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JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

# Event-specific Method for the Quantification of Maize MON 87403 Using Real-time PCR

# Validation Report

European Union Reference Laboratory for
Genetically Modified Food and Feed

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EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Health, Consumers & Reference Materials
Food & Feed Compliance Unit

# Event-specific Method for the Quantification of Maize MON 87403 Using Real-time PCR

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**20 April 2018**

**European Union Reference Laboratory for GM Food and Feed**

## Executive Summary

In line with its mandate[a] the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), validated an event-specific real-time polymerase chain reaction (qPCR) method for detecting and quantifying maize event MON 87403 (unique identifier MON-874Ø3-1). The validation study was conducted according to the EURL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and the relevant internationally accepted guidelines [1-5].

In accordance with current EU legislation[b], Monsanto Company represented by Monsanto Europe S.A. provided the detection method and the positive and negative control samples (genomic DNA extracted from homogenised seeds of MON 87403 maize as positive control DNA, and genomic DNA extracted from homogenised seeds of conventional maize as negative control DNA). The EURL GMFF verified the method performance data provided by the applicant, where necessary experimentally, prepared the validation samples (calibration samples and blind samples at different GM percentage [copies GM/total maize genome copies]), organised an international collaborative study and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL, in line with the provisions of Annex III-3.C.2 to Regulation (EU) No 503/2013, and it fulfils the analytical requirements of Regulation (EU) No 619/2011[c]. This validation report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

[a] Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed".
[b] Regulation (EC) No 503/2013 of 3 April 2013 "on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006".
[c] Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired.

## Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: BELAC 268-TEST (Flexible Scope for DNA extraction, DNA identification and real-time PCR) and ISO 17043:2010 accredited (certificate number: BELAC 268-PT, proficiency test provider).

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.


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# Content

# 1. Introduction

In line with Regulation (EC) No 1829/2003, Monsanto Company represented by Monsanto Europe S.A. provided the EURL GMFF with an event-specific method for detection and quantification of maize event MON 87403 (unique identifier MON-87403-1) together with genomic DNA as positive and negative control samples.

The dossier was found complete (step 1 of the EURL GMFF validation procedure) and the scientific dossier assessment (step 2) concluded that the reported method performance characteristics, assessed against the ENGL method acceptance criteria[d], allowed moving the method forward to step 3 of the procedure (experimental testing), where the EURL GMFF verified the purity of the control samples provided and conducted an in-house testing of samples and method.

The positive and negative control DNA, submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Regulation (EC) No 1829/2003, were found of good quality in terms of molecular weight and degree of purity.

Step 3 was completed with the conclusion that the method could be submitted to the collaborative study (step 4). This study confirmed that the method is well suited for quantifying genomic DNA of GM maize MON 87403, appropriately extracted from food or feed, down to a GM content level of 0.1% m/m.

# 2. Step 1 (dossier acceptance) and step 2 (scientific dossier assessment and bioinformatics analysis)

Documentation and data supplied by the applicant were evaluated by the EURL GMFF for completeness (step 1) and compliance with the ENGL method acceptance criteria (step 2).

The specificity of the event-specific assay was verified by the applicant and confirmed by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

Specificity assessment by the applicant

The specificity of the event-specific assay was assessed by the applicant in real-time PCR reactions, according to the method described and using 200 ng genomic DNA extracted from maize MON 87403 seeds: rapeseed RT73, MON 88302; maize MON 87403, NK603, MON 810, MON 863, MON 88017, MON 89034, MON 87460, MON 87427; cotton MON 531, MON 15985, MON 1445, MON 88913, MON 88701; soybean 40-3-2, MON 89788, MON 87769, MON 87701, MON 87705, MON

[d] EURL/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm)

87708; alfalfa J101, J163, KK179 and conventional rapeseed, conventional maize, conventional cotton, conventional soybean, conventional wheat, conventional alfalfa, lentils, sunflower, rice, quinoa, and millet. The real-time PCR reactions were performed in duplicate for each extract.

According to the method developer the MON 87403 event-specific assay did not react with any sample except the positive control.

The taxon-specific assay is based on the detection and quantification of the *high mobility group* (*hmg)* maize endogenous gene and is found to be fully specific to maize. The *hmg* assay amplifies a single copy target sequence per haploid genome equivalent.

Its specificity was assessed by the method developer in real-time PCR reactions, according to the method described, using 200 ng genomic DNA extracted from maize MON 87403 seeds: rapeseed RT73, MON 88302; maize MON 87403, NK603, MON 810, MON 863, MON 88017, MON 89034, MON 87460, MON 87427; cotton MON 531, MON 15985, MON 1445, MON 88913, MON 88701; soybean 40-3-2, MON 89788, MON 87769, MON 87701, MON 87705, MON 87708; alfalfa J101, J163, KK179 and conventional rapeseed, conventional maize, conventional cotton, conventional soybean, conventional wheat, conventional alfalfa, lentils, sunflower, rice, quinoa, and millet. The real-time PCR reactions were performed in duplicate for each extract.

According to the method developer the maize-specific reference system did not react with any sample except the positive control maize lines.

Bioinformatics specificity assessment by the EURL GMFF

The detection method spans the 3' insert-to-plant junction in maize MON 87403. The forward primer "87403 QF" binds to the insert. The reverse primer "87403 QR" binding site was found in the maize (*Zea mays*) genomic border adjacent to the insertion. The probe "87403 QP" binds to the junction between the insert and the 3' genomic region of *Zea mays*.

The amplicon size expected to be 88 bp was consistent with the sequence reported by the applicant. The sequence of the amplicon was analysed by BLASTN program search (NCBI) against local copies of the nucleotide and patent databases, and no significant similarity was found with any other published sequence. In addition, the primers were tested against the sequences of the GMO events present in the Central Core Sequence Information System (CCSIS) of the JRC, as well as the whole genomes of more than 80 plant species (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays*) using the e-PCR prediction tool (NCBI), and no potential amplicon was identified.

Verification of the ENGL acceptance parameters

The parameters of four calibration curves (slope, $R^2$ coefficient) prepared from a standard DNA solution (S1) of 10% maize event MON87403 genomic DNA (expressed as mass fraction of GM-material) which was serially diluted in 0.2x TE Buffer fourfold to obtain the standard solutions S2, S3 and S4 and fivefold to obtain the standard solution S5, were reported as provided by the applicant (Table 1).

Table 1. Slope and $R^2$ values* obtained by the applicant

| **MON 87403** | | ***hmg*** | |
|---|---|---|---|
| **Slope** | ***R²*** | **Slope** | ***R²*** |
| -3.50 | 0.9983 | -3.36 | 1.0000 |
| -3.49 | 0.9988 | -3.40 | 0.9999 |
| -3.49 | 0.9969 | -3.38 | 0.9997 |
| -3.40 | 0.9999 | -3.39 | 1.0000 |

* Numbers are not rounded but are presented as reported by the applicant

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.6 to -3.1 and the $R^2$ coefficient shall be ≥ 0.98.

Table 1 indicates that the slope and $R^2$ coefficient of the standard curves for the GM-system (MON 87403) and the maize-specific *high mobility group* (*hmg*) system, as established by the applicant, were within the ENGL acceptance criteria.

Precision and trueness of the method were also established by the applicant and are reported in two tables, one containing the results of the experiment for the determination of the trueness (Table 2) and one with the results of the experiments for the determination of the precision (Table 3). Table 2 is based on one real-time PCR run and averages of 14 replicate values for each of 3 GM levels (expressed as mass fraction of GM-material) are reported as provided by the applicant. The values reported in Table 3 are based on three real-time PCR runs, each with 13-15 replicate values for each of the 3 GM levels. Precision and trueness values for the three GM levels are reported as provided by the applicant.

Both parameters were within the ENGL acceptance criteria (trueness ± 25%, $RSD_r$ ≤ 25% across the entire dynamic range).

Table 2. Mean %, precision and trueness values* provided by the applicant in the experiment for the determination of trueness

| | Test results | | |
|---|---|---|---|
| **Expected GM %** | **0.085** | **1.0** | **10.0** |
| Measured mean GM % | 0.0747 | 0.810 | 8.33 |
| Precision ($RSD_r$ %) | 10.72 | 2.92 | 3.23 |
| Trueness (bias %) | -12.11 | -18.97 | -16.67 |

* Numbers are not rounded but are presented as reported by the applicant

Table 3. Mean %, precision and trueness values* provided by the applicant in the experiment for the determination of precision

| | Test results | | |
|---|---|---|---|
| **Expected GM %** | **0.085** | **1.0** | **10.0** |
| Measured mean GM % | 0.0741 | 0.88 | 8.58 |
| Precision ($RSD_r$ %) | 3.83 | 2.28 | 1.33 |
| Trueness (bias %) | -12.77 | -11.52 | -14.22 |

* Numbers are not rounded but are presented as reported by the applicant

# 3. Step 3 (experimental testing of the samples and method)

## 3.1 DNA extraction

Genomic DNA was isolated by the applicant from ground maize seeds, using a "CTAB-based" protocol coupled with PEG purification previously submitted for detection of maize event MON 88017.

This protocol had already been validated by the EURL GMFF. The protocol for DNA extraction and a report on testing were published in 2018 at http://gmo-crl.jrc.ec.europa.eu/summaries/CRL-VL-16-05-XP-Corrected-version-2.pdf.

In agreement with the ENGL position, endorsing the modularity principle (see also Annex III to Reg. (EU) No 503/2013), and given the similarity in the matrix, the EURL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for maize event MON 87403.

Whenever DNA is extracted from more complex matrices, a thorough control of the quality of the DNA is recommended in order to ensure that the DNA has the required quality for subsequent PCR analysis.

## 3.2 Method protocol for the PCR analysis

The PCR method provided by the applicant is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event MON 87403 DNA to total maize DNA. The procedure is a simplex system, in which a maize specific assay targeting the endogenous gene *high mobility group* (*hmg*), and the GM target assay for MON 87403 are performed in separate wells. The validated method protocol is described as "validated method" in Annex 1 and is published by the EURL GMFF at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

For the detection of GM event MON 87403, an 88 bp fragment of the region spanning the 3' insert-to-plant junction in maize MON 87403 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of GM event MON 87403, a maize taxon-specific system amplifies a 79 bp fragment of a maize *high mobility group* (*hmg*) endogenous gene, using *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM as reporter dye at its 5' end and TAMRA as quencher dye at its 3' end.

Standard curves are generated for both the MON 87403 and the *hmg* systems by plotting the Cq values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves.

For the relative quantification of event MON 87403 DNA in a test sample, the MON 87403 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value (GM % = MON 87403/*hmg* x 100).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (expressed in picograms) by the published average 1C value for the maize genome (2.73 pg) [(6)]. The copy number values used in the quantification, the GMO contents of the calibration samples, and the total DNA quantity used in the PCR reactions are listed in Table 4.

*Note: Numerical values presented in the following tables were rounded keeping three digits for values ≤ 0.1, two digits for values between 0.1 and 1, one digit for values between 1 and 10 and no digit for values ≥ 10, unless otherwise stated. The calculations in the MS Excel files however were done over not rounded data. This approach might create small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.*

Table 4. Copy number values of the standard curve samples

| Sample code | S1 | S2 | S3 | S4 | S5 |
|---|---|---|---|---|---|
| Total amount of maize DNA in the reaction (ng) | 200 | 50 | 13 | 3.1 | 0.78 |
| Target taxon *hmg* copies | 73260 | 18315 | 4579 | 1145 | 286 |
| Target MON 87403 copies | 7326 | 1832 | 458 | 114 | 29 |

## 3.3 EURL GMFF experimental testing

### 3.3.1 Determination of the zygosity ratio in the positive control sample

Annex II of Regulation (EU) No 619/2011 requires that "when results are primarily expressed as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EURL GMFF." This requires knowledge of the zygosity of the event. In order to satisfy this requirement, the EURL GMFF assessed the zygosity (GM-target to reference target ratio) in the positive control sample submitted by the applicant.

The copy number of the MON 87403 and of the *hmg* targets in the positive control sample were determined by digital PCR (dPCR) performed on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Reaction mixes were prepared for five replicates to a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix (Applied Biosystems, cat. number 4318157), 1X GE sample loading reagent (Fluidigm PN 85000746), primers and probes at the concentrations indicated in the validated method in Annex 1 and 1 µL of DNA at a concentration of 4 ng/µL; the DNA concentration was adjusted to avoid panel saturation (optimal between 200<positive partitions<700).

Loading of the digital chip was performed according to the manufacturer's instructions by using the IFC controller (Fluidigm). Approximately 4.6 µL of the reaction mixes were loaded into each well and distributed into the 765 partitions constituting one panel. The experiment was repeated three times for a total number of thirteen data sets for the GM target and fifteen for the reference target. 'No template controls' were included. Amplification conditions were as reported in Annex 1. Data analysis and copy number calculations were performed using the BioMark digital PCR Analysis software. The range of Cq retention was from 15 to 35.

Calculations of means and variances were carried out according to the procedure outlined for random variables in the Annex 4 of the ENGL guidance document 'Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods'[e].

### 3.3.2 In-house verification of the method performance against ENGL method acceptance criteria

The method performance characteristics were verified by quantifying on a copy number basis five blind test samples distributed over a range of GM levels (0.058 %-5.0 %). The experiments were performed on an ABI 7500, an ABI 7900HT and a Roche LC480 real-time platform under repeatability conditions and followed the protocol provided by the applicant. Test samples with GM levels 0.40 %, 0.9 %, 2.0 % and 5.0 % were tested in two real-time PCR runs with two replicates for each GM level on each plate (total of four replicates per GM level). The test sample with GM level 0.058% (corresponding to 0.10 % in mass fractions of GM material) was tested in 15 replicates in an additional run for each platform. On Roche LC480 platform test samples with GM levels 0.40 %, 0.9 %, 2.0 % and 5.0 % were run at 45 cycles and analysed with the second derivative maximum method; the test sample with GM level 0.058 % was run at 40 cycles and analysed with the fit point method. Average values of the slope and of the $R^2$ coefficient of the standard curves and method trueness and precision over the dynamic range were evaluated against the ENGL method acceptance criteria.

## 3.4 International collaborative study (step 4)

The international collaborative trial involved twelve randomly selected laboratories, all being "National reference laboratories, assisting the EURL for testing and validation of methods for detection", as listed in annex to Regulation (EU) No 120/2014 who had expressed their interest in participating. The study was carried out in accordance with the following internationally accepted guidelines:

- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) (1)
- ISO 5725 "Accuracy (trueness and precision) of measurement methods and results", Part 1 and Part 2 (ISO, 1994); ISO 5725-1:1994/Cor 1 (ISO 1998) and ISO 5725-2:1994/Cor 1 (ISO, 2002) (2-5)

[e] Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods. Version 2. European Network of GMO Laboratories (ENGL), 2017. http://gmo-crl.jrc.ec.europa.eu/ENGL/docs/WG-MV-Report-version-2.pdf

The objective of the international collaborative study was to verify in experienced laboratories the trueness and precision of the PCR analytical method provided by the applicant and verified in-house by the EURL GMFF.

### 3.4.1 List of participating laboratories

The twelve laboratories participating in MON 87403 international collaborative study (see Table 5) were randomly selected from the 32 National Reference Laboratories (NRLs) that offered to participate.

Clear guidance was given to the selected laboratories for strictly following the validation protocol provided.

Table 5. Laboratories participating in the validation of the detection method for maize event MON 87403

| Laboratory | Country |
|---|---|
| Agricultural Technology Center Augustenberg - Forchheim | DE |
| Crop Research Institute - Reference Laboratory for GMO Detection and DNA Fingerprinting | CZ |
| General Chemical State Laboratory (GCSL), Food Division - Athens | GR |
| INIAV UEISTSA - Laboratório de Microbiologia Agro-Industrial | PT |
| Italian National Institute for Health - Department of Veterinary Public Health and Food Safety - Unit GMOs and Mycotoxins | IT |
| Laboratorio Arbitral Agroalimentario | ES |
| Landeslabor Schleswig-Holstein - Food, Veterinary and Environmental Diagnostic Institute | DE |
| National Food and Veterinary Risk Assessment Institute Molecular Biology and GMO Department | LT |
| Office for Consumer Protection of the German Federal State Saarland- Saarbrücken | DE |
| State Institute of Chemical and Veterinarian Analysis - Freiburg | DE |
| State Office for Agriculture, Food safety and Fisheries - Mecklenburg Western Pomerania- Rostock | DE |
| Walloon Agricultural Research Centre - Department Valorisation des productions (D4) - Unit 16 - Authentication and traceability | BE |

### 3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: three laboratories used ABI 7500, one used ABI PRISM 7700, two ABI 7900HT, one ABI StepOnePlus, one ABI QuantStudio 6 Flex, one Bio-Rad CFX, one Qiagen Rotor-Gene Q, one Stratagene Mx3005P, and one used Roche LC480 II.

This variability of equipment, with its known potential influence on PCR results, reflects the real-life situation in the control laboratories and provides additional assurance that the method is robust and usable under real conditions.

### 3.4.3 Materials used in the international collaborative study

For the validation of the quantitative event-specific method, test samples were provided by the EURL GMFF to the participating laboratories. They were derived from:

*i)* genomic DNA extracted by the applicant from homogenized heterozygous maize seeds harbouring the MON 87403 event, and
*ii)* genomic DNA extracted by the applicant from homogenized conventional maize seeds genetically similar to those harbouring the MON 87403 event.

The control samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11[f].

The control samples were used by the EURL GMFF to prepare standards (of known GMO content) and test samples (of undisclosed GM content = blind samples) by mixing MON 87403 maize DNA and non-GM maize DNA.

The calibration sample S1 was prepared by mixing the appropriate amount of MON 87403 genomic DNA with control non-GM maize genomic DNA to obtain a 10 % (in copy number ratio) GM sample. Calibration samples S2-S5 were prepared by 4.0-fold serial dilutions from the S1 sample in TE Buffer.

The twelve NRLs participating in the validation study received the following materials:

- ✓ Five calibration samples with known concentrations of GM-event (125 µL of DNA solution each) labelled from S1 to S5 (Table 4).
- ✓ Twenty blinded test DNA samples (62.5 µL of DNA solution, each at 45 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 6)

[f] Control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample).

Table 6. MON 87403 blinded samples GM % contents

| MON 87403 GM %<br>GM copy number/maize genome copy number x 100 |
|---|
| 5.0 |
| 2.0 |
| 0.90 |
| 0.40 |
| 0.058 |

Reaction reagents:

- TaqMan® Universal PCR Master Mix (2x), one vial: 8 mL
- distilled sterile water, one vial: 4 mL

✓ Primers and probes (1 tube each) as follows:

*hmg* taxon-specific assay

- hmg primer 1 (10 µM): 240 µL
- hmg primer 2 (10 µM): 240 µL
- hmg probe (10 µM): 128 µL

MON 87403 assay

- 87403 QF (10 µM): 360 µL
- 87403 QR (10 µM): 340 µL
- 87403 QP (10 µM): 160 µL

### 3.4.4 Design of the collaborative study

Participating laboratories received a detailed validation protocol that included the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the MON 87403 event-specific system and for the *hmg* taxon-specific system. In total, two plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for the maize event MON 87403 and the *hmg* assay in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per pre-determined plate layout.

The amplification reaction followed the cycling program specified in the protocol. Participants determined the GM % in the test samples according to the instructions and also reported the raw data to the EURL GMFF on an Excel sheet that was designed, validated and distributed by the EURL GMFF. All data are stored by the EURL GMFF on a dedicated and protected server.

The EURL GMFF analysed the data against the parameters and the limits set by the ENGL, i.e. trueness, precision, amplification efficiency and linearity.

### 3.4.5 Deviations reported from the protocol

Eight laboratories reported no deviations from the validation protocol. Two laboratories reported technical/pipetting problems in one well; one laboratory reported pipetting errors in two wells; another laboratory reported that the run for plate B stopped due to instrument connection problems 8 minutes after the start. The run was restarted with a new cycling program without the step at 50°C (which had already been performed before the run) and with 7 minutes of initial denaturation at 95°C instead of 10. The rest of the run was performed as described in the validation protocol.

# 4. Results

## 4.1 EURL GMFF experimental testing

### 4.1.1 Zygosity ratio in the positive control sample

The results of the digital PCR analysis conducted by the EURL GMFF on the MON 87403 and *hmg* targets to determine the zygosity ratio in the positive control samples are shown in Table 7.

Table 7. Zygosity ratio of the MON 87403 and *hmg* targets in the positive control sample.

| | |
|---|---|
| Mean ratio (MON 87403/*hmg*) | 0.58 |
| Standard deviation | 0.051 |
| $RSD_r$ (%) | 8.7 |
| Standard error of the mean | 0.014 |
| Upper 95% CI of the mean | 0.61 |
| Lower 95% CI of the mean | 0.56 |

The mean ratio (MON 87403/*hmg*) equals 0.58. The 95% confidence interval (CI) spans around 0.582, the expected ratio for a maize heterozygous control sample with a GM parental contribution of female origin and an assuming single-copy endogenous gene target. Therefore, the measured mean ratio is not significantly different from the expected ratio, for an alpha = 0.05.

Hence:

0.058 GM % in DNA copy number ratio = 0.1 GM % in mass fraction

### 4.1.2 In-house verification of method performance against ENGL method acceptance criteria

Samples with GM levels from 0.40% to 5.0% were tested in two real-time PCR runs on three different instruments with two replicates for each GM level on each plate (total of four replicates

per GM level). The sample at 0.058% GM level (corresponding to 0.10% in mass fractions of GM material) was tested for precision in 15 replicates with a separate run for each instrument.

Tests were conducted on ABI 7500, ABI 7900HT and Roche LC480 platforms.

The corresponding standard curve parameters and the results of efficiency, linearity, trueness and precision are shown in Tables 8A, 8B, 9, 10 and 11.

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.6 to -3.1 and the $R^2$ coefficient shall be $\geq$ 0.98. Tables 8A and 8B document that the slopes of the standard curves and the $R^2$ coefficients were within the limits established by the ENGL. The EURL GMFF in-house results confirm the data provided by the applicant.

Table 8A. Standard curve parameters of the real-time PCR tests carried out on ABI 7500, ABI 7900HT, and Roche LC480 for quantifying GM levels 0.40 % to 5.0 %. Slope and $R^2$ coefficient values were rounded to two digits.

| | **MON 87403 system** | | | ***hmg* system** | | |
|---|---|---|---|---|---|---|
| | Slope | PCR efficiency %* | $R^2$ | Slope | PCR efficiency %* | $R^2$ |
| Run A | -3.49 | 93 | 1.00 | -3.38 | 98 | 1.00 |
| Run B | -3.50 | 93 | 1.00 | -3.35 | 99 | 1.00 |
| Run C | -3.42 | 96 | 1.00 | -3.37 | 98 | 1.00 |
| Run D | -3.39 | 97 | 1.00 | -3.36 | 98 | 1.00 |
| Run E | -3.39 | 97 | 1.00 | -3.35 | 99 | 1.00 |
| Run F | -3.39 | 97 | 1.00 | -3.30 | 101 | 1.00 |

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$
Runs A-B were carried out on ABI 7500; Runs C-D were carried out on ABI 7900HT; Runs E and F were carried out on Roche LC480.

Table 8B. Standard curve parameters of the real-time PCR tests carried out on ABI 7500, ABI 7900HT, and Roche LC480 for quantifying the GM level 0.058 % in 15 replicates. Slope and $R^2$ coefficient values were rounded to two digits.

| | **MON 87403 system** | | | ***hmg* system** | | |
|---|---|---|---|---|---|---|
| | Slope | PCR efficiency %* | $R^2$ | Slope | PCR efficiency %* | $R^2$ |
| Run G | -3.49 | 93 | 0.99 | -3.39 | 97 | 1.00 |
| Run H | -3.54 | 92 | 1.00 | -3.44 | 95 | 1.00 |
| Run I | -3.26 | 103 | 1.00 | -3.25 | 103 | 1.00 |

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$
Run G was carried out on ABI 7500; Run H was carried out on ABI 7900HT; Run I was carried out on Roche LC480.

According to the ENGL method acceptance criteria the method trueness (measured as bias in % of the target GM level) should be within ±25 % of the accepted reference value over the entire dynamic range. The method precision, expressed as $RSD_r$ % (relative standard deviation of repeatability), should be ≤25%, also over the entire dynamic range.

Tables 9, 10 and 11 show that trueness and precision were within the limits established by the ENGL for the PCR machines used.

Table 9. Values of trueness and precision as established by the EURL GMFF by in-house verification using an ABI 7500 instrument. GM % expressed in copy/copy.

| **Target GM levels %** | **Measured GM level %** | **Bias % of the target GM level** | **Precision ($RSD_r$ %)** |
|---|---|---|---|
| 5.0 | 5.1 | 2.8 | 2.9 |
| 2.0 | 1.9 | -5.4 | 3.8 |
| 0.90 | 0.86 | -4.1 | 2.8 |
| 0.40 | 0.38 | -4.0 | 2.9 |
| 0.058 | 0.060 | 2.6 | 15 |

Table 10. Values of trueness and precision as established by the EURL GMFF by in-house verification using an ABI 7900HT instrument. GM % expressed in copy/copy.

| **Target GM levels %** | **Measured GM level %** | **Bias % of the target GM level** | **Precision ($RSD_r$ %)** |
|---|---|---|---|
| 5.0 | 5.3 | 6.2 | 3.0 |
| 2.0 | 1.9 | -7.2 | 6.1 |
| 0.90 | 0.89 | -0.66 | 6.1 |
| 0.40 | 0.35 | -11 | 6.9 |
| 0.058 | 0.067 | 14 | 13 |

Table 11. Values of trueness and precision as established by the EURL GMFF by in-house verification using a Roche LC480 instrument. GM % expressed in copy/copy.

| **Target GM levels %** | **Measured GM level %** | **Bias % of the target GM level** | **Precision ($RSD_r$ %)** |
|---|---|---|---|
| 5.0 | 5.3 | 5.5 | 7.5 |
| 2.0 | 2.1 | 4.0 | 9.0 |
| 0.90 | 0.94 | 4.2 | 4.8 |
| 0.40 | 0.40 | 0.91 | 7.5 |
| 0.058 | 0.051 | -13 | 24 |

## 4.2 Results of the international collaborative study

### 4.2.1 PCR efficiency and linearity

The PCR efficiency (%) and $R^2$ values (expressing the linearity of the regression) for the standard curve, reported by participating laboratories are reported in Table 12. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

$$\text{Efficiency (\%)} = (10 * (-1/\text{slope})) - 1) \times 100$$

Table 12. Values of slope, PCR efficiency and $R^2$ obtained during the international collaborative trial. Slope and $R^2$ coefficient values were rounded to two digits.

| | | **MON 87403** | | | ***hmg*** | | |
|---|---|---|---|---|---|---|---|
| **Lab** | **Plate** | **Slope** | **PCR Efficiency (%)** | ***R²*** | **Slope** | **PCR Efficiency (%)** | ***R²*** |
| **1** | A | -3.63 | 88 | 1.00 | -3.52 | 92 | 1.00 |
| | B | -3.47 | 94 | 0.99 | -3.53 | 92 | 1.00 |
| **2** | A | -3.39 | 97 | 1.00 | -3.39 | 97 | 1.00 |
| | B | -3.11 | 109 | 1.00 | -3.22 | 104 | 0.99 |
| **3** | A | -3.64 | 88 | 0.99 | -3.61 | 89 | 1.00 |
| | B | -3.64 | 88 | 0.99 | -3.61 | 89 | 1.00 |
| **4** | A | -3.83 | 83 | 0.99 | -3.82 | 83 | 1.00 |
| | B | -3.86 | 82 | 0.99 | -3.67 | 87 | 0.99 |
| **5** | A | -3.58 | 90 | 1.00 | -3.51 | 93 | 1.00 |
| | B | -3.32 | 100 | 1.00 | -3.34 | 99 | 1.00 |
| **6** | A | -3.62 | 89 | 0.99 | -3.45 | 95 | 0.99 |
| | B | -3.47 | 94 | 0.99 | -3.44 | 95 | 0.99 |
| **7** | A | -3.64 | 88 | 1.00 | -3.57 | 91 | 1.00 |
| | B | -3.73 | 85 | 1.00 | -3.58 | 90 | 1.00 |
| **8** | A | -3.65 | 88 | 0.99 | -3.76 | 84 | 1.00 |
| | B | -3.68 | 87 | 1.00 | -3.73 | 86 | 1.00 |
| **9** | A | -3.60 | 90 | 1.00 | -3.65 | 88 | 1.00 |
| | B | -3.70 | 86 | 1.00 | -3.64 | 88 | 1.00 |
| **10** | A | -3.56 | 91 | 0.99 | -3.60 | 90 | 1.00 |
| | B | -3.54 | 92 | 0.99 | -3.46 | 95 | 1.00 |
| **11** | A | -3.59 | 90 | 1.00 | -3.48 | 94 | 1.00 |
| | B | -3.51 | 93 | 0.99 | -3.45 | 95 | 1.00 |
| **12** | A | -3.47 | 94 | 0.99 | -3.37 | 98 | 1.00 |
| | B | -3.53 | 92 | 0.99 | -3.43 | 96 | 0.99 |
| | **Mean** | -3.57 | 91 | 0.99 | -3.53 | 92 | 1.00 |

Table 12 indicates that the efficiency of amplification for the MON 87403 system ranges from 82% to 109 % and the linearity from 0.99 to 1.00; the amplification efficiency for the maize-specific system ranges from 83 % to 104% and the linearity from 0.99 to 1.00 as well. The mean PCR

efficiency was 91 % for the MON 87403 assay and 92% for *hmg*. The average $R^2$ was 0.99 and 1.00 for the MON 87403 and *hmg* assays, respectively. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

These results confirm the appropriate performance characteristics of the methods in terms of efficiency and linearity.

### 4.2.2 GMO quantification

Table 13 reports the values of quantification for the four replicates of each GM level as reported by each of the twelve participating laboratories.

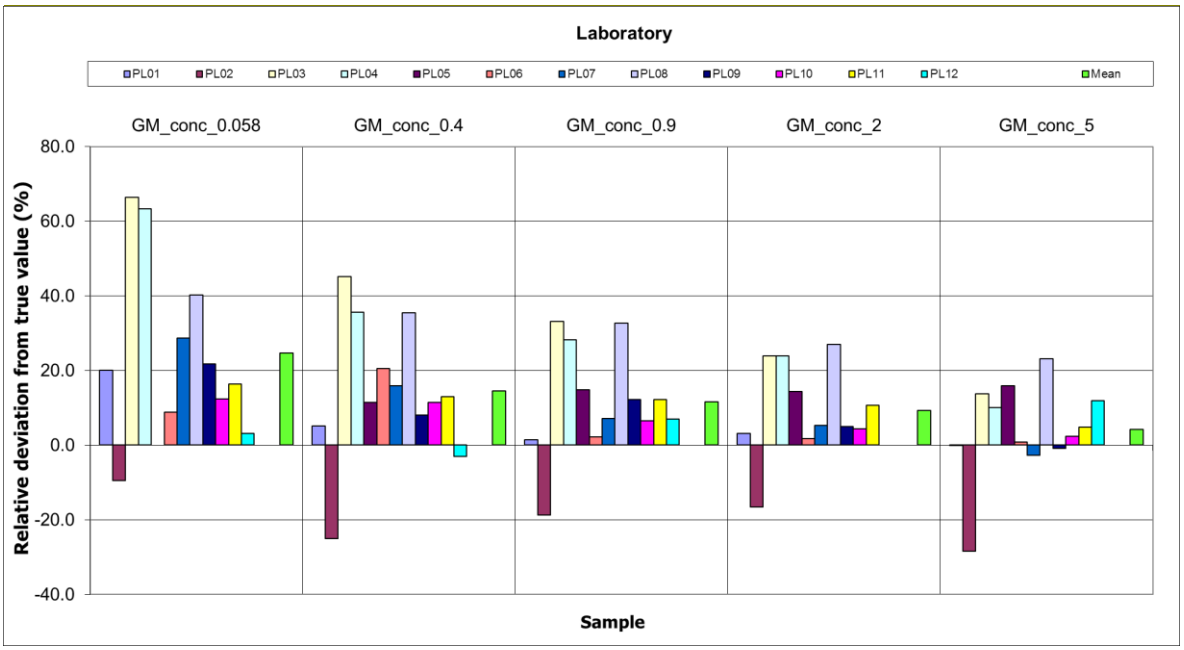
Table 13. GM % values determined by laboratories for test samples

| | GMO content (%) * | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **LAB** | **0.058** | | | | **0.40** | | | | **0.90** | | | | **2.0** | | | | **5.0** | | | |
| | REP 1 | REP 2 | REP 3 | REP 4 | REP 1 | REP 2 | REP 3 | REP 4 | REP 1 | REP 2 | REP 3 | REP 4 | REP 1 | REP 2 | REP 3 | REP 4 | REP 1 | REP 2 | REP 3 | REP 4 |
| **1** | 0.07 | 0.06 | 0.08 | 0.07 | 0.46 | 0.41 | 0.36 | 0.45 | 0.83 | 0.98 | 0.97 | 0.87 | 2.25 | 1.98 | 2.15 | 1.86 | 4.80 | 5.32 | 5.20 | 4.65 |
| **2** | 0.05 | 0.03 | 0.06 | 0.06 | 0.30 | 0.32 | 0.25 | 0.33 | 0.80 | 0.61 | 0.80 | 0.71 | 1.75 | 1.85 | 1.45 | 1.62 | 3.02 | 3.42 | 3.78 | 4.11 |
| **3** | 0.10 | 0.11 | 0.10 | 0.08 | 0.56 | 0.57 | 0.66 | 0.53 | 1.16 | 1.22 | 1.23 | 1.19 | 2.54 | 2.64 | 2.48 | 2.25 | 5.43 | 6.56 | 5.59 | 5.16 |
| **4** | 0.10 | 0.09 | 0.09 | 0.10 | 0.53 | 0.55 | 0.53 | 0.55 | 1.18 | 1.09 | 1.16 | 1.18 | 2.37 | 2.32 | 2.45 | 2.77 | 5.48 | 5.14 | 5.54 | 5.85 |
| **5** | 0.17 | 0.06 | 0.07 | 0.07 | 0.48 | 0.38 | 0.42 | 0.51 | 0.92 | 1.12 | 1.01 | 1.09 | 2.13 | 2.45 | 2.22 | 2.35 | 6.73 | 5.87 | 5.36 | 5.22 |
| **6** | 0.05 | 0.06 | 0.08 | 0.07 | 0.50 | 0.61 | 0.38 | 0.44 | 0.85 | 0.84 | 1.05 | 0.94 | 1.92 | 1.97 | 2.01 | 2.23 | 4.83 | 5.12 | 5.05 | 5.17 |
| **7** | 0.07 | 0.08 | 0.08 | 0.07 | 0.43 | 0.45 | 0.51 | 0.47 | 0.96 | 1.01 | 0.94 | 0.94 | 2.44 | 1.97 | 2.07 | 1.95 | 4.86 | 4.55 | 5.22 | 4.83 |
| **8** | 0.08 | 0.08 | 0.09 | 0.07 | 0.57 | 0.49 | 0.59 | 0.52 | 1.16 | 1.15 | 1.23 | 1.24 | 2.36 | 2.28 | 2.90 | 2.60 | 5.84 | 6.08 | 6.76 | 5.94 |
| **9** | 0.07 | 0.08 | 0.07 | 0.07 | 0.42 | 0.45 | 0.46 | 0.39 | 1.00 | 1.02 | 1.05 | 0.98 | 2.12 | 2.08 | 2.13 | 2.07 | 4.75 | 4.90 | 5.11 | 5.08 |
| **10** | 0.07 | 0.06 | 0.07 | 0.07 | 0.46 | 0.45 | 0.40 | 0.48 | 1.06 | 0.94 | 0.93 | 0.91 | 2.07 | 2.08 | 2.10 | 2.11 | 5.02 | 4.80 | 5.35 | 5.29 |
| **11** | 0.07 | 0.07 | 0.06 | 0.07 | 0.43 | 0.48 | 0.40 | 0.49 | 1.02 | 0.96 | 1.03 | 1.03 | 2.28 | 2.21 | 2.31 | 2.05 | 5.21 | 5.34 | 5.21 | 5.22 |
| **12** | 0.05 | 0.06 | 0.07 | 0.06 | 0.49 | 0.31 | 0.44 | 0.32 | 0.88 | 0.88 | 0.99 | 1.11 | 1.72 | 1.68 | 2.05 | 2.90 | 4.67 | 4.95 | 6.06 | 6.69 |

* GMO % = (GMO copy number/maize genome copy number) x 100
n.a. not available

A graphical representation of the data reported in Table 13 is provided in Figure 1 showing the relative deviation from the true value for each GM level tested for the participating laboratories. The coloured bars represent the deviation of the GM level measured by the respective laboratory in % of the true GM level; the green bar on the right represents the mean relative deviation over all data after eliminating outliers (PL5, 0.058 % level; PL12, 2.0 % level) for each GM level.

Figure 1. Relative deviation (%) from the true value of each GM level *




* For PL05 level 0.058% and PL12 level 2.0% the corresponding histograms are not displayed in Figure 1 because their values were eliminated as outliers. PL = participating laboratory.

Overall, the mean relative deviations from the true values were within a maximum of ±25% for most laboratories. At GM level 0.058 % seven laboratories were within the limit, at GM level 0.40 % eight and at GM level 0.9 % nine laboratories; at GM level 2.0 % ten and at GM level 5.0 % eleven laboratories were within the limit. Four laboratories overestimated GM level 0.058 % by more than 25 %, similarly, three laboratories overestimated GM levels 0.40% and 0.9 % by more than 25 %. Overall a trend can be observed for overestimation of the GM content for almost all laboratories at all GM levels.

### 4.2.3 Method performance requirements

Among the method performance requirements established by ENGL and adopted by the EURL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), repeatability and reproducibility are to be assessed through an international collaborative trial. Table 14 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study (see Table 5 for a list of the participant laboratories).

According to the ENGL method performance requirements the relative reproducibility standard deviation ($RSD_R$), that describes the inter-laboratory variation, should be below 35% at the target concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 14, the method satisfies this requirement at all GM levels tested. Indeed, the highest value of $RSD_R$ (%) is 22 % at the 0.058 % GM level, thus within the acceptance criterion.

Table 14. Summary of validation results expressed as GM copy numbers in relation to target taxon copy numbers. Standard deviation values and absolute bias values are rounded to three digits.

| | **Test Sample Expected GMO %** | | | | |
|---|---|---|---|---|---|
| | **0.058** | **0.40** | **0.9** | **2.0** | **5.0** |
| Laboratories having returned valid results | 12 | 12 | 12 | 12 | 12 |
| Samples per laboratory | 4 | 4 | 4 | 4 | 4 |
| Number of outliers | 1 | 0 | 0 | 1 | 0 |
| Reason for exclusion | C* | - | - | C* | - |
| Mean value | 0.073 | 0.46 | 1.0 | 2.2 | 5.2 |
| Relative repeatability standard deviation, $RSD_r$ (%) | 13 | 12 | 6.8 | 7.7 | 8.8 |
| Repeatability standard deviation | 0.010 | 0.053 | 0.068 | 0.168 | 0.457 |
| Relative reproducibility standard deviation, $RSD_R$ (%) | 22 | 19 | 14 | 13 | 15 |
| Reproducibility standard deviation | 0.016 | 0.088 | 0.145 | 0.291 | 0.759 |
| Bias** (absolute value) | 0.014 | 0.058 | 0.104 | 0.187 | 0.211 |
| Bias (%) | 25 | 14 | 12 | 9.3 | 4.2 |

* C= Cochran's test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

** Bias is estimated according to ISO 5725 data analysis protocol.

Table 14 also documents the relative repeatability standard deviation ($RSD_r$) estimated for each GM level. In order to accept methods for collaborative study, the EURL GMFF and ENGL require that the $RSD_r$ value indicated by the applicant and confirmed by the EURL GMFF in its in-house experiments is below 25% (see ENGL document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). As it can be observed from the values reported, the repeatability standard deviation is below 25% at all GM levels, with the highest value of 13% at the 0.058% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 25% (24.7%) at the 0.058% GM level.

# 5. Compliance of the method for detection and quantification of event MON 87403 with the requirements of Regulation (EU) No 619/2011

To verify the compliance of the method under validation with the requirements of Regulation (EU) No 619/2011, the following steps were carried out and their outcome is summarised in Table 15:

- at step 2 of the validation process (scientific assessment of the dossier), the EURL GMFF acknowledged that the $RSD_r$ % value at the 0.085 % level shown by the applicant's dossier (expressed as mass fraction of GM-material) was 3.83 %, hence below the maximum value of 25 % required by the ENGL. The value was calculated on 3 real-time PCR runs each with 13-15 replicates (Table 3). The EURL GMFF therefore concluded that it could accept the applicant's data on method performance;

- at step 3 of the validation process (experimental testing of samples and methods), the EURL GMFF determined the $RSD_r$ % value at the level of 0.1% in mass fraction of GM-material (corresponding to 0.058 % expressed in terms of copy number ratio). The experiments were carried out under repeatability conditions on fifteen replicates. The $RSD_r$ resulted to range between 13 % and 24 % (Table 9, 10 and 11) depending on the qPCR platform applied, hence also below 25 %;

- the collaborative study (step 4 of the validation process) established that over the twelve participating laboratories at the level of 0.1 % related to mass fraction of GM-material the $RSD_r$ of the method was 24.7 %, therefore also below 25 % and well in line with the previous data.

The outcome of the different steps is summarised in Table 15.

Table 15. Precision of the event-specific method for quantitative detection of MON 87403 at or around 0.1% level related to mass fractions of GM material.

| **Source** | **$RSD_r$ %** | **GM %** |
|---|---|---|
| Applicant's method optimisation | 3.83 | 0.085 |
| EURL GMFF tests | 13-24 | 0.1 |
| Collaborative study | 25 | 0.1 |

Based on the results of the EURL GMFF in-house verification and of the international collaborative study, it is concluded that the method $RSD_r$ % is lower than 25% at the level of 0.1% related to mass fraction of GM material, hence the method meets the requirement laid down in Regulation (EU) No 619/2011.

# 6. Conclusion

The method provided by the applicant has been validated in accordance to the EURL GMFF validation process, respecting all requirements of the relevant EU legislation and international standards for method validation.

This validation study confirms that the method is applicable to the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex III-3.C.2 to Commission Regulation (EU) No 503/2013 and (EU) No 619/2011 and meets all method performance requirements established by the ENGL and the EURL GMFF. The method is therefore valid to be used for regulatory purposes, including the quantification of low levels [0.1% (mass/mass)] of the GM event. It can be assumed that it is applicable to any appropriately extracted maize genomic DNA.

In any case the user of the method is advised to verify the quality of the extracted genomic DNA in order to ensure that it is suitable for the subsequent PCR analysis. This is particularly relevant for more complex matrices of samples from food and feed products.

The validated method is described in detail as "Validated Method" at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1.

# 7. References


1. Horwitz W. Protocol for the design, conduct and interpretation of method- performance studies, *Pure and Appl. Chem.* 1995; 67: 331-343.
2. International Standard (ISO) 5725-1, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 1: General principles and definitions. International Organization for Standardization, Genève, Switzerland.
3. ISO 5725-1:1994/Cor 1:1998.
4. International Standard (ISO) 5725-2, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method. International Organization for Standardization, Genève, Switzerland.
5. ISO 5725-2:1994/Cor 1:2002.
6. Plant DNA C-values Database, http://data.kew.org/cvalues/

# Annex 1: Event-specific Method for the Quantification of maize MON 87403 by Real-time PCR

# Validated Method

**Method development:**

Monsanto Company S. A.

# 1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR (polymerase chain reaction) procedure for the determination of the relative content of maize event MON 87403 (unique identifier MON-874Ø3-1) genomic DNA to total maize genomic DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assays, in particular in case of complex and difficult matrices. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are also recommended to ensure suitability of the extracted DNA.

For the specific detection of GM event MON 87403, an 88 bp fragment of the region spanning the 3' insert-to-plant junction in maize MON 87403 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein), as reporter dye at its 5' end, and TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of maize GM event MON 87403 DNA, a maize taxon-specific reference system amplifies a 79 bp fragment of a maize *high mobility group* (*hmg*) endogenous gene (GeneBank accession number AJ131373.1), using *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM as reporter dye at its 5' end and TAMRA as quencher dye at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Cq" value. For quantification of the amount of MON 87403 genomic DNA in a test sample, Cq values for the MON 87403 and the *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON 87403 genomic DNA to total maize genomic DNA.

# 2. Validation and performance characteristics

## 2.1 General

The method was optimised for suitable DNA extracted from genetically modified and conventional maize seeds. Precision and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

## 2.2 Collaborative trial

The method was validated in an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF). The study was undertaken with twelve participating laboratories in June 2016.

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

### 2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.040 % (related to mass fraction of GM material) in 200 ng of total suitable maize DNA. The relative LOD was not assessed in the collaborative study.

### 2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.085 % (related to mass fraction of GM material) in 200 ng of total suitable maize DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1 % (mass fraction of GM-material).

### 2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 3' insert-to-plant junction in maize MON 87403 and is therefore event-specific for the event MON 87403. This was confirmed by bioinformatics analyses.

## 3. Procedure

### 3.1 General instructions and precautions

- Laboratory organisation, e.g. "forward flow direction" during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.

- PCR reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.

- All the equipment should be sterilised prior to use and any residue of DNA should have been removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.

- Filter pipette tips protected against aerosol should be used.

- Powder-free gloves should be used and changed regularly

- Laboratory benches and equipment should be cleaned periodically, with e.g. 10 % sodium hypochlorite solution (bleach).

- Pipettes should be checked regularly for precision and calibrated, if necessary.

- All handling steps, unless specified otherwise, should be carried out at room temperature.

- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

## 3.2 Real-time PCR for quantitative analysis of maize event MON 87403

### 3.2.1 General

The real-time PCR set-up for the taxon (*hmg*) and the GMO (event MON 87403) target sequences are carried out in separate vials. Multiplex qPCR (using differential fluorescent labels for the probes) has not been tested or validated by the EURL GMFF.

The method is developed for a total volume of 25 µL per reaction mixture for the GM (event MON 87403) and the taxon (*hmg*) assay with the reagents as listed in Table 2 and Table 3.

### 3.2.2 Calibration

To establish the calibration curve at least five samples should be prepared and analysed. The range of GM contents in the calibration curve should be equal to or included in the range validated during the international collaborative study, as given in Table 1. In the trial the first point of the calibration curve (S1) was established for a sample containing 10 % (relative to copy number fractions) maize MON 87403 genomic DNA in a total of 200 ng maize genomic DNA (corresponding to 73260 maize genome copies with one haploid genome assumed to correspond to 2.73 pg of maize genomic DNA) [(1)]. Standards S2 to S5 were prepared by fourfold serial dilutions according to Table 1 below.

Table 1. Copy number values of the standard curve samples

| Sample code | S1 | S2 | S3 | S4 | S5 |
|---|---|---|---|---|---|
| Total amount of maize DNA in reaction (ng) | 200 | 50 | 12.5 | 3.1 | 0.78 |
| *hmg* copies | 73260 | 18315 | 4579 | 1145 | 286 |
| MON 87403 copies | 7326 | 1832 | 458 | 114 | 29 |

A calibration curve is produced by plotting the Cq values against the logarithm of the target copy number for the calibration points. This can be done by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software of the real-time PCR platform.

The copy number measured for each unknown sample DNA is obtained by interpolation from the standard curves.

### 3.2.3 Real-time PCR set-up

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.

2. In two tubes on ice, add the components in the order mentioned below (except DNA) to prepare the amplification reaction mixes for the MON 87403 maize specific system (Table 2) and the *hmg* reference gene system (Table 3).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 87403 event-specific assay.

| Component | Final concentration | µL/reaction |
|---|---|---|
| TaqMan® Universal PCR Master Mix (2x) | 1x | 12.5 |
| 87403 QF (10 µM) | 450 nM | 1.125 |
| 87403 QR (10 µM) | 450 nM | 1.125 |
| 87403 QP* (10 µM) | 200 nM | 0.50 |
| Nuclease free water | - | 5.75 |
| DNA (max 200 ng) | - | 4.0 |
| Total reaction volume: | | 25 µL |

*TaqMan® probe labelled with 6-FAM at its 5'-end and TAMRA at its 3'-end

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* taxon-specific assay.

| Component | Final concentration | µL/reaction |
|---|---|---|
| TaqMan® Universal PCR Master Mix (2x) | 1x | 12.5 |
| hmg primer 1 (10 µM) | 300 nM | 0.75 |
| hmg primer 2 (10 µM) | 300 nM | 0.75 |
| hmg probe* (10 µM) | 160 nM | 0.40 |
| Nuclease free water | - | 6.6 |
| DNA (max 200 ng) | - | 4.0 |
| Total reaction volume: | | 25 µL |

*TaqMan® probe is labelled with 6-FAM at its 5'-end and TAMRA at its 3'-end

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for maize event MON 87403 and one for the *hmg* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the amount of reaction mix for 3.5 PCR repetitions (73.5 µL for the *hmg* system and 73.5 µL for the MON 87403 maize system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (14 µL DNA). The additional 0.5 repetition included will ensure adequate volume when loading the samples.
6. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
7. Spin down the tubes. Aliquot 25 µL for the MON 87403 system and for the *hmg* reference system in each well.
8. Place an optical cover on the reaction plate and briefly centrifuge the plate at low speed (e.g. approximately 250 x g for 1 minute) to spin down the reaction mixture.
9. Place the reaction plate in the real-time PCR apparatus (possibly apply a compression pad, depending on the model), according to the manufacturer's instructions and your Standard Operating Procedures.
10. Select FAM as reporter dye and TAMRA as quencher dye for the MON 87403 and the *hmg* reference systems. Select ROX as the passive reference dye. Enter the correct reaction volume (25 µL).
11. Run the PCR with the cycling program described in Table 4. Users who plan to use the second derivative maximum analysis method (an option e.g. on Roche LC480 instruments) are advised to program 45 cycles instead of 40, in order to be able to quantify down to Cq 40.

Table 4. Cycling program for MON 87403/*hmg* assays.

| Step | Stage | | T (°C) | Time (s) | Acquisition | Cycles |
|---|---|---|---|---|---|---|
| 1 | UNG* | | 50 | 120 | No | 1 |
| 2 | Initial denaturation | | 95 | 600 | No | 1 |
| 3 | Amplification | Denaturation | 95 | 15 | No | 40** |
| | | Annealing & Extension | 60 | 60 | Yes | |

*UNG: Uracil-N-glycosylase
** see comment above for users of second derivative maximum analysis method

### 3.3 Data analysis

After the real-time PCR, analyse the run following the procedure below:

a) Analyse data: automatic baseline and threshold settings have given the best results at the EURL GMFF.

b) Save the settings and export all the data for further calculations.

### 3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification, the instrument's software calculates the Cq values for each reaction.

The standard curves are generated both for the *hmg* and the MON 87403 specific assays by plotting the Cq values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.
Thereafter, the standard curves are used to estimate the DNA copy number in the unknown samples.

To obtain the percentage value of event MON 87403 DNA in the unknown sample, the MON 87403 copy number is divided by the copy number of the maize endogenous gene *hmg* and multiplied by 100 (GM% = MON 87403/*hmg* x 100).

## 4. Equipment and Materials

### 4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition) and appropriate analysis software
- 96-well reaction plates
- Optical caps/adhesion covers
- Micropipettes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex
- Racks for reaction tubes, also cooled
- 0.5, 1.5 mL and 5 or 15 mL DNAse free reaction tubes

### 4.2 Reagents

- TaqMan® Universal PCR Master Mix. Applied Biosystems Part No 4318157.
- Nuclease free water

### 4.3 Primers and Probes

Table 5. Primers and probes for the MON 87403 and *hmg* methods

| | Name | DNA Sequence (5' to 3') | Length (nt) |
|---|---|---|---|
| MON 87403 | | | |
| Forward primer | 87403 QF | CTT TCT TTT TCT CCA TAT TGA CCA TCA TAC | 30 |
| Reverse primer | 87403 QR | TAC TCC GGA ATG AGT GCT CTG TAT C | 25 |
| Probe | 87403 QP | 6-FAM- TCA TTG CGA TCC ACA TTT CCC TAC ATG G-TAMRA | 28 |
| *hmg* | | | |
| Forward primer | hmg primer 1 | TTG GAC TAG AAA TCT CGT GCT GA | 23 |
| Reverse primer | hmg primer 2 | GCT ACA TAG GGA GCC TTG TCC T | 22 |
| Probe | hmg probe | 6-FAM- CAA TCC ACA CAA ACG CAC GCG TA-TAMRA | 23 |

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine;

# 5. References

1. Plant DNA C-values Database. Royal Botanic Gardens, Kew, http://data.kew.org/cvalues/

## JRC Mission

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