Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of plasmidic DNA containing MON 810 maize DNA fragments

Certified Reference Materials ERM®-AD413

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CERTIFICATION REPORT

Certification of plasmidic DNA containing MON 810 maize DNA fragments

Certified Reference Materials ERM®-AD413

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ABSTRACT

This report describes the preparation, homogeneity, stability and certification studies of a plasmidic deoxyribonucleic acid (DNA) (ERM-AD413) containing a defined DNA fragment specific for a genetic modification present in *Zea mays* MON 810 event [1] as well as a defined DNA fragment specific for the *Zea mays* taxon.

The CRM was processed in 2005 and certified in 2007 by the European Commission, Directorate General Joint Research Centre, Institute for Reference Materials and Measurements (IRMM) in Geel, Belgium. The CRM is available in plastic tubes containing 500 µL of the plasmidic DNA in 1 mmol/L Tris, 0.01 mmol/L EDTA pH 7.0 buffer. Each tube contains approximately 10^9 copies of the ERM-AD413 plasmid which correspond approximately to 3.41 ng of DNA.

The plasmid contains a 170 bp fragment of the MON 810 *5′ plant-P35S* junction and a 351 bp fragment of the maize endogenous high mobility group gene (*hmg*) [2]. Both DNA sequences originated from DNA extracted from MON 810 seeds supplied by RAGT Semences (Rodez, France).

The certified value is the number of each cloned DNA fragments per plasmid. The number ratio between those two DNA fragments is given as an indicative value measured by duplex and simplex real-time PCR.

substance	**Certified value [number]**	**Uncertainty**
Fragment of *5′ plant-P35S* junction DNA / plasmid[1)]	1	negligible
Fragment of *hmg* DNA / plasmid	1	negligible

	Indicative value [number ratio]	**Uncertainty [4] [number ratio]**
Ratio between the numbers of *5′ plant-P35S* junction and *hmg* fragments in the plasmid	1.00 [2)] 1.04 [3)]	0.06 0.06

[1)] The 100 % sequence identity has been confirmed by dye terminator cycle sequencing of the *hmg* and *5′ plant-P35S junction* fragments both present in *Zea mays* MON-∅∅81∅-6.

[2)] The number ratio for the two DNA fragments has been characterised by duplex real-time PCR targeting each cloned fragments.

[3)] The number ratio for the two DNA fragments has been characterised by simplex real-time PCR targeting each cloned fragments.

[4)] The uncertainty is the expanded uncertainty estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) with a coverage factor k = 2, corresponding to a level of confidence of about 95 %.

Following the dilution protocol given in **Table 6**, the minimum sample intake is 50 µL to perform the dilution series. In the PCR tubes 5 µL are used per PCR reaction.

The intended use of this CRM concerns ONLY the calibration of the method ISO 21570:2005(E) D2 [3] for the quantification of the MON 810 event. The material must be used as calibrant to determine the number ratio of copies of the MON 810 per haploid genome as recommended in the Commission Recommendation (EC) No 787/2007 [4]. The CRM can alternatively be used to identify the MON-∅∅81∅-6 maize event as notified by

Monsanto Services International S.A./N.V. and registered in the EC Community Register of GM Food and Feed [1] according to Regulation (EC) No 65/2004 of 14.01.2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms [5].

GLOSSARY

$\bar{x}$	average
6-FAM	6-carboxylfluorescein dye
A	absorbance
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
bp	base pair
Bt	*Bacillus thuringiensis*
cp	number of copies
CRM	Certified Reference Material
Cry1A(b)[1]	δ endotoxin from *Bacillus thuringiensis*
CTAB	cetyltrimethylammonium bromide
Ct-value	number of PCR cycles to pass a set threshold
CV	coefficient of variation
DNA	deoxyribonucleic acid
ε	PCR efficiency
EDTA	ethylenediamine tetraacetic acid
*Eco*RI, *Hind*III, *Xba*I	restriction endonucleases
GMFF CRL	Community Reference Laboratory for Genetically Modified Food and Feed
GMO	genetically modified organism
hmg	high mobility group gene from *Zea mays* (taxon specific gene)
IncQ	plasmid incompatibility group Q
IRMM	Institute for Reference Materials and Measurements
LB	Luria-Bertani
LOD	limit of detection
LOQ	limit of quantification
MON 810	GM *Zea mays* event MON 810
MOPS	3-(N-morpholino)propanesulfonic acid
M	Molar mass
n	number of samples analysed
N	number of tubes analysed
N_A	Avogadro number
NCBI	National Center for Biotechnology Information
NIH	National Institute of Health
OD	optical density
oriV	plasmid origin of replication
PCR	polymerase chain reaction
R^2	correlation coefficient
RSD	relative standard deviation
rt-PCR	real-time PCR
s	standard deviation
S	size of a DNA fragment expressed in number of bp
SI	International system of units
RNA	ribonucleic acid
TAMRA	6-carboxytetramethylrhodamine
TE	Tris EDTA
TET	6-carboxy-2',4,7,7'-tetrachlorofluorescein (fluorescent dye)
U	expanded uncertainty
u	standard uncertainty
u^*_{bb}	standard uncertainty related to the between-tubes heterogeneity that can be hidden by the method repeatability
u_{lts}	standard uncertainty contributed by the long-term stability of the material
UNG	uracil N-glycosylase
UV	ultra violet
VIC™	fluorescent dye from Applied Biosystems
35S	35S promoter, derived from cauliflower mosaic virus

[1] Following international nomenclature the capital letter at the beginning refers to the protein, whereas lower case italic letters are used for the 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 [6]. 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. Until recently, GMO CRMs produced by IRMM or other providers have been produced by mixing GM powder with non-GM powder and certified for their GM mass fraction. The certified value was therefore also traceable to the SI.

In October 2004 the European Commission recommended to express the content of genetically modified (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 [4]. As a consequence, calibrants for the determination of the ratio of copies of transgenic and taxon specific genes are required.

Pure plasmid DNA molecules targeting MON 810 *5' plant-P35S* junction and *hmg* have proved to represent suitable calibrants for the quantification of gene copies of samples containing MON 810 maize [7]. Therefore, a pure DNA calibrant containing a maize DNA sequence specific to the MON 810 event and DNA sequence containing a *Zea mays* taxon specific sequence has been prepared in such a way that the ratio between the sequences is equal to 1.

The identity of both sequences has been confirmed by the dye terminator cycle sequencing method. The nucleotide sequence of the *5' plant-P35S* junction cloned into the plasmid calibrant was identical with the nucleotide sequence referred to as AF434709 in the NIH genetic sequence database (GenBank®) and corresponds to the synthetic construct transgenic *Zea mays* with Cauliflower mosaic virus genomic sequence present in MON 810 maize [8].

Likewise, the nucleotide sequence of the *hmg* fragment cloned into the plasmid calibrant was identical to the nucleotide sequence referred to as AJ131373 in the NIH genetic sequence database (GenBank®) and corresponds to the *Zea mays hmg* gene present in *Zea mays* species [2].

The transgenic maize line MON 810 *Zea mays* L. (Maize) Yieldgard® was genetically engineered to resist the European corn borer (*Ostrinia nubilalis*) by producing its own insecticide and is commercialised by Monsanto Company. This line was developed by introducing the *cry1A(b)* gene, isolated from the common soil bacterium *Bacillus thuringiensis* (Bt), into the maize cultivar Hi-II by particle acceleration (biolistic) transformation. The *cry1A(b)* gene produces the insect control protein Cry1A(b), a δ-endotoxin.

2. DESIGN OF THE PROJECT AND CERTIFICATION PROCEDURE

The major objectives of the project were the design, construction and processing of a plasmidic DNA reference material for the real-time PCR calibration and the consequent characterisation and assessment of the homogeneity, stability and commutability of the material.

The material is intended to be used as calibrant for the quantification of the MON 810 event expressed as the ratio between the number of copies of the MON 810 event and the number of copies of a taxon specific *Zea mays* gene. The plasmid calibrant is certified for containing one single DNA fragment of those 2 genes per plasmid. The cloned DNA fragments are also certified to be identical to the sequences published in the NIH genetic sequence database (GenBank®).

The relative number of copies of the MON 810 event per haploid genome of *Zea Mays* that is present in a DNA extract can be calculated applying the ISO 21570:2005(E)D2 quantification method using the ERM-AD413 calibrant.

The timing of the project was as follows:

- Processing of the material
  - Construction of the plasmidic DNA: May 2005
  - Processing and bottling: November 2006
- Commutability study completed: October 2006
- Storage of the sample for stability study: November 2006
- Homogenetity : January 2007
- Characterisation study: February 2007
- Stability study
  - Short term: November 2006 – December 2006
  - Long term 6 months: November 2006 – May 2007

3. PARTICIPANTS

Construction and processing, homogeneity and stability of the plasmid ERM-AD413

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Characterisation and commutability of the plasmid ERM-AD413 by rt-PCR

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*Measurements within the scope of accredidation to ISO/IEC 17025

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Evaluation and uncertainties

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4. PROCESSING OF THE MATERIAL

4.1. DESCRIPTION OF THE STARTING MATERIAL

Genomic DNA extracted from ground MON 810 *Zea mays* (cultivar DK 513 delivered by RAGT Semences, Rodez, France) seeds received for the processing of ERM-BF413 certified reference material was used as source of DNA to construct plasmid pIRMM-0011.
A 170 bp fragment containing part of the maize chromosome and part of the MON 810 transgenic sequence (*5' plant-P35S* junction sequence) was amplified by PCR using the gDNA extracted from MON 810 as template. The PCR product was purified using Qiagen PCR purification kit (Venlo, NL) and ligated into ligation-ready pT-Adv vector using a TA cloning kit (Invitrogen, Carlsbad, CA, USA). Transformants (50 μL ligation mixture) were selected on LB plates containing 10 μg/mL kanamycin and identified by PCR-insert screening. Positive clones were confirmed by restriction enzymatic digestion with *Eco*RI and by PCR with primers P-0271 and P-0272 on plasmid minipreps of pIRMM-0011 clones. The sequence of the primers is given in **Annex 1**. The *EcoR*I fragment of pIRMM-0011 was further amplified with the *EcoR*I cloning primers P-0342 and P-0343, digested with EcoRI and cloned into pUC18 (pIRMM-0014) to give plasmid pIRMM-0012. The plasmid pIRMM-0012 was digested by *Hin*dIII/*Xba*I and generated the correct bands confirming the insertion site of the fragment. Furthermore, the sequence identity of the cloned fragment was verified by dye terminator cycle sequencing which confirmed a 100 % sequence identity with the published sequence [8].

Additionally, a 351 bp fragment of the endogenous high mobility group (*hmg*) gene from maize was amplified by the high fidelity polymerase *Pfx* polymerase using the P-0063 and the P-0064 primers and genomic DNA extracted from MON 810 *Zea Mays* as template. The amplicon was cloned in pCR®2.1 (Invitrogen, Carlsbad, CA, USA) to obtain the plasmid called pIRMM-0034. This plasmid was digested with *Hind*III and *Xba*I to release the fragment specific for the *hmg* gene. Subsequently this fragment was ligated in the *Hind*III/*Xba*I restricted pIRMM-0012 using the rapid DNA ligation kit (Roche, Mannheim, DE) to yield the dual target plasmid (pIRMM-0036) (**Fig. 1**). The screening of the transformed *E. coli* colonies was performed by PCR using 2 primers flanking the border regions of the cloned fragments and the correct insertion was confirmed by restriction enzymatic digestion using *Eco*RI, *Xba*I, *Hind*III.

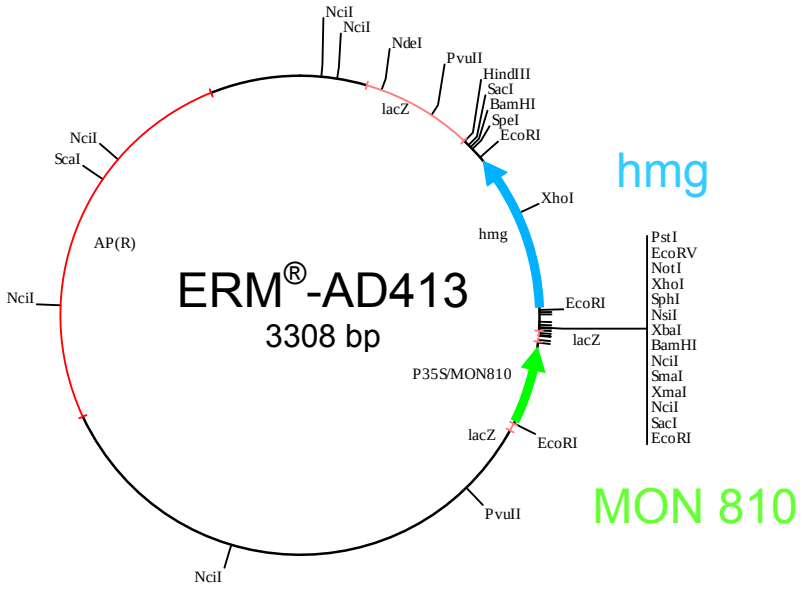


Figure 1: Circular map of ERM-AD413 representing the *5'plant-P35S* junction and *hmg* inserts as well as the enzymatic restriction sites.

4.2. PURITY OF THE MATERIAL

The purity of the plasmid pIRMM-0036 preparation was analysed by agarose gel electrophoresis and no other DNA bands than those expected after restriction of the pIRMM-0036 with *Eco*RI (**Fig 2.** lane 1-2) or *Hin*dIII/*Xba*I (**Fig 2.** lane 4-5) could be seen after ethidium bromide staining. This confirmed the correct cloning of the fragments.

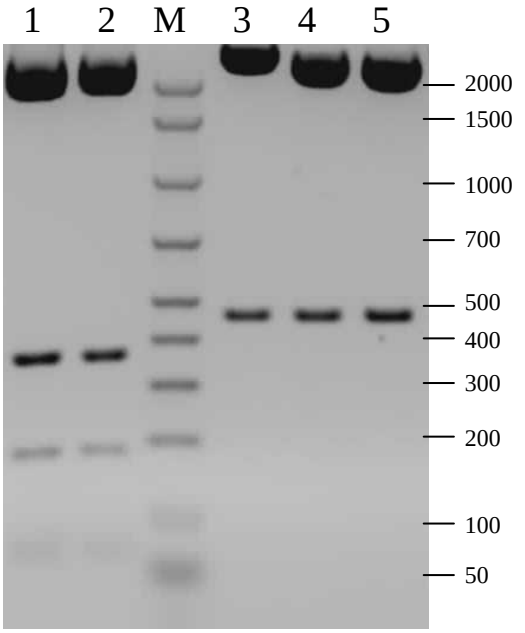


Figure 2: Restriction analysis of ERM-AD413
Lane 1-2: pUC18 vector (containing both targets) restricted with *Eco*RI (expected fragments: 72 + 186 + 367 + 2683 bp); Lane M: molecular DNA marker (BioRad 50-2000 bp); Lane 3: pCR2.1 vector (containing endogene target) restricted with *Hin*dIII/*Xba*I (expected fragments 461 + 3819 bp); Lane 4-5: pUC18 vector (containing both targets) restricted with *Hin*dIII/*Xba*I (expected fragments: 461 + 2847 bp).

As no smear was visible after restriction with *Eco*RI restriction enzyme of the plasmidic preparation and no RNA band was visible, it can be reasonably concluded that the plasmidic preparation was not contaminated with external genomic DNA or a relatively large amount of RNA molecules. However traces of genomic DNA from host bacterial cell or traces of RNA molecules can not be excluded in the final plasmidic preparation.
Such traces do not influence the target sequence ratio. Indeed a BLAST 2.0 analysis of the cloned target sequences did not reveal any nucleic acid sequences identity with a bacterial host DNA source present in the NCBI databases (data not shown).

Remaining traces of genomic DNA or RNA would not affect the measurement as the primers and probe used are highly specific for the targeted sequence and will not hybridise to other DNA fragments that could be present in the final preparation. Such traces represent, however, a bias in the DNA quantification of the plasmid solution by UV and therefore an erronous estimation of the number of plasmid copies in the tube. For that reason the DNA concentration in each tube can only be given with an indicative value.

During the ligation of the *Hind*III/*Xba*I fragment into pIRMM-0012 restricted by *Hind*III/*Xba*I, it cannot be excluded that a few undigested pIRMM-0012 and undigested pIRMM-0034 containing the *hmg* fragment were still present in the ligation mixture, even if a complete digestion of pIRMM-0012 was performed and no undigested plasmids were visible on agarose gel.

The *E. coli* cells could, consequently be transformed with 3 populations of plasmids: pIRMM-0036 present in large concentration, undigested pIRMM-0012, and undigested pCR2.1. However, as those 3 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 unique 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 both target DNAs were present and correctly cloned.

The specificity of the cloned sequences was verified by examining the PCR products from ERM-AD413 using several GM maize validated PCR methods. A sequence identification and amplification was confirmed with the MON 810 event-specific method [2] and, as expected, no amplification was observed with other methods targeting events such as Bt176, Bt11, NK603, MON 863, 3272, GA21, MIR604, 59122, and 1507 maize. The *hmg* target sequence was confirmed for all the maize events tested (**Annex 2**).

4.3. PROCESSING OF THE MATERIAL

2.5 L of LB media containing 100 µg/mL of Ampicillin were inoculated with 5 mL of a pre-culture of *E. coli* containing the plasmid pIRMM-0036 and shaken vigorously overnight at 37 °C. The culture was then centrifuged at 4 °C to recover a pellet of bacteria that was washed twice in 0.01 mol/L $MgCl_2$ and stored at -20 °C. This operation was repeated to obtain a pellet of bacteria from an initial culture of 5 L. The bacterial pellet was lysed in the presence of RNase following the recommended protocol for the QIAfilter GIGA kit (QIAGEN Benelux B.V., NL), purified on an anion-exchange column (GIAGEN-tip 10000) and eluted in buffer (50 mmol/L MOPS; pH 7.0; 15 % volume fraction isopropanol). The eluate was precipitated with isopropanol to remove remaining salt traces and further washed with 70 % volume fraction ethanol, air-dried and finally the purified DNA was resuspended in 16 mL of TE buffer 10 mmol/L pH 8.0. That plasmid solution was aliquoted and stored at -20°C.

The plasmid DNA solution was verified by UV spectrophotometry and fluorometry. A ratio of $OD_{260/280}$ equal to 1.73 and a DNA concentration of 3.19 mg/mL was measured.

This concentration was used to calculate the number of copies of plasmid that were present per µL, knowing the precise size of the plasmid and the average Molar mass (M) of a base using the following formula:

1 g of DNA contains *x* copies of a plasmid

With $x = N_A / [2 * M * S]$

where,

N_A = Avogadro number,
M = average molar mass of a nucleotide (expressed in g/mol),
S = size of the plasmid molecule (expressed in number of bp),

[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.

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

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

A solution of 2.5 L containing 2 x 10^6 cp/µL of the plasmid, corresponding to 6.8 pg DNA/µL was prepared by diluting the stock plasmid solution in a background of Col E1 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.

4.4. BOTTLING OF SAMPLES

The above plasmid solution was sterilised by filtration through a filter with a mesh of 0.2 µm and 5000 pre-labelled high recovery polypropylene tubes were filled manually each with 500 µL of the plasmidic solution under sterile conditions. Each tube contains therefore approximately 10^9 copies of the ERM-AD413 plasmid which correspond approximately to 3.41 ng of DNA.
The tubes were placed in cardboard boxes each containing 100 tubes. Samples for the homogeneity, short and long term stability studies, and for some additional characterisations were selected randomly from the 50 boxes. Each box was then sealed under light vacuum in a plastic pouch and frozen either at -20 °C or at -70 °C.

4.5. DISPATCHING OF SAMPLES

Samples were dispatched to the participating laboratories performing the commutability study and to two independent 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. METHOD USED FOR STABILITY AND HOMOGENEITY STUDIES

Simplex and duplex real-time quantitative PCR targeting a 92 bp fragment of the MON 810-*plant 5'-junction* region (further referred to as MON 810 event) and a 79 bp fragment of the *hmg* gene was used to identify and quantify the amount of both targets DNA present in the sample.

5.2. METHODS USED FOR CHARACTERISATION

DNA samples (10 μL total volume) were analysed by gel electrophoresis using a Minicell System (Bio-Rad Labs. Hercules. CA. USA) in a 1 % to 2 % volume fraction agarose gel containing ethidium bromide at a mass concentration of 0.5 μg/mL. Gels were run for 45 min at 100 V in a 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).

The sequencing was performed in-house on an ABI Prism® 3130xl Genetic Analyser (Applied Biosystems, Lennik, BE) using the BigDye® Terminator v1.1 Cycle Sequencing kit DNA sequence analysis.

The sequence identification and quantification of the MON 810 transgenic target was performed using an event-specific method targeting a 92 bp fragment of the MON810-plant 5'-junction region and a 79 bp fragment of the *hmg* gene used as normaliser. The final primer amount of substance concentrations in the PCR reaction was 300 nmol/L, while the amount of substance concentration of the probe was 160 nmol/L. TaqMan® Universal PCR analyses were carried out according to the manufacturer's instructions (Applied Biosystems, Lennik, BE) with universal 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 MasterMix containing AmpliTaq Gold® DNA polymerase and AmpErase® UNG to protect against subsequent re-amplifications of PCR products and to minimise carry-over contamination was used.

Five µL of template DNA was used in 25 µL final reaction volume for the simplex rt-PCR and the duplex rt-PCR. The MON 810 and the *hmg* probes were labelled with 6-FAM in the simplex PCR, whereas the *hmg* probe was labelled with VIC™ for the duplex rt-PCR.

In a duplex PCR reaction, both targets are simultaneously amplified with two sets of primers specific for the two nucleic acid target sequences. In addition, 2 TaqMan® probes specific for a small region located between the 2 primers, each labelled with a different reporter dye emitting light at different wavelengths are use in the real-time duplex PCR reaction. The fluorescence intensity detected is proportional to the number of target DNA sequences. The fluorescence is recorded on-line (in real-time) during the PCR amplification process.

5.3. METHODS USED FOR ADDITIONAL CHARACTERISATION MEASUREMENTS

The extracted DNA was quantified using the Picogreen® dsDNA Quantitation Kit (Molecular Probes, Eugene, USA) according to the kit 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 or Biophotometer Eppendorf). The ratios of the absorbance at 260 nm and 280 nm ($A_{260/280}$) and of the absorbance at 260 nm and 230 nm ($A_{260}/_{230}$) were calculated to provide an estimation of the quality of the extracted DNA.

6. HOMOGENEITY STUDY

6.1. PLANNING

The homogeneity of ERM-AD413 with respect to the copy number ratio was investigated by duplex rt-PCR using tubes selected according to a random stratified procedure. There was no reason to assume that the ratio of the two sequences would vary among the batch as it is an intrinsic property of the DNA sequence that does not differ. However, the quantity of plasmid present in the different tubes could vary from one tube to the other. The duplex-PCR allows to identify and quantity both targets at the same time. The ratios of those two targets were used to evaluate the homogeneity of the ERM-AD413.

For ERM-AD413, the homogeneity study consisted of twenty samples which were analysed under repeatability conditions using a sample intake of 5 µL. The plasmid solution was diluted in water to generate a calibration curve with the following plasmid number concentrations: 100000, 20000, 10000, 2000, 1000, 200 and 5 cp of the plasmid per µL. For each plasmid concentration the corresponding Ct value was measured in triplicate by real-time PCR for both gene targets. The Ct values correspond to the number of cycles needed to generate a fluorescent signal above a fixed threshold. The Ct values are inversely related to the number of copies of the plasmid present in the PCR well. The homogeneity of the ratio of both targets was also calculated by dividing the Ct obtained for the MON 810 sequence by the Ct obtained for the *hmg* sequence for each point of the calibration curves.

6.2. EVALUATION

Grubbs-tests were performed to detect potentially outlying individual results as well as outlying tube average results. No outlying individual result was detected for ERM-AD413 at a 95 % confidence level.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. No trends were observed for ERM-AD413.

It was furthermore checked whether the individual data and tube averages followed a normal or unimodal distribution using normal probability plots and histograms respectively. The individual data and the tube averages for ERM-AD413 followed a normal distribution. ANOVA statistics were used to calculate the between tube standard deviation (s_{bb}) and the maximum uncertainty related to the inhomogeneity that can be hidden by the method repeatability (u^*_{bb}), using the formulas:

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

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

Both values were converted into relative uncertainties by dividing them by the average of the study (**Table 1A** and **1B**). The same calculations were performed on the ratio of the two sequences. The uncertainty due to the heterogeneity between tubes ($u^*_{bb.\ rel}$) of the MON 810 transgenic/*hmg* sequence ratio of ERM-AD413 analysed by duplex rt-PCR using a sample intake of 5 µL (N = 20, n = 24) was 0.065 % whereas the relative between tube heterogeneity ($s_{bb.\ rel}$) was 0.72 %. The relative maximum hidden heterogeneity was included into the overall uncertainty of the certified values (**Section 8**).

Table 1: Estimation of uncertainties due to heterogeneity between tubes of the MON 810 transgenic sequence and the *hmg* sequence of ERM-AD413, analysed by duplex rt-PCR using a sample intake of 5 µL (N = 20, n = 3).

A- Relative maximum hidden heterogeneity ($u^*_{bb,\,rel}$) expressed in [%] for different number concentrations [cp/µL].

	cp / µL							
	100000	20000	10000	2000	1000	200	20	5
Target sequences								
hmg	0.25	0.15	0.13	0.15	0.12	0.15	0.14	0.37
MON 810 event	0.23	0.15	0.12	0.14	0.11	0.20	0.13	0.38

B- Relative between-tube heterogeneity ($s_{bb.\,rel}$) expressed in [%] for different number concentrations [cp/µL].

	cp / µL							
	100000	20000	10000	2000	1000	200	20	5
Target sequences								
hmg	1.01	0.96	0.67	0.91	0.91	1.10	0.60	0.53
MON 810 event	1.01	0.56	0.45	0.49	0.54	0.78	0.60	n/a

The material produced is sufficiently homogeneous and contains both targets.

7. STABILITY

7.1. SHORT-TERM STABILITY STUDY

7.1.1. Design of the short-term stability study

The short term stability of ERM-AD413 was evaluated by analysing 5 tubes stored at -20°C, 4 °C, 18 °C or 60 °C for 1, 2, and 4 weeks. Three sub-samples from each tube were analysed (N = 5, n = 3) at several dilution levels. A similar number of samples was stored at -70 °C as reference. Each sample was analysed in triplicate by duplex rt-PCR to reveal changes in the amount of the 2 sequences present in the test.

7.1.2. Results of the short-term stability study

The ratio of both sequences was also analysed but did not indicate any significant change at 60°C as both targets degrade at the same rate (**Fig. 3** and **Fig. 4**). However, it was important to determine the storage temperature at which no significant lost of material was observed. When scrutinising the data, no outliers were detected by the Grubbs-tests. Regression analysis was done for each of the storage temperatures to reveal any trend in Ct in relation to the time of incubation. A *t*-test showed a significant change over the time period of 4 weeks (95 % confidence interval) for the material kept at 18 °C and 60 °C. The same significant slopes of the linear regression were observed for the two analysed sequences. No significant slope (95 % confidence interval) was observed for both sequences at -20 °C and 4 °C, when testing the plasmids at a final concentration of 20000 or 4 cp/µL. The significant slope observed for the tubes kept at 18 °C, does not represent any loss of material as the Ct values are slightly lower than the control values measured for the tubes kept at -70 °C. The instability observed at 60 °C after 4 weeks affected both targets in the same proportion that the ratio between both targets remained unchanged (average ratio of 1.02 for 20000 cp/µL).. However, after storage at 60 °C, 200 cp/µL could not be amplified after 4 weeks (data not shown). The increased Ct values reflected here probably resulting from a partial lost or degradation of plasmids exposed to high temperatures.

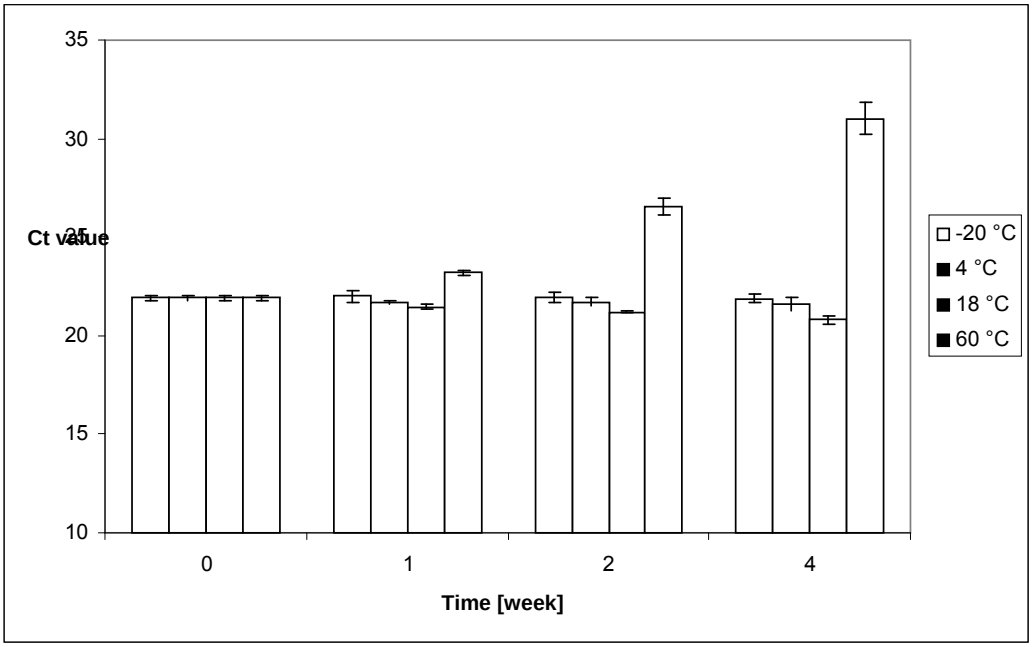


Figure 3: Short-term stability of ERM-AD413 stored at different temperatures for 0, 1, 2 and 4 weeks and analysed by *hmg* specific rt-PCR. The bars indicate the interval $\bar{x} \pm s$ for $N = 5$; $n = 3$.

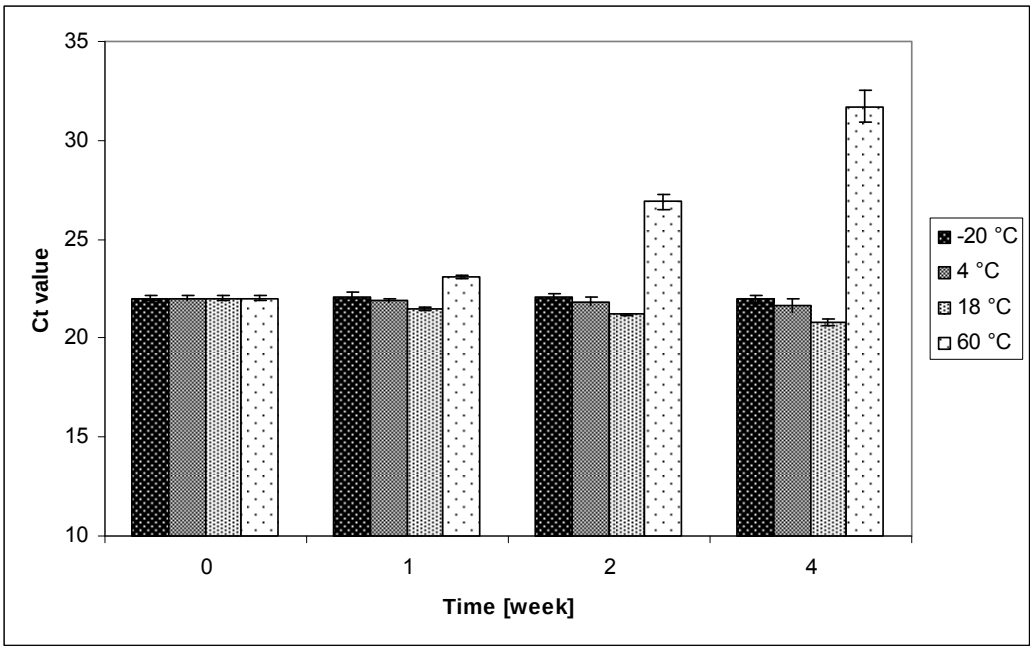


Figure 4: Short-term stability of ERM-AD413 stored at different temperatures for 0, 1, 2 and 4 weeks and analysed by MON 810 specific rt-PCR. The bars indicate the interval $\bar{x} \pm s$ for $N = 5$; $n = 3$.

7.1.3. Conclusion of the short-term stability study

It was concluded that the degradation during dispatch at temperatures above 18 °C is not negligible. It was therefore decided to ship ERM-AD413 frozen.

7.2. LONG-TERM STABILITY STUDY

7.2.1. Design of the long-term stability study

The long-term stability study was performed by placing tubes at -70 °C (reference temperature), -20°C and at 18 °C for time periods of 2, 4 and 6 months. Five different tubes were tested in triplicate (5 µL pDNA per PCR reaction) at each temperature by rt-PCR and the triplicate average is displayed in **Fig. 5** for both targets. The data were scrutinised and no outliers were detected with the Grubbs test. Fixing a maximum relative uncertainty (u_{lts}) for the long term stability study of 2.5 %, the shelf lives were calculated (**Fig. 6** and **Fig. 7**).

7.2.2. Results of the long-term stability study

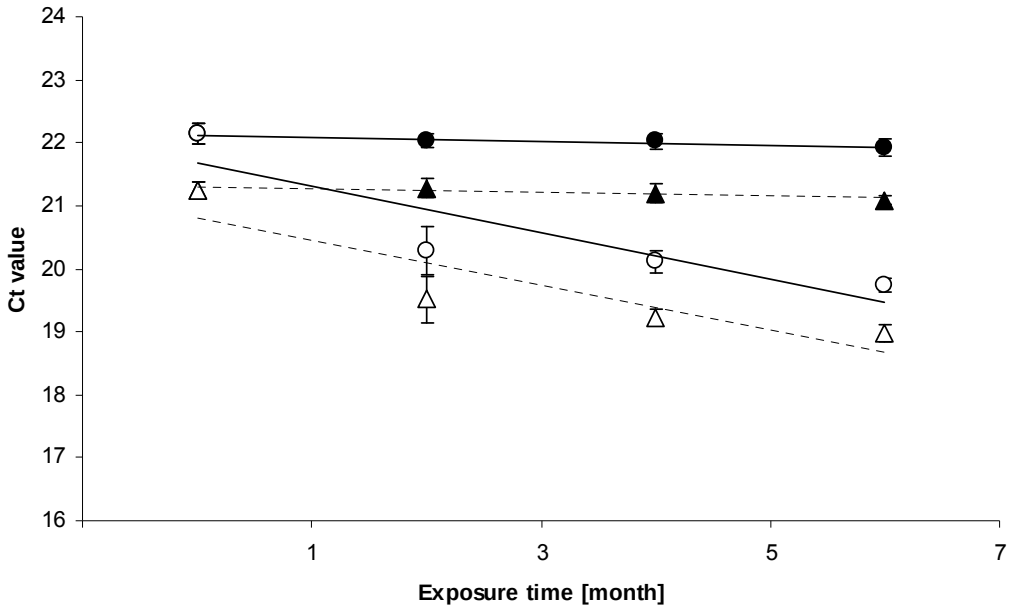


Figure 5: Long-term stability of ERM-AD413 stored for 2, 4 and 6 months at -20 °C (closed symbols) and 18 °C (open symbols). The average Ct values are given for the MON 810 fragments (circle) and the *hmg* fragments (triangle) at the concentration of 100000 cp/µL. (*N* = 5, *n* = 3).

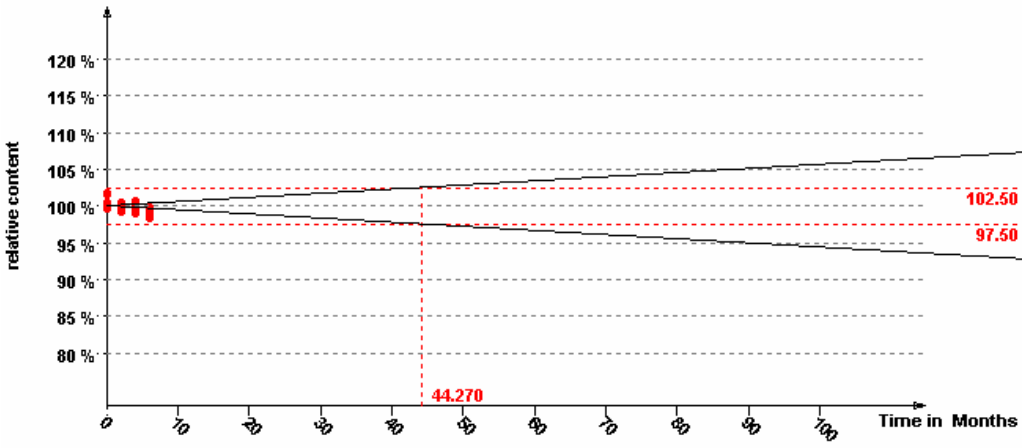


Figure 6: Calculated shelf life of MON 810 fragments kept at -20 °C with a relative uncertainty of 2.5 %

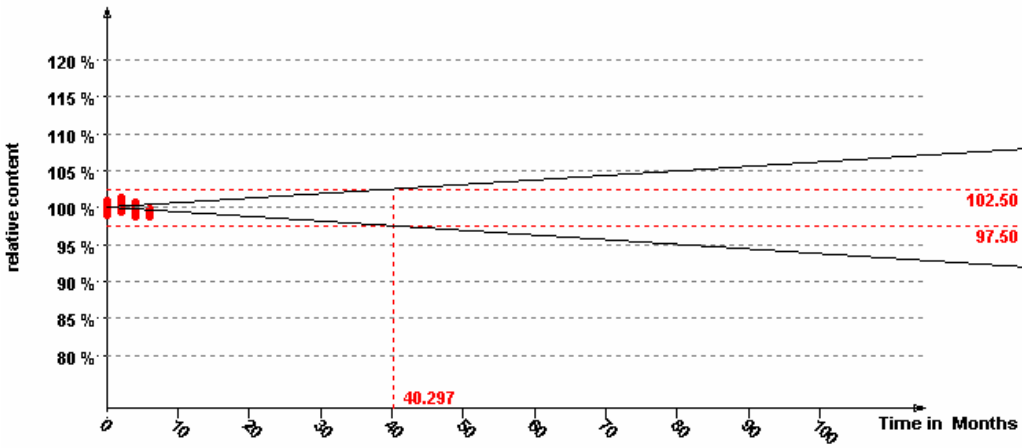


Figure 7: Calculated shelf life of *hmg* fragments kept at -20 °C with a relative uncertainty of 2.5 %

7.2.3. Conclusions of the long-term stability study

The material can be stored at -20°C with a minimum shelf life of 40 months taking into account a relative uncertainty (u_{lts}) of 2.5 %.

8. BATCH CHARACTERISATION

8.1. PLANNING

To verify the correct number ratio between the two cloned DNA fragments in the ERM-AD413 calibrant, the full sequence of the plasmid pIRMM-0036 was determined by 3 independent laboratories. The sequencing was performed in-house on an ABI Prism® 3130xl Genetic Analyser (Applied Biosystems, Lennik, BE) using the BigDye® Terminator v1.1 Cycle Sequencing kit. The companies QIAGEN Sequencing Services, (Hilden, DE) and Eurofins (Medigenomic GmbH, DE) were also asked to provide the full sequence of the ERM-AD413. The three sequences were identical and showed 100 % sequence identity (**Annex 3**) with the published sequences. The total size of the ERM-AD413 plasmid is 3308 bp (**Fig. 1**). The DNA sequence also confirms the ratio of 1 between the *hmg* and the MON 810 cloned fragments.

The determination of the number ratio between the two sequences was determined with the data generated by the homogeneity study performed by duplex rt-PCR as well as with the data generated from the commutability study and performed by simplex PCR. For the simplex PCR, twenty calibration curves comprising each 8 points were generated from twenty individual tubes. The Ct values for each data point were measured in triplicate for both gene targets. The ratios between the Ct values were calculated independently and represented the sequence ratio as determined experimentally. An average ratio ($\bar{x} \pm s$) of 1.004 ± 0.014 (N = 20, n = 24) was obtained. For the simplex PCR, 102 calibration points were measured for both targets and an average ratio ($\bar{x} \pm s$) of 1.04 ± 0.007 (N = 51, n = 2) was obtained. Both ratios correspond to the ratio of 1 as demonstrated by the analysis of the plasmid sequence.

8.2. ASSIGNMENT OF A CERTIFIED VALUE

The material is certified to contain one 170 bp *5' plant-P35S* junction fragment and one 351 bp *hmg* fragment per plasmid based on DNA sequencing. This number ratio was experimentally confirmed by simplex and duplex rt-PCR and given as an indicative value.

8.3. ADDITIONAL CHARACTERISATION MEASUREMENTS

8.3.1. Commutability study

Forty-three laboratories were selected on the basis of proven experience and quality assurance systems in place to investigate the commutability of the calibrant used for calibration of the rt- PCR measurements. The PCR efficiencies as well as the linearity of the calibration curves were compared between the plasmidic calibrant (ERM-AD413) and the gDNA extracted from certified maize powders prepared gravimetrically by dry-mixing of MON 810 GM powder and non-GM powder.

No significant differences between PCR efficiencies of gDNA and pDNA calibrants were found for the endogenous target *hmg* (p = 0.6) and for the transgenic target (p = 0.1) with the event-specific detection method (**Table 2**) indicating a similar PCR efficiency of the calibrant as compared to real samples. There was also no significant difference between both calibrants with respect to the ratios of PCR efficiencies of the transgenic to the endogenous target sequence (data not shown).

Table 2. Comparison of the PCR efficiencies (ε) of gDNA extracted from seed powder and pDNA calibrants by means of two-sided *t*-tests assuming unequal variances as the F-tests revealed significant differences between variances. *n* indicates the number of data sets; *p*, probability.

Target sequence	Average ε and *s* [%]		*p* for difference in ε between the 2 calibrants	*n*
	Seeds gDNA	Plasmid pDNA		
hmg	96.0 ± 7.7	95.4 ± 3.7	0.6	30
MON 810	92.1 ± 6.9	90.1 ± 5.3	0.1	30

The correlation coefficient (R^2) provides information about the fitting of data to a linear calibration curve. A comparison of the correlation coefficients of pDNA and gDNA calibration curves did not show significant differences for both PCR methods (p = 0.5 for all targeted sequences) (**Table 3**). However, it must be mentioned that entire data sets from single laboratories exhibiting a R^2 value below 0.98 for one of the calibration curves had been excluded beforehand as recommended in the definition of Minimum Performance Requirements for Analytical Methods of GMO Testing method validation of the Community Reference Laboratory for GM Food and Feed [9]. Such a low correlation coefficient was not accepted as it may reflect erroneous dilutions or inappropriate PCR amplification.

Table 3. *t*-tests to compare R^2 coefficients of pDNA and gDNA calibration curves for event-specific real-time PCR method. *p*, probability.

	Mean R^2		Variance		*n*	*p* [a]
	pDNA	gDNA	pDNA	gDNA		
hmg	0.999	0.999	4.02×10^{-7}	1.10×10^{-6}	30	0.5
MON 810	0.998	0.998	2.90×10^{-6}	2.04×10^{-6}	30	0.5

[a] At a 95 % confidence level, two-sided *t*-tests assuming unequal variances were performed as the F-tests revealed significant differences between variances.

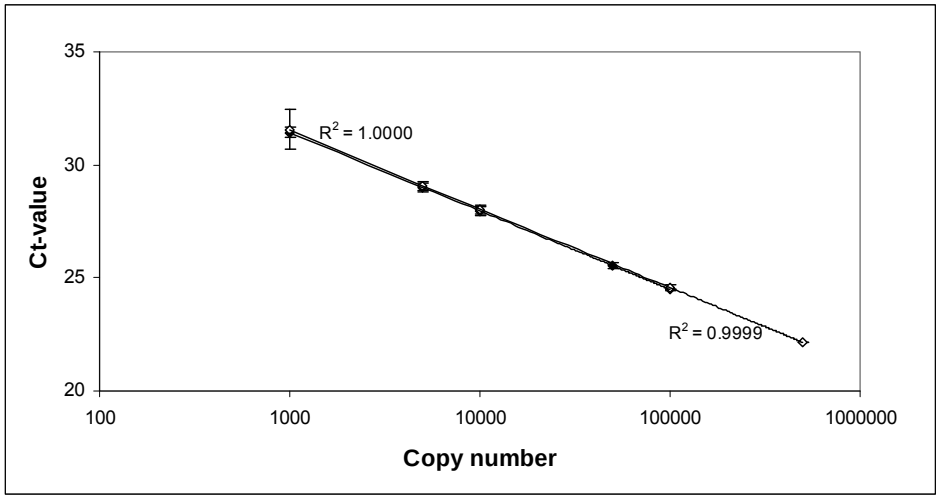
Subsequent multiple alignments of the PCR efficiencies of pDNA and gDNA are depicted in **Table 4**. Tukey and Newman-Keuls tests confirmed the observations of the two-sided *t*-tests summarised in **Table 2**.
There was no effect of the type of calibrant on the PCR efficiency for the transgenic MON 810 target sequence (p = 0.2 and 0.1 for Tukey and Newman-Keuls, respectively), nor for the hmg target (p = 0.8 and 0.6 for Tukey and Newman-Keuls, respectively).

Table 4. Probability *p* for differences in PCR efficiencies calculated by multiple alignments of PCR efficiencies (ε) of pDNA calibrants and gDNA calibrants.

	Tukey	Newman-Keuls
	p for difference in ε between pDNA and gDNA	*p* for difference in ε between pDNA and gDNA
hmg	0.8	0.6
MON 810	0.2	0.1

The statistical analyses have shown that pDNA and gDNA calibrants extracted from MON 810 maize leave tissues behave in a similar way for the parameters studied. pDNA and gDNA calibrants provided the same good linearity of calibration curves as can be also seen in **Fig 7**.

A



B

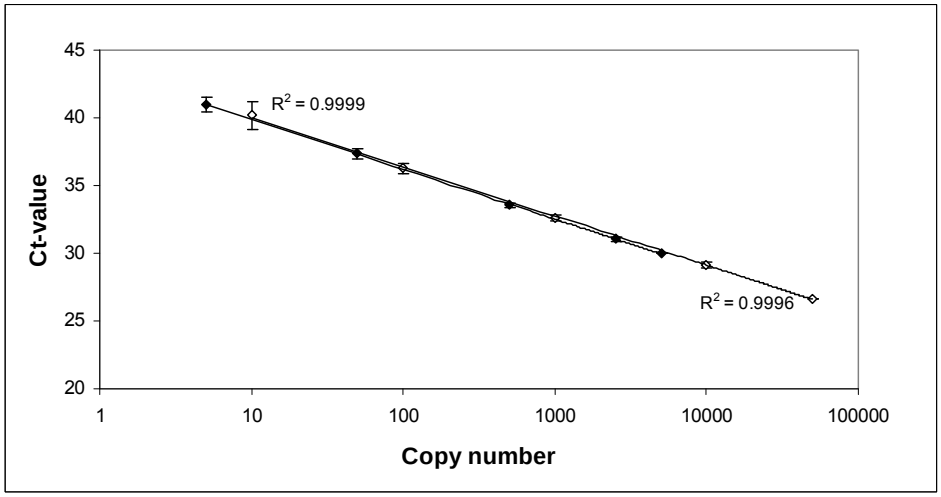


Figure 7. Alignment of pDNA (◊) and gDNA (♦) calibration curves (*n* = 58) for the endogenous and transgenic target sequences *hmg* (A) and MON 810 (B) of the event-specific detection method tested in simplex [2].

9. UNCERTAINTY BUDGET AND CERTIFIED UNCERTAINTY

9.1. UNCERTAINTY EVALUATION

9.1.1. Conceptual considerations

The plasmidic calibrant is characterised for two properties, the number of each specific fragment per plasmid and the ratio between the number of those two fragments. Based on the sequence analyses, it can be concluded that the MON 810 event and the *hmg* DNA fragments are present as a single copy per plasmid. Three independent laboratories provided exactly the same sequence which was 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 3308 bp analysed. The uncertainty on the sequencing under those conditions can be considered as negligible.The number ratio between the 2 fragments is also 1 as only 1 copy of each target was cloned and found in the plasmid ERM-AD413. This ratio could be measured experimentally by simplex and duplex real-time PCR. As the calibrant will be used for the calibration of PCR measurement, the experimental proof that an effective ratio of 1 can be measured is provided in this report. There is a marginal uncertainty on this ratio which does not depend on the nature of the calibrant itself but on the experimental procedure used for the measurement. This uncertainty on the ratio has been calculated and is reported in this Section.

9.1.2. Uncertainty source "homogeneity"

In order to verify a Gaussian distribution, normal probability plots were prepared of the homogeneity data versus their quantile values (data not shown). The straight lines obtained in these plots were an indication of a Gaussian distribution of the data.

The relative maximum hidden heterogeneity (u^*_{rel} bb) did not vary considerably across the calibration curves and varied in a similar way for both analysed targets (**Table 1**).The relative between tube heterogeneity ($S_{bb.rel}$) was higher than the relative maximum hidden heterogeneity and therefore also taken to determine the uncertainty of the homogeneity. Individual heterogeneity was determined separately for both sequences as well as the ratio heterogeneity of the ratio between the two targets. The relative between tube heterogeneity of that ratio was taken into consideration for the calculation and represented 0.0072 expressed in number fraction.

9.1.3. Uncertainty source "stability"

The material can be stored at -20 °C or below with a shelf life of 40 months. The calculated uncertainty associated to this shelf life is 0.025 expressed in number fraction. ERM-AD413 will be submitted to intensive stability monitoring, the shelf life may be extended as further evidence of stability becomes available.

9.1.4. Uncertainty source "batch characterisation"

An average ratio ($\bar{x} \pm s$) of 1.004 ± 0.014 (N = 20, n = 24) was obtained during the characterisation of the batch by duplex real time PCR. The uncertainty of this ratio was u_{char} = [0.014/√480] = 0.0007. The batch was further characterised during the commutability study by simplex PCR. An average ratio ($\bar{x} \pm s$) of 1.037 ± 0.007 (N = 51, n = 2) was then obtained with an uncertainty u_{char} = [0.007/√102] = 0.0007. The uncertainties associated to the two PCR methods applied were identical.

9.1.5. Uncertainty budget

The combined standard uncertainty of the indicative value comprises contributions from the the between-tubes inhomogeneity at the recommended sample intake of 5 µL, from the long-term stability of the material (calculated for 6 months) and the batch characterisation (**Table 5**). To calculate the expanded uncertainty corresponding to a 95 % level of confidence a coverage factor of 2 was used.

Table 5: Uncertainty budget for the number fraction of maize event MON 810 in ERM-AD413

CRM	Standard uncertainty contribution Number fraction			Expanded uncertainty U (k = 2) Number fraction
	u_1 a)	u_2 b)	u_3 c)	
ERM-AD413	0.0072	0.0251	0.0007	**0.06**

a) standard uncertainty contribution resulting from the homogeneity assessment.
b) standard uncertainty resulting from the stability of frozen plasmid during storage, extrapolated to 40 months.
c) standard uncertainty resulting from the characterisation measured either by simplex or duplex PCR.

9.2. CERTIFIED VALUES

The cloned DNA sequences are certified to be identical to the respective *hmg* and MON 810 PCR targets from the method validated by the GMFF CRL and to be present each as one single copy per plasmid. Based on the sequence and purity assessment the theoretical number ratio between the two targets is equal to 1.
When measured by rt-PCR and taking into account the PCR efficiency of both reactions it can be verified to be equal to 1.00 in duplex rt-PCR and to 1.04 in simplex rt-PCR. The uncertainty on that value depending mainly from the uncertainty component of the long-term stability is given in **Table 5**.

10. METROLOGICAL TRACEABILITY

The indicative number ratios are traceable to dye terminator cycle sequencing of the *hmg* and MON 810 DNA, to the duplex or simplex rt-PCR and the procedure described in references [8-9].

11. INSTRUCTIONS FOR USE

11.1. HANDLING AND INTENDED USE

The ERM-AD413 calibrant is intended to be used exclusively with the quantification method as carried out by the German Federal Institute for Risk Assessment (Berlin, DE) and assessed by the GMFF CRL (14/03/2006). The plasmid tube should be opened and diluted under a laminar flow to reduce the risk of contamination. It contains approximatively 2 x 10^6 cp/µL. To prepare the calibration curve, label 9 empty tubes from 5 x 10^5 cp/µL to 5 cp/µL. 1 mmol/L Tris, 0.01 mmol/L EDTA pH 8.0 buffer should be used as plasmid dilution buffer. Proceed with the dilution series as described in **Table 6**.

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

Starting concentration cp / µL	**Resulting concentration cp / µL**	[3]	[4]	**Dilution factor**	**DNA [µL]**	**Plasmid dilution buffer [µL]**
2 x 10^6	5 x 10^5			4	50	150
5 x 10^5	10^5	E		5	50	200
10^5	2 x 10^4	E		5	50	200
2 x 10^4	10^4		T	2	100	100
10^4	2000	E	T	5	50	200
2000	1000	E		2	100	100
1000	200	E	T	5	50	200
200	20		T	10	50	450
20	5		T	50	50	150

The dilution series should always be prepared freshly prior to a run. Each ERM-AD413 calibrant is sufficient to prepare 10 calibration curves for each target.

11.2. TRANSPORT AND STORAGE

The ERM-AD413 should be dispatched on dry-ice and kept at -20 °C upon arrival. The serial dilutions of the plasmid should be prepared freshly prior the real-time PCR measurement.

11.3. MINIMUM SAMPLE INTAKE

Following the dilution protocol given in **Table 6**, the minimum sample intake is 50 µL to perform the dilution series. In the PCR tube 5 µL are used per PCR reaction.

[3] "E" refers to the concentrations of the pDNA calibrant that will be used in rt-PCR for the detection of the endogenous target (namely 100000 cp/µL, 20000 cp/µL, 2000 cp/µL, 1000 cp/µL and 200 cp/µL).

[4] "T" refers to the concentrations of the pDNA calibrant that will be used in rt-PCR for the detection of the transgenic target (namely 10000 cp/µL, 2000 cp/µL, 200 cp/µL, 20 cp/µL and 5 cp/µL).

12. REFERENCES

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http://ec.europa.eu/food/dyna/gm_register/gm_register.cfm?gm_id=2

[2] 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

[3] ISO 21570:2005(E) Foodstuffs-Methods of analysis for the detection of genetically modified organisms and derived products-quantitative nucleic based methods. Annex D2 Event-specific method for the relative quantitation of maize line MON 810 DNA using real-time PCR. p 93-99.

[4] European Commission. 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

[5] Regulation (EC) No 65/2004 of 14.01.2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms.

[6] 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.

[7] D. Charels, S. Broeders, P. Corbisier, S. Trapmann, H. Schimmel, H. Emons, Towards metrological traceability for DNA fragment ratios in GM quantification Part 3: Suitability of DNA calibrants studied with a MON 810 corn model, J. Agric. Food Chem. 55 (2007) 3268-3274

[8] A. Holck, M. Vaitilingom, L. Didierjean, K. Rudi, 5'-nuclease PCR for quantitative event specific detection of the genetically modified Mon810 MaisGard maize. (2002) www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=18034386

[9] Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing. http://gmo-crl.jrc.it/guidancedocs.htm.

13. ACKNOWLEDGEMENTS

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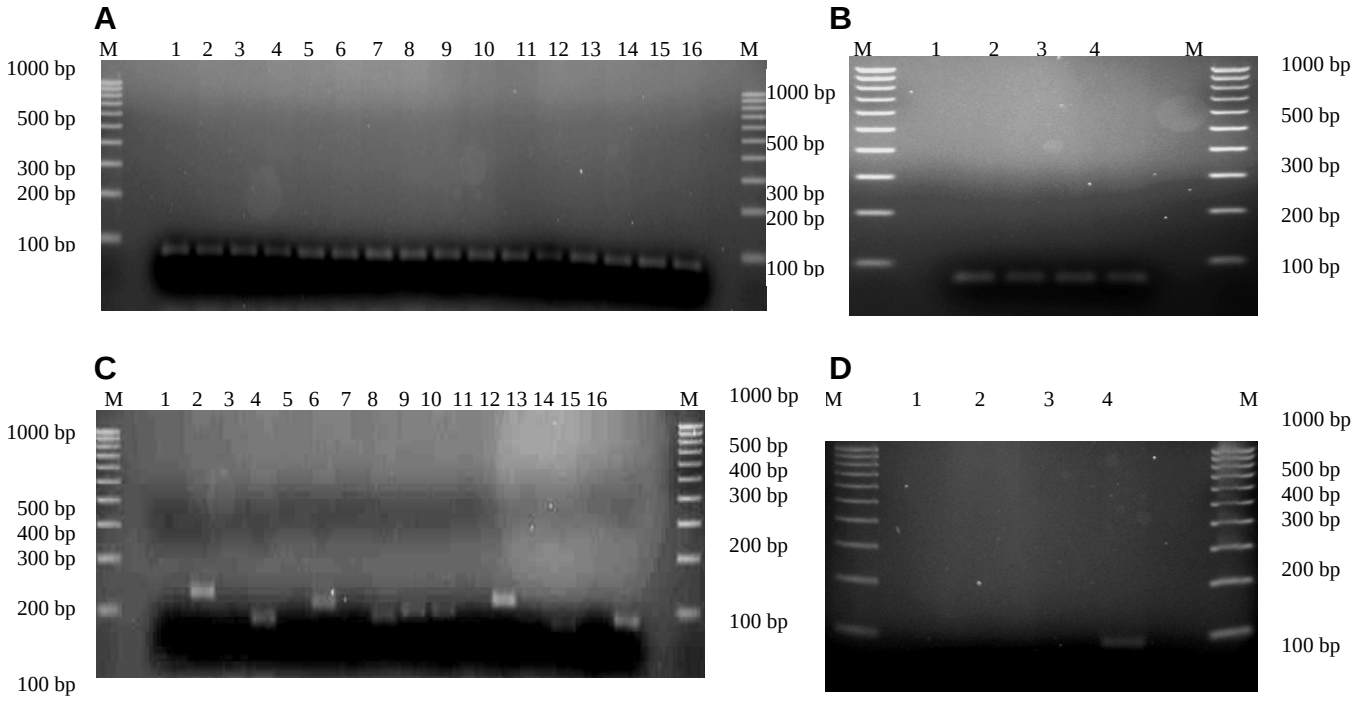
Annex 1 : Primer and probe sequences used for the quantification of the MON 810 event-specific rt-PCR and for the construction of ERM-AD413.

	Sequence (5' to 3')	**Reference**
MON 810 primer 1	TCg AAg gAC gAA ggA CTC TAA CgT	[2]
MON 810 primer 2	gCC ACC TTC CTT TTC CAC TAT CTT	[2]
MON 810 probe	6-FAM- AAC ATC CTT TgC CAT TgC CCA GC -TAMRA	[2]
P-0063	gAT TCC CCT CTC CTg gTC gA	this report
P-0064	CAA CAC ATg gTT CAg TAA gCA TAC g	this report
P-0271	TCg AAg gAC gAA ggA CTC TAA Cg	this report
P-0272	TCC ATC TTT ggg ACC ACT gTC g	this report
P-0342	CgA gCT Cgg ATC CAC Tag TAA	this report
P-0343	gCC AgT gTg ATg gAT ATC TgC	this report
ZM1 probe	6-(FAM)-CAA TCC ACA CAA ACg CAC gCg TA-TAMRA	[2]
ZM1-F primer 1	TTg gAC TAg AAA TCT CgT gCT gA	[2]
ZM1-R primer 2	gCT ACA TAg ggA gCC TTg TCC T	[2]

Annex 2 : ERM-AD413 specificity The specificity of the cloned sequences was verified by examining the PCR products from ERM-AD413 using several GM maize validated PCR methods.



The *hmg* endogenous gene for maize was evaluated by agarose gel electrophoresis. 20 µL of rt-PCR products were analysed on 2 % gel agarose. A band around 79 pb appeared for all of each samples using the *hmg* forward P-0302 and reverse P-0303 primers.

A : Lanes 1, 3, 5, 7, 9, 11, 13, 15: ERM-AD413; Lane 2: Bt176; Lane 4: Bt11; Lane 6: NK 603; Lane 8: MON 863; Lane 10: MON 810; Lane 12: GA21; Lane 14: MIR 604; Lane 16: 59122; M: DNA ladder 100 bp

B: Lanes 1, 3: ERM-AD413; Lane 2: maize 1507; Lane 4: maize 3272; M: DNA ladder 100 bp

Event-specific fragments targeting Bt176, Bt11, NK 603, MON 863, MON 810, GA21, MIR 604, maize 1507, maize 3272 where analysed using event specific primers. 20 µL of rt-PCR products were analysed on 2 % gel agarose.

C: Lanes 1, 3, 5, 7, 9, 11, 13, 15: ERM-AD413, Lane 2: Bt176; Lane 4: Bt11; Lane 6: NK 603; Lane 8: MON 863; Lane 10: MON 810; Lane 12: GA21; Lane 14: MIR 604; Lane 16: 59122; M: DNA ladder 100 bp

D: Lanes 1, 3: ERM-AD413; Lane 2: maize 1507; Lane 4: maize 3272; M: DNA ladder 100 bp

ANNEX 3: Sequence of ERM-AD413. The *hmg* fragment is highlighted in blue, whereas the 5' plant-P35S junction (MON 810 event) fragment is highlighted in green.

```
1     TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCG
101   TCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATA
201   CCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTAT
301   TACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAA
401   GCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGGCTTCAACACATGGTTCAGTAAGCATACGTATTTGAATAAATGACC
501   AGCAGGAGCAGTCTATATACACATTAAGTAACAACGCAATTGAAGCATCAGTTTCCCTACCTCGCCCTTGTTGTAGGCAGCGATGGCCTTGTTGTACTCG
601   AGCTTGAGCTTGTTAGCCTTGGCTACATAGGGAGCCTTGTCCTACAATCCACACAAACGCACGCGTAAAACAATTAATCAGCACGAGATTTCTAGTCCAA
701   TCAACTACTACTTTAGTACAGAAGATGGAGCTTACCGACTCGCTCAGGGATTTCCACCTGTCGCCAGCAGCTTTCCCCACCTGAAAAGTTCGACCAGGAG
801   AGGGGAATCAAGCCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGGCTTTCC
901   ATCTTTGGGACCACTGTCGGCAGAGGCATCTTCAACGATGGCCTTTCCTTTATCGCAATGATGGCATTTGTAGGAGCCACCTTCCTTTTCCACTATCTTC
1001  ACAATAAAGTGACAGATAGCTGGGCAATGGCAAAGGATGTTAAACGTTAGAGTCCTTCGTCCTTCGAAAGCCGAATTCGTAATCATGGTCATAGCTGTTT
1101  CCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACAT
1201  TAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTAT
1301  TGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCC
1401  ACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGC
1501  TCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTC
1601  CCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGG
1701  TATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTG
1801  AGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAA
1901  GTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCC
2001  GGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTA
2101  CGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATG
2201  AAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGT
2301  TCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCT
2401  CACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTG
2501  TTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATG
2601  GCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCA
2701  GAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGA
2801  GTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTA
2901  AAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACT
3001  GATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTG
3101  AATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAA
3201  ATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGC
3301  CCTTTCGT
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Abstract
This report describes the preparation, homogeneity, stability and certification studies of a plasmidic deoxyribonucleic acid (DNA) (ERM-AD413) containing a defined DNA fragment specific for a genetic modification present in *Zea mays* MON 810 event [1] as well as a defined DNA fragment specific for the *Zea mays* taxon.

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