the stability of ERM-AD415 during long-term storage, data obtained from the post-certification monitoring of ERM-AD413 for a period of 24 months was used. Tubes containing ERM-AD413 have been stored at (-20 ± 5) °C and (18 ± 5) °C to be analysed at those respective times (Figure 5).

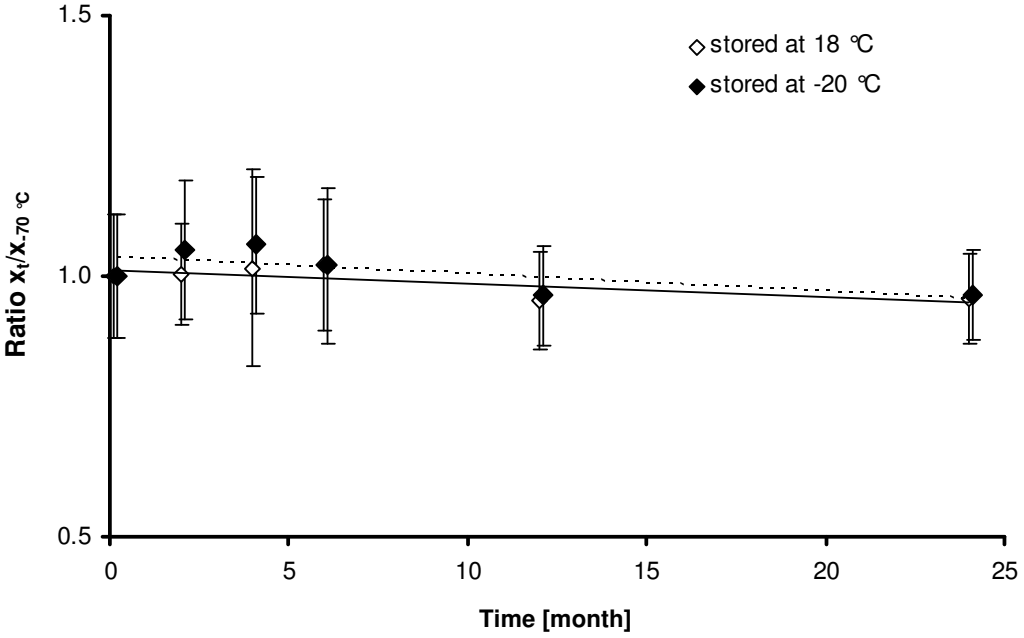

Figure 5: Long-term stability of plasmid DNA (ERM-AD413, MON810 calibrant) stored at (-20 ± 5) °C and (18 ± 5) °C for 24 months based on real-time PCR measurements. The stability is expressed as the ratio between the copy number ratios of MON810 and *hmg* fragments per plasmid in tubes stored at the indicated temperatures and the copy number ratios of the two fragments from tubes stored for the same period at the reference temperature [(-70 ± 10) °C]. Each bullet represents the mean ± standard deviation *s* of a minimum of 5 measurements. The dashed line is the regression line generated on the basis of the (-20 ± 5) °C data points, whereas the full line is the regression line generated on the basis of the (18 ± 5) °C data points.

7.2.2 Conclusions from the long-term stability study

Based on the stability study of ERM-AD413, a minimum shelf-life of 24 months at (-20 ± 5) °C can be ensured for real-time PCR applications. The ratio between the two inserted targets measured by real-time PCR remains unaffected, and the material can be,

therefore, considered as suitable for use in real-time PCR measurements.The total amount of material present in the tubes does not decrease as the Ct values for the respective targets remains unchanged upon storage.

The long-term stability study does not show differences for the material storage at (-20 ± 5) °C or at (18 ± 5) °C in terms of copy number ratio. We recommend nevertheless to store the material at (-20 ± 5) °C, if the material needs to be stored for several months. Furthermore, yearly post-certification monitoring is planned to provide an additional control of the stability of ERM-AD415 and to extend the shelf-life of this CRM.

8 BATCH CHARACTERISATION

8.1 PLANNING

To determine the number of the cloned DNA fragments in the ERM-AD415 calibrant, sequencing of the regions of DNA insertion in pIRMM-0086 was performed at IRMM on an ABI Prism® 3130*xl* Genetic Analyser (Applied Biosystems, Lennik, BE) using the BigDye® Terminator v1.1 Cycle Sequencing kit. Additionally, the full sequence of the plasmid was determined by two independent laboratories. The companies QIAGEN Sequencing Services (Hilden, DE) and Eurofins Medigenomix GmbH (Martinsried, DE) were asked to provide the full sequence of pIRMM-0086. The plasmid was sequenced on both strands by the two independent laboratories to insure a very high accuracy of the sequence generated and a full characterisation of the molecular composition of the plasmid DNA. The three sequences were compared to each other and submitted to GenBank database (NCBI, NIH, Bethesda, MD, US) for BLASTN homology searches [16]. All three determined sequences were identical confirming the correct cloning of the five fragments and the identity with published sequences. The pIRMM-0086 plasmid could be accurately sized and consists of 4050 bp (Figure 1). The DNA sequences also confirm that each plasmid contains one single copy of the *zssIIb*, NK603, Zm*adh1*, *adh1* and *hmg* fragments. The uncertainty related to the sequence determination can be considered as negligible as the probability to report a wrong base was calculated to be less than 1/63000000. As a clear consequence, the structurally defined ratio between the numbers of 3' insertion-specific DNA and *hmg* fragments in the plasmid pIRMM-0086 is 1.

8.2 ASSIGNMENT OF A CERTIFIED VALUE

The material is certified to contain one 108 bp 3' insert-plant junction fragment and one 79 bp *hmg* fragment per pIRMM-0086 plasmid[3] based on DNA sequencing. This certified value is based on the DNA sequence of pIRMM-0086 provided by three independent laboratories.

8.3 SUITABILITY STUDY

Twenty-one laboratories were selected on the basis of proven experience and quality management systems in place to investigate the analytical behaviour of the calibrant used for calibration of the real-time PCR measurements. With this interlaboratory comparison, the determination of the copy number ratio related to the ERM-BF415e matrix was performed using simplex real-time PCR. Within one requested analysis, measurements were calibrated using two types of calibrants, pDNA ERM-AD415 and genomic DNA extracted from hybrid maize NK603 plant leaves. Measurement results from the same analysis obtained using a different calibrant were grouped into a separate data set. A total of 42 analyses were requested that resulted in 84 datasets (a detailed description of the methodology of the study is given in [13]). Within one data set of the study, each laboratory carried out two independent analyses, on two different days. Consequently, the experimental setup on each day was based on two calibration curves, namely, the transgenic and endogeneous calibration curve, each of them including five concentration levels. PCR efficiencies as well as the linearity of the dilution series were calculated on the basis of serial dilutions of the plasmid calibrant (ERM-AD415) in $T_1E_{0.01}$ buffer, dilutions of gDNA extracted from leaves from germinated NK603 maize seeds, labeled in the figures and tables as gDNA$_\ell$, as well as dilutions of gDNA extracted from ERM-BF415e (containing 19.6 ± 0.9 g/kg of NK603 maize seed powder in non-GM maize powder) and labeled as gDNA$_s$.

[3] The certified value expressed as number of DNA fragments per plasmid DNA molecule is an entitic number.

To evaluate the analytical behaviour of the plasmid and genomic DNA, two parameters derived from the regression line generated by DNA serial dilution were compared and statistically analysed.

The first parameter was the PCR efficiency estimated for both transgenic and endogenous targets, using the three DNA types. PCR efficiencies were only compared if the respective data sets passed the selection criteria defined beforehand, namely the coefficient of determination (R^2) of the calibration curve, and PCR efficiency (estimated on the basis of the slope of the calibration curve). These selection criteria were applied to avoid interferences of technically weak results generated by the participating laboratories [17-18]. Firstly, a R^2 below 0.98 was not accepted within the study as it may reflect erroneous dilutions or inappropriate PCR amplification. Secondly, the PCR efficiencies interval was defined on the basis of pDNA and gDNAℓ efficiencies (both materials used as calibrants in the study). The means of PCR efficiencies were calculated for the endogenous and transgenic targets using either the pDNA or gDNAℓ calibrant. Four intervals were generated accordingly based on the mean ± 1 standard deviation (1 *s*) (not shown). Minimum and maximum values of the resulting eight cut-off points were then used to define a lower and a higher limit of the PCR efficiency of the study, *i.e.* 77 and 99 % respectively. These values were consistent with the performance of the simplex real-time PCR method for quantification of the NK603 maize event observed with in-house performed studies and from results obtained from the interlaboratory comparison.

The means of the PCR efficiencies varied between 83 % and 95 % (Table 1). For both targets, PCR efficiencies were highest using the pDNA and lowest for the gDNAℓ calibrant. Comparing the targets, PCR efficiencies were higher for the endogenous gene *hmg* than for the transgene NK603 (Table 1).

A difference in PCR efficiencies between the pDNA and the gDNAℓ calibrants was observed (2.8 % for the endogenous and 4.6 % for the transgenic targets) (Table 1). The PCR efficiencies of pDNA and gDNAℓ calibrants were found to be significantly different in the cases of both targets (ANOVA: *hmg:* $p = 2.2 \times 10^{-8}$, $\alpha = 0.05$; NK603: $p = 3.6 \times 10^{-14}$, $\alpha = 0.05$) using a simplex real-time PCR detection method.

The distributions of the various PCR efficiencies for pDNA/gDNAℓ and gDNAs were compared and showed a large overlap for both targets for the three DNA types (Figure 6). There was no significant difference between the PCR efficiencies of both the endogenous and transgenic targets for pDNA and gDNAs (Kruskal-Wallis test[4] for the endogenous target: $p = 0.59$, $\alpha = 0.05$; ANOVA for the transgenic target: $p = 0.25$, $\alpha = 0.05$). All other comparisons led to significantly different results in the cases of both (endogenous and transgenic) real-time PCR targets (Figure 6 and Table 1).

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

Table 1: Comparison of the real-time PCR efficiencies of gDNA extracted from leaves or seeds and pDNA; N_{ds} is the number of accepted data sub-sets under repeatability conditions, *s* is the standard deviation.

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

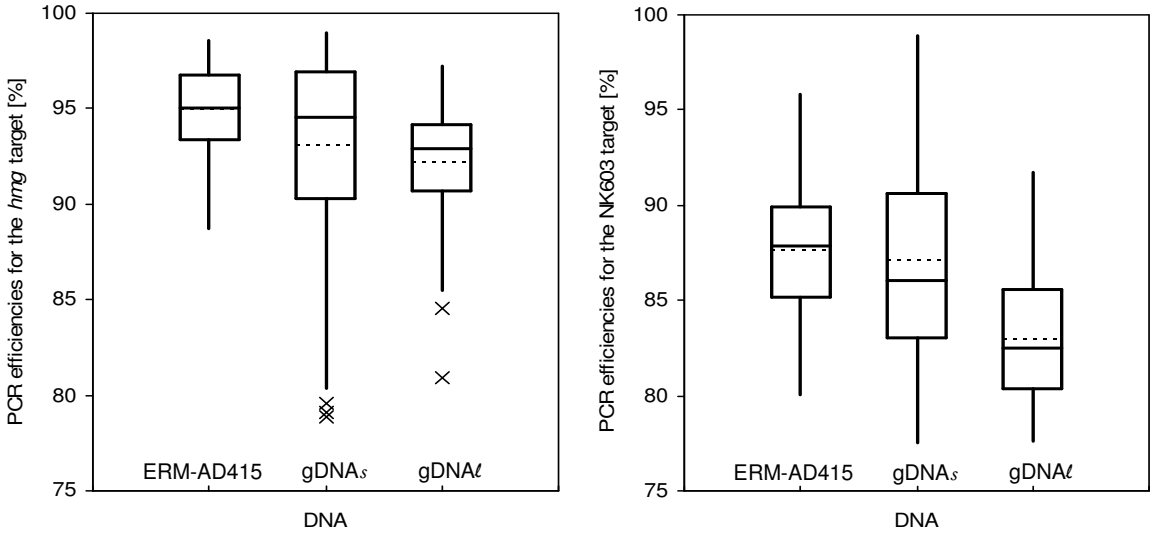

Figure 6: Box-and-whisker diagram illustrating the PCR efficiencies for *hmg* and NK603 based on the dilution series performed using either ERM-AD415 calibrant or gDNA extracted from NK603 seeds or NK603 leaves. The bottom and top of the box are the 1st and 3rd quartiles, respectively; the full line near the middle of the box is the median. The ends of the whiskers are determined by subtracting 1.5 times the interquartile range (IQR) from the 1st quartile, and adding 1.5 times the IQR to the 3rd quartile, respectively. The dashed line corresponds to the mean of each group, whereas values outside the 95 % confidence interval are marked with x.

The second parameter studied was the coefficient of determination (R^2) that provides information about the fitting of data to a linear regression line obtained for both targets and the three types of DNA as calibrant (Figure 7 and Table 2).

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

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

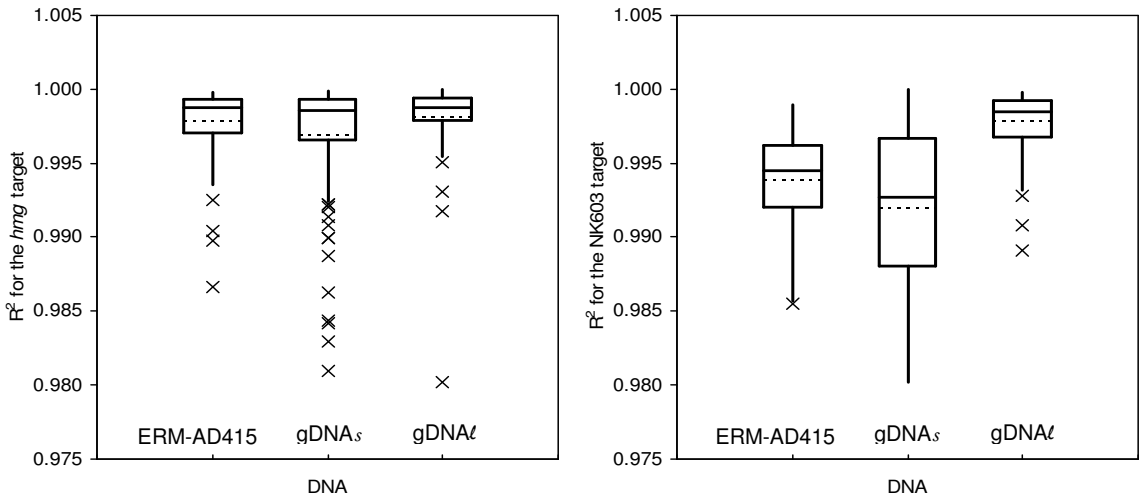

Figure 7: Box-and-whisker diagram representing the coefficients of determination (R^2) for *hmg* and NK603 based on the dilution curves performed using either ERM-AD415 calibrant or gDNA extracted from NK603 seeds or NK603 leaves. A description of a box-and-whisker diagram is given in Figure 6.

A comparison of the coefficients of determination of pDNA and gDNAℓ calibration curves showed no major influence of the type of the DNA calibrant in the case of the endogenous target (Figure 7). For the transgenic target the distribution of the coefficient of determination displayed a large variation. However, the coefficients of determination of gDNA*s* dilution curves distributed closer to those of the pDNA calilbrant in the case of both targets.

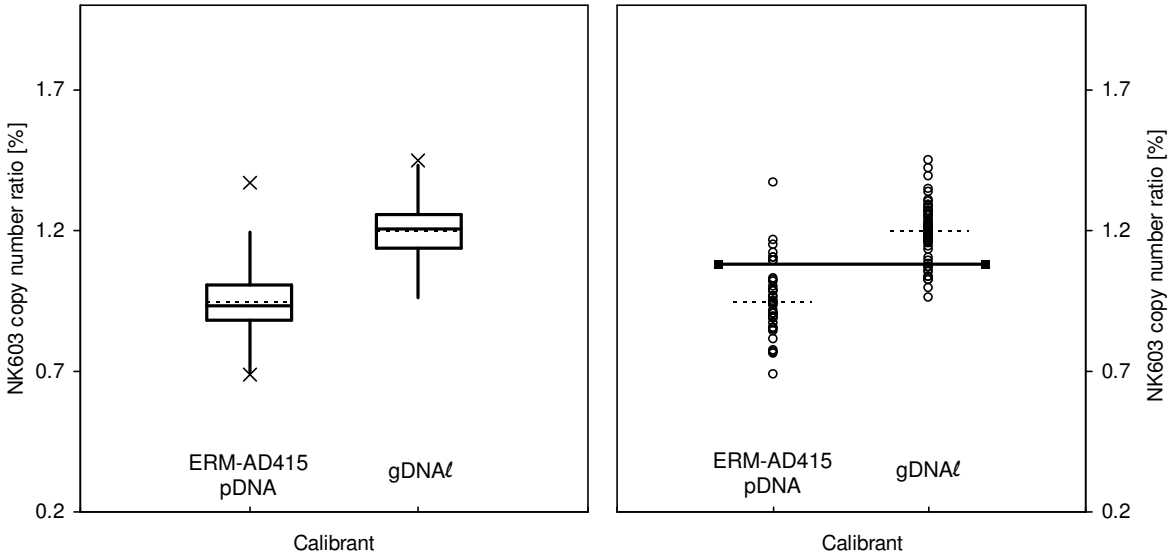

Figure 8: Box-and-whisker diagram and distribution graph of the NK603 copy number ratio in ERM-BF415e using either ERM-AD415 or gDNA extracted from NK603 leaves (gDNAℓ) as calibrant. A description of a box-and-whisker diagram is given in Figure 6. The right graph represents the distribution of the individual results. The horizontal line is the mean value between the two groups of calibrants.

Finally, the NK603 GM copy number ratio of ERM-BF415e (19.6 ± 0.9 g/kg by mass fraction) was compared using either ERM-AD415 or gDNAℓ extracted from leaves as calibrants (Figure 8 and Table 3). The data sets from both calibrants follow a normal distribution. Though the GM copy number ratios obtained for ERM-BF415e by the two different calibrants overlap, the mean GM copy number ratios are very different. Single factor ANOVA confirms that the data set calibrated with ERM-AD415 is significantly different from the data set calibrated with gDNA from leaves (ANOVA: $p = 1.9 \times 10^{-17}$,

α = 0.05). Therefore, GM copy number data calibrated with ERM-AD415 or gDNA from leaves cannot be pooled.

Table 3: GM content in ERM-BF415e expressed in copy number ratio and *s* [%] and calibrated either with ERM-AD415 or genomic DNA extracted from NK603 leaves; N_{ds} indicates the number of accepted data sub-sets under reproducibility conditions, *s* is the standard deviation.

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

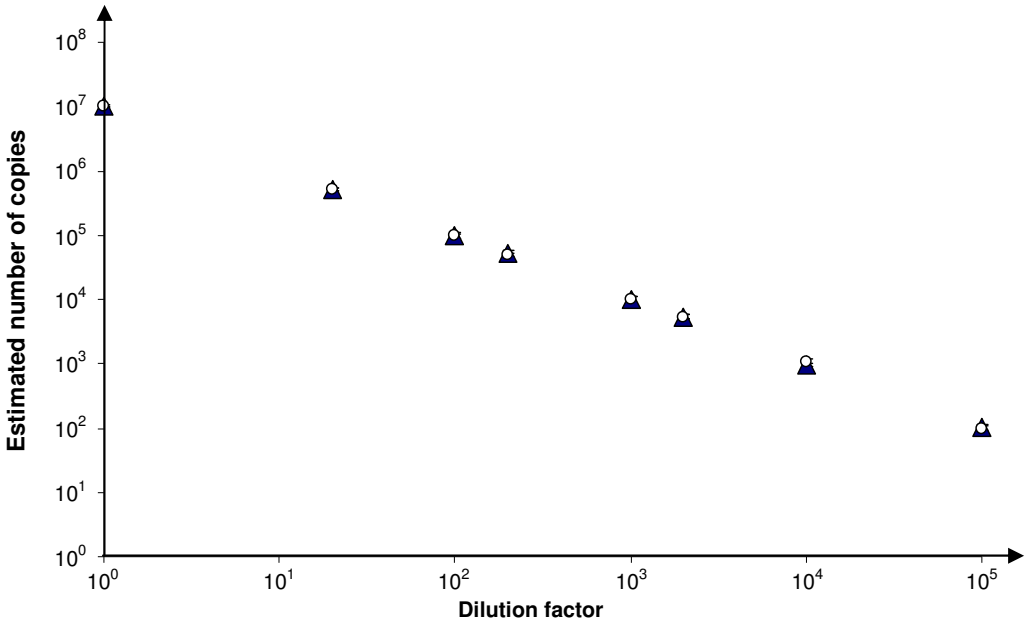

Figure 9: Comparison of estimated number of copies of *hmg* (○) and NK603 (▲) target sequences by the simplex real-time PCR method applied for a serial dilution of ERM-AD415 (N = 18) in $T_1E_{0.01}$. The copy numbers were estimated using the PicoGreen dsDNA quantitation kit measurements to measure the initial DNA mass concentration of the plasmid pIRMM-0086 solution used for processing.

In the study reported here, statistical analyses have shown that pDNA and gDNAℓ calibrants behave in a different way with respect to the PCR efficiencies of the transgenic and endogenous target sequences (Figure 6). The individual PCR efficiency of each target sequence has a significant impact on GM quantification by real-time PCR. Therefore, the effect of a low difference in PCR efficiencies of the transgenic and endogenous targets on GM quantification by real-time PCR can generate a rather large difference in copy number due to the exponential nature of the PCR amplification. Consequently, such differences in PCR efficiencies of the calibrants may explain the difference of GM copy number ratios observed when the mean values are compared (Table 3).

Based on this study, it can be concluded that both calibrants, the ERM-AD415 pDNA and the gDNA from plant leaves are equally suited to calibrate the quantitative PCR method applied here. However, one should be aware that the choice of calibrant influences the measured copy number ratio and leads to significantly different results (Table 3).

Due to the biology of maize it is impossible to establish a reliable copy number ratio value based on the GM mass fractions. Using the pDNA calibrant ERM-AD415 or the gDNA calibrant from leaves might not approach a true value.

The results of this study indicate that the most suitable approach is to set a reference system based on the pDNA. This is described here for the copy number ratio together with the modified GM quantification method published by the EURL-GMFF [3-4] and leads to comparable measurement results in GM quantification.

As shown in Figure 9 the material ERM-AD415 can be used for establishing dilutions down to 1/100000 and can be easily quantified for both targets.

9 CERTIFIED VALUES AND UNCERTAINTY

9.1 CERTIFIED VALUES

The plasmid calibrant is characterised for the number of each of the two specific fragments per plasmid, NK603 and *hmg*. The two cloned DNA fragments for which ERM-AD415 is certified, the NK603 event- and the *hmg*-specific sequences, were found to be identical to the respective PCR targets published with the validated method for quantification of the maize NK603 event by the EURL-GMFF [4] modified according to Broothaerts et al. [3] and certified to be present as one single copy per pIRMM-0086 plasmid each. Based on the sequence and purity assessment the theoretical number ratio between the two targets is equal to 1.

9.2 UNCERTAINTY EVALUATION

Based on the sequence analyses, it can be concluded that each of the DNA fragments, the NK603 event and *hmg*, is present as a single copy per pIRMM-0086 plasmid. Two independent laboratory analyses provided exactly the same sequences which were also the expected sequences as published in the NIH genetic sequence database (GenBank). The DNA sequencing performed by forward and backward sequencing (on both strands) generated the correct sequence for the 4050 bp analysed. The uncertainty on the sequencing under those conditions can be considered as negligible. The estimated error probability of the sequence identification of each fragment is smaller than 0.0002 %. In addition, sequencing of the regions of DNA insertion in pIRMM-0086 generated during plasmid construction was performed at IRMM.

10 METROLOGICAL TRACEABILITY

The certified value is expressed as a number of DNA fragments per plasmid. ERM-AD415 is certified for the number of 108 bp fragments of 3' insert-plant specific (NK603 event) DNA and the number of 79 bp *hmg* DNA fragments in the plasmid pIRMM-0086. This number is determined on the basis of the full sequence of the plasmid pIRMM-0086 and is traceable to SI.

The purity of the preparation and the number of each cloned fragment was confirmed by endonuclease restriction. The use of identical vectors to construct pIRMM-0086 originating from single colonies ensures the presence of only one type of plasmid bearing one copy of the NK603 event and the *hmg* DNA fragments each. The sequence analysis confirmed the presence of only one type of plasmid in the pIRMM-0086 preparation.

End-point PCR followed by agarose gel electrophoresis (data not shown) as well as digital PCR were used to investigate the purity of ERM-AD415. No evidence for contamination of ERM-AD415 with respect to its certified properties was found. However, the ability of the methods applied to investigate the purity of the material was limited and can only ensure a purity of at least 90 %.

11 COMMUTABILITY

The two calibrants tested during the suitability study, pDNA ERM-AD415 and genomic DNA extracted from plant leaves (gDNA$_\ell$), led to significant differences in the measured copy number ratio value of ERM-BF415e (Table 3). Commutability [19] problems should be considered for the copy number ratio measurements of DNA extracted from food and feed samples. It could not be proven during the suitability study that one of the calibrants (pDNA/gDNA$_\ell$) behaves more similar to the gDNA extracted from seed powder (gDNA$_s$).

However, based on the traceability chain described above, as well as on additional practical reasons, which include full sequence characterisation, reproduction of additional batches of calibrant and availability, the pDNA ERM-AD415 is selected as the calibrant of choice to be used for the calibration of the event-specific method for the quantification of the NK603 event. The user should be therefore aware, that values measured with the NK603 event-specific real-time PCR method and calibrant ERM-AD415 are reproducible, however, values obtained with this measuring system may not be close to the true value.

12 INSTRUCTIONS FOR USE

12.1 INTENDED USE

ERM-AD415 is intended to be used as a calibrant exclusively with the method for quantification of the maize NK603 event validated by the EURL-GMFF [4] and modified with respect to the reference gene target according to Broothaerts et al. [3]. ERM-AD415 can therefore also be used for quantification of the NK603 event present in food and feed products containing this event.

When using ERM-AD415 for the calibration of real-time PCR for the quantification of the maize NK603 event, the value estimated from the real-time PCR measurement and its related ucertainty should be taken into consideration.

The recommended minimum sample intake is 50 µL to perform a dilution series (see Annex 2). A minimum sample intake of 5 µL is recommended to be used per real-time PCR assay.

12.2 HANDLING

The plasmid tube should be opened and handled under a laminar flow to reduce the risk of contamination. ERM-AD415 has a number concentration of approximately 2×10^6 cp/µL of the pIRMM-0086 plasmid. Dilution series should always be prepared freshly prior to a real-time PCR run. $T_1E_{0.01}$ buffer should be used as plasmid dilution buffer. Calibration curves can be prepared as recommended in ERM Application Note 5 [20]. A proposed dilution scheme for the preparation of a calibration curve is presented in Annex 2.

ERM-AD415 can be treated using *Sap*I or *Sac*I restriction endonucleases (unique restriction sites in the plasmid) so that the two cloned fragments present on pIRMM-0086 will remain intact. Restriction with *Sap*I will linearise the circular DNA molecule outside the space between the NK603 event and *hmg* fragments, whereas *Sac*I will digest pIRMM-0086 in the space between the two fragments so that they will be located at both extremities of the linearised plasmid.

12.3 TRANSPORT AND STORAGE

ERM-AD415 shall be dispatched with cooling elements and has to be kept at 4 °C or lower upon arrival. However, for long-term storage, keeping the material at -20 °C is recommended. The serial dilutions of the plasmid should be prepared freshly prior to the real-time PCR measurement.

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REFERENCES

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

[2] European Commission Recommendation (EC) N° 787/2004 of 4.10.2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003. Off J Eur Union L 348 (2004) 18-26.

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

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

[5] E.S. Dennis, W.L. Gerlach, A.J. Pryor, J.L. Bennetzen, A. Inglis, D. Llewellyn, M.M. Sachs, R.J. Ferl, W.J. Peacock. Molecular analysis of the alcohol dehydrogenase (Adh1) gene of maize Nucleic Acids Res 12(9) (1984) 3983-4000.

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

[7] M.E. Knight, C. Harn, C.E. Lilley, H. Guan, G.W. Singletary, C. MuForster, B.P. Wasserman, P.L. Keeling. Molecular cloning of starch synthase I from maize (W64) endosperm and expression in Escherichia coli. Plant J 14(5) (1998) 613-22.

[8] Living Modified Organism (LMO) Registry of the Biosafety Clearing-House (https://bch.cbd.int/database/lmo-registry/).

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

[10] P. Corbisier, S. Bhat, L. Partis, V.R. Xie, K.R. Emslie. Absolute quantification of genetically modified MON810 maize (Zea mays L.) by digital polymerase chain reaction. Anal Bioanal Chem 396(6) (2010) 2143-2150.

[11] P.G. Held. Measure your purity: assessment of nucleic acid purity via UV Absorbance. GIT Laboratory J 5-6/2008 (2008) 9-11.

[12] R.P. Novick. Plasmid incompatibility. Microbiol Rev 51(4) (1987) 381-395.

[13] E. De Andrade Silva, B. Jeynov, W. Broothaerts, A.M. Kortekaas, S. Vincent , S. Trapmann, P. Corbisier, H. Emons. Certification of a NK603 Maize Reference Material for its DNA copy number ratio. Certified Reference Material ERM®-BF415e (2011) ISBN 978-92-79-19074-2. EC certification report EUR 24699 EN.

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

[15] P. Corbisier, S. Broeders, D. Charels, S. Gioria, S. Trapmann, S. Vincent, H. Emons. Certification of plasmidic DNA containing MON 810 maize DNA fragments. Certified Reference Material ERM®-AD413. (2007) ISBN 978-92-79-07139-3. EC certification report EUR 22948 EN.

[16] Z. Zhang, S. Schwartz, L. Wagner, W. Miller. A greedy algorithm for aligning DNA sequences. J Comput Biol 2000 7(1-2) (2000) 203-14.

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

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

[19] ISO/IEC Guide 99-12:2007 International Vocabulary of Metrology – Basic and General Concepts and Associated Terms, VIM. Available via http://www.bipm.org/utils/common/documents/jcgm/JCGM_200_2008.pdf.
[20] P. Corbisier. Use of certified reference materials for quantification of GMO in DNA copy number. ERM Application Note 5 (2007). (http://www.erm-crm.org/ERM_products/application_notes/application_note_5/Pages/index.aspx)

ANNEX 1

Table 4: Primer and probe sequences used for the quantification of the NK603 event-specific real-time PCR.

	Sequence	**Reference**
ZM1-F:	5'-TTGGACTAGAAATCTCGTGCTGA-3'	[6]
ZM1-R:	5'-GCTACATAGGGAGCCTTGTCCT-3'	[6]
Probe ZM1:	5'-(6-FAM)-CAATCCACACAAACGCACGCGTA-(TAMRA)-3'	[6]
NK603 primer F:	5'-ATGAATGACCTCGAGTAAGCTTGTTAA-3'	[4]
NK603 primer R:	5'-AAGAGATAACAGGATCCACTCAAACAC-3'	[4]
NK603 probe:	5'-(6-FAM)-TGGTACCACGCGACACACTTCCACTC-(TAMRA)-3'	[4]

Table 5: Changes to the real-time PCR protocol published in the method validated by the EURL-GMFF [4].

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

ANNEX 2

An example for the preparation of calibration curves for the endogenous and transgenic targets is given in Table 6. Each ERM-AD415 calibrant is sufficient to prepare 10 calibration curves for each target (see also ERM Application Note 5 [20]).

Table 6: Preparation of calibration curves for the endogenous and transgenic targets

Starting concentration [cp/µL]	Resulting concentration [cp/µL]	PCR target[1)]	PCR target[2)]	Dilution factor	DNA [µL]	Plasmid dilution buffer [µL]
2 000 000	500 000			4	50	150
500 000	100 000	E		5	50	200
100 000	20 000	E		5	50	200
20 000	10 000		T	2	100	100
10 000	2 000	E	T	5	50	200
2 000	1 000	E		2	100	100
1 000	200	E	T	5	50	200
200	20		T	10	50	450
20	5		T	4	50	150

[1)] "E" refers to concentrations of the pDNA calibrant that will be used in rt-PCR for the detection of the endogenous target (namely 100 000 cp/µL, 20 000 cp/µL, 2 000 cp/µL, 1 000 cp/µL and 200 cp/µL).
[2)] "T" refers to concentrations of the pDNA calibrator that will be used in rt-PCR for the detection of the transgenic target (namely 10 000 cp/µL, 2 000 cp/µL, 200 cp/µL, 20 cp/µL and 5 cp/µL).

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Abstract

This report describes the preparation, characterisation, stability and suitability studies of the certified reference material (CRM) ERM®-AD415 which contains a plasmid (pIRMM-0086) carrying a defined deoxyribonucleic acid (DNA) fragment specific for a genetic modification present in *Zea mays* L. line NK603 as well as defined DNA fragments specific for the *Zea mays* taxon, of which one has been certified. The maize NK603 event is registered in the EU register of GM Food and Feed according to Regulation (EC) No 65/2004 and has received the unique identifier maize MON-ØØ6Ø3-6.

The CRM was processed in 2009 and certified in 2010 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE. The CRM is available in plastic tubes containing 500 µL of the plasmid DNA in 1 mmol/L Tris, 0.01 mmol/L EDTA pH 8.0 buffer ($T_1E_{0.01}$) and 1 ng/µl ColE1 plasmid used as background DNA. Each tube contains approximately 10^9 copies of the ERM-AD415 plasmid which correspond approximately to 4.1 ng of DNA.

The plasmid contains a 108 bp fragment of the region that spans the 3' insertion locus of the event NK603 and four fragments specific for the maize taxon: a 79 bp fragment of the maize endogenous *high mobility group* gene (*hmg*), a 151 bp fragment of the maize endogenous *starch synthase IIb* gene (*zssIIb*), a 135 bp fragment which targets to the 5'-untranslated region of the maize endogenous *alcohol dehydrogenase 1* gene (Zm*adh1* target) and a 70 bp fragment which targets to the junction between the second intron and the third exon of the maize endogenous *alcohol dehydrogenase 1* gene (*adh1* target).

The certified values are the numbers of cloned DNA fragments for the NK603 and *hmg* PCR targets per plasmid.

The minimum sample intake is 50 µL to perform the dilution series. A minimum sample intake of 5 µL is used per real-time PCR assay.

The intended use of this CRM is for calibration of the event-specific method for the quantification of the NK603 event validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), "Event-specific method for the quantitation of maize line NK603 using real-time PCR", available on the homepage of the European Union Reference Laboratory for Genetically Modified Food and Feed (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) published on 10/01/2005. With respect to the findings reported by Broothaerts et al. (2008) J Agric Food Chem 56(19): 8825-31, the method for the quantification of the maize NK603 event described here targets a 79 bp fragment from the endogenous maize gene *hmg* instead of the 70 bp fragment of the maize endogenous gene *adh1* used in the method validated by the EURL-GMFF.

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