European Commission

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Event-specific Method for the Quantification of Maize MON 87419 Using Real-time PCR

Validation Report

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European Union Reference Laboratory for
Genetically Modified Food and Feed

2019

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JRC Science Hub
https://ec.europa.eu/jrc

JRC 117701

Ispra: European Commission, 2019

How to cite this report: European Union Reference Laboratory for GM Food and Feed, Joint Research Centre. "Event-specific Method for the Quantification of Maize MON 87419 Using Real-time PCR", 2019. http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers & Reference Materials (Geel/Ispra)
Food and Feed Compliance

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14 August 2019

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate[1] 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 87419 (unique identifier MON-87419-8). 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[2–6].

In accordance with current EU legislation[7], Monsanto Company provided the detection method and the positive and negative control samples (genomic DNA from ground seeds of MON 87419 maize as positive control DNA, and genomic DNA from ground 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 haploid 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[7], and it fulfils the analytical requirements of Regulation (EU) No 619/2011[8]. This validation report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

Content

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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1. Introduction

In line with Regulation (EC) No 1829/2003[1], Monsanto Company provided the EURL GMFF with an event-specific method for detection and quantification of maize event MON 87419 (unique identifier MON-87419-8) 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[9], 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.

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 87419, appropriately extracted from food or feed, down to a GM content level of 0.1 % m/m.

The preparation of the report (step 5) was aligned with the timeline communicated by EFSA for its risk assessment.

2. Dossier reception and acceptance (step 1)

Monsanto Company submitted the detection methods, data demonstrating their adequate performance when applied to genomic DNA extracted from the event, and the corresponding control samples of DNA extracted from the GM event maize MON 87419 and from non GM maize.

The dossier was found to be complete and thus was moved to step 2.

3. Scientific assessment and bioinformatics analysis (step 2)

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL and with regard to their documentation and reliability.

Three requests of complementary information regarding reagents, samples preparation, data analysis and experiments for the assessment of the specificity were addressed to the applicant. The EURL GMFF verified the data and the complementary information received and accepted the received clarifications as satisfactory.

3.1 Specificity assessment conducted by the applicant

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.

The specificity of the event-specific assay was assessed by the applicant by real-time PCR according to the method described in Annex 1 and using genomic DNA extracted from: alfalfa J101, J163, KK179; oilseed rape RT73, MON 88302, 73496, Ms1, Ms8, Rf1, Rf2, Rf3, T45, Topas19/2; cotton MON 531, MON 15985, MON 1445, MON 88913, MON 88701, MON 88702, LLCotton25, COT102, 281-24-236 x 3006-210-23, GHB614; maize MON 87419, NK603, MON 810, MON 863, MON 88017, MON 89034, MON 87460, MON 87427, MON 87411, MON 87403, 3272, 98140, Bt11, Bt176, 1507, 59122, 40278, VCO-01981-5, DP-004114-3, GA21, T25, MIR162, MIR604; potato EH92-527-1, AM04-1020, AV43-6-G7; rice LLRICE62; soybean GTS40-3-2, MON 89788, MON 87769, MON 87701, MON 87705, MON 87708, MON 87751, DAS-81419-2, DAS 68416-4, 356043, 305423, DAS-44406-6, FG72, A2704-12, A5547-127, BPS-CV127-9; sugar beet H7-1; conventional alfalfa, conventional cotton, conventional oilseed rape, conventional potato, conventional rice, conventional soybean, conventional sugar beet, conventional wheat, lentils, sunflower, quinoa, millet, peanut and two conventional maize samples, one of which was isogenic to the MON 87419.

According to the method developer, the MON 87419 assay did not react with any of the samples except for the positive control.

In addition, the applicant performed an in-silico specificity analysis by using the amplicon sequence as a query for BLASTn search against public sequence of NCBI database. No sequence showed the alignment of both forward and reverse primers or with the full length of the probe.

A previously validated maize-specific PCR method (http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27_MON87460_validated_Method.pdf), which amplifies a 79 base pair (bp) fragment of the *high mobility group* gene (*hmg*) of *Zea mays*, was used as a reference method.

The specificity of the taxon-specific assay was assessed by the applicant by real-time PCR, according to the method described, using the DNA samples listed above.

According to the method developer the *hmg* assay did not react with any of the samples except for the DNA extracted from maize.

Multiple sub-samples of conventional potato and conventional sugar beet from certified reference materials tested positive with low-level maize contamination. However, according to the applicant, this was not indicative of non-specificity of the *hmg* assay, as the *hmg*-specific PCR did not amplify GM potato or GM sugar beet DNA.

3.2 Specificity assessment conducted by the EURL GMFF

The detection method spans the 5' insert-to-plant junction in maize MON 87419. The forward primer 87419 QF binds to the maize genomic border adjacent to the insertion 5' end of the insert. The reverse primer 87419 QR binding site was found in the DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA. The 87419 QP probe binds to the junction between the 5' end of the insert and the genomic region of *Zea mays*.

The amplicon size is expected to be 97 bp, consistent to what reported by the applicant. The sequence of the amplicon was analysed by BLAST (NCBI) against local copies of the "nt" and "patents" databases, and no significant similarity was found with any other published sequence. In addition, the primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System (CCSIS) of the JRC, as well as the whole genomes of more than 80 plants (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 with the exception of the 3rd insertion site of the GM Event Florigene Moonvista (123.8.8) Carnation (FLO-40685-2). In particular, a potential amplicon of 144 bp was predicted by the annealing of the primer 87419 QR (with 2 mismatches) in an inverted repeat of T-DNA sequences. However, the alignment of the probe showed that 5 bases at the 5' do not anneal the amplicon sequence; therefore, the probability to get a signal in qPCR is limited.

The *in silico* assessment of the specificity of the MON 87419 method on the sequence of the insert of MON 87419 showed a potential occurrence of two amplicons: the one expected of 97 bp and a second of 253 bp. The latter may be produced by the recognition by the 87419 QF primer of a binding site at the 5' of the 97 bp amplicon, although two mismatches are identified at the 2nd and 18th base of the primer. The applicant showed that no amplification products are observed after 45 and 55 PCR cycles except for the one expected of 97 bp.

3.3 Verification of the ENGL acceptance parameters

The applicant submitted data generated by the method developer (reported as laboratory 1) and from the transferability study of the method to another laboratory (reported as laboratory 2).

The applicant prepared the calibration curve from a DNA solution (S1) of 10 % maize event MON 87419 genomic DNA (expressed as copy number ratio) with 200 ng of genomic DNA per reaction. The calibration curve was generated from serial dilution (1:4) of S1 to obtain solutions S2, S3, S4 and S5. The parameters (slope, R^2 coefficient) of five runs of the calibration curve as provided by the applicant and three runs from the transferability study to another laboratory are reported (Table 1).

Table 1. Summary of the slope and R^2 values obtained by the applicant (laboratory 1) and from another laboratory (laboratory 2) in the transferability study.

	MON 87419		***hmg***	
Laboratory	**Slope**	**R^2**	**Slope**	**R^2**
1	-3.34	0.9999	-3.4	0.9999
1	-3.29	0.9997	-3.40	0.9997
1	-3.38	0.9995	-3.41	0.9998
1	-3.44	0.9988	-3.38	0.9999
1	-3.34	0.9992	-3.42	0.9998
2	-3.34	0.9997	-3.38	0.9994
2	-3.42	0.9993	-3.48	0.9983
2	-3.33	0.9996	-3.40	0.9996

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 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 87419) and the maize-specific high mobility group gene (*hmg*) system, as established by the applicant, were within the ENGL acceptance criteria.

Also precision and trueness of the method were established by the applicant and 15 values for each of three GM levels (expressed as haploid genome copy fraction of GM-material) were provided. Table 2 reports precision and trueness values for the three GM-levels 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 (laboratory 1) and from another laboratory (laboratory 2) in the transferability study.

	Results					
	Laboratory 1			**Laboratory 2**		
Expected GM %	**10%**	**1%**	**0.085%**	**10%**	**1%**	**0.085%**
Measured mean GM %	9.83	0.94	0.073	9.32	0.98	0.075
Precision (RSD_r %)	7.11	6.62	13.38	7.77	8.43	15.35
Trueness (bias %)	-1.62	-6.48	-14.01	-6.77	-2.25	-11.40

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

The limit of detection (LOD) of the method was assessed in 60 PCR replicates by the applicant and to be at least 5 copies of MON 87419 per reaction. The result was confirmed in the transferability study. Therefore, the LOD is in line with the ENGL acceptance criteria (< 25 copies with a level of confidence of 95%).

The robustness of the method was assessed on the Applied Biosystems 7500 and Bio-Rad CFX96 Touch instruments in combination with the following variations: exact/-30 % primer concentration, exact/-30 % probe concentration, exact/-10 % master mix concentration, +/-1 °C in annealing temperature, +/-1 µL master mix volume. The RSDr and trueness calculated for a combination of changes did not exceed 30 %, thus meeting the ENGL requirement for robustness.

3.4 DNA extraction

Genomic DNA was isolated from ground maize seeds, using a modified DNA extraction method from Rogers and Bendich (1985)[10] previously submitted for detection of maize event MON 88017 and already validated in-house by the EURL GMFF. The protocol for DNA extraction and a report on testing are published at http://gmo-crl.jrc.ec.europa.eu/summaries/CRL-VL-16-05-XP-Corrected-version-2.pdf.

Consequently, the EURL GMFF did not verify the DNA extraction method proposed by the applicant.

Annex III to Reg. (EU) No 503/2013[7] requires the applicant to discuss the validity and limitations of the detection methods in the various types of foods and feeds (matrices) that are expected to be placed on the market. To this regard the applicant stated that the applicability of the quantitative real-time PCR methods developed for MON 87419 depends *"on the isolation of sufficient quantity and quality of purified DNA."*

"The provided DNA extraction method is intended for extraction of genomic DNA from seed which results in primarily high molecular weight DNA, indicating that the DNA is intact with limited fragmentation." The applicant also informed the EURL GMFF that during the processing of maize grains into food and feed ingredients a number of "*treatments may influence the quality and the intactness of the DNA contained in the final product* [11,12]*. In some cases, additional rounds of processing in order to clean up the DNA and eliminate PCR inhibitors may be required in order to achieve a quality of genomic DNA suitable for PCR* [13,14] *".*

The applicant also informed that, *"regardless of the DNA extraction method employed, studies have shown that the processing steps for maize result in the significant degradation of high molecular weight DNA and failure to PCR amplify products greater than a few 100 base pairs* [11,12]*; in addition, random DNA fragmentation is known to lead to variability in quantitating DNA by qPCR*[15]*, thus affecting the ability to accurately quantify the presence of a GM event in processed fractions."*

In agreement with the ENGL position, endorsing the modularity principle (see also Annex III to Reg. (EU) No 503/2013)[7], 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 87419.

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

4. Materials and methods

4.1 Samples

The following positive and negative control samples were provided by the applicant to the EURL GMFF:

- genomic DNA extracted from heterozygous maize ground seeds harbouring the MON 87419 event, and
- genomic DNA extracted from conventional maize ground seeds genetically similar to those harbouring the MON 87419 event.

4.2 Method 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 87419 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 87419 are performed in separate wells. The validated method protocol is published by the EURL GMFF at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and can be found in Annex 1 to this report.

For the detection of GM event MON 87419, a 97 bp fragment of the region spanning the 5' insert-to-plant junction in maize MON 87419 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 87419, a maize taxon-specific system amplifies a 79 bp fragment of the maize *high mobility group* (*hmg*) endogenous gene, using *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

Standard curves are generated for both the MON 87419 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 87419 DNA in a test sample, the MON 87419 copy number is divided by the copy number of the maize haploid genome and multiplied by 100 to obtain the percentage value (GM % = [MON 87419 / maize haploid genome] x 100).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (nanograms) by the published average 1C value for the maize genome (2.73 pg)[16]. 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 3.

Note: Numerical values presented in the following tables were rounded keeping two digits for values ≤ 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 3. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of maize DNA in the reaction (ng)	250	63	16	3.9	0.98
Target taxon haploid genome copies	91575	22894	5723	1431	358
Target MON 87419 copies	9158	2289	572	143	36

4.3 EURL GMFF experimental testing

4.3.1 Determination of the zygosity ratio in the positive control sample

Annex II of Regulation (EU) No 619/2011[8] 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 87419 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 in order to assess the zygosity in 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 85000820), primers and probes at concentrations indicated in the corresponding validated method (87419 QF and 87419 QR primers at 300 nM each, 87419 QP probe at 200 nM; hmg primer 1 and hmg primer 2 at 300 nM each, hmg probe at 160 nM), and 1 µL of DNA at a concentration of 3.5 ng/µL; the DNA concentration was chosen in order 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 fifteen 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 or in the Validated Method document at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx. 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 interlaboratory validated methods - Version 2' [17].

4.3.2 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 (0.05 % - 10 %). The experiments were performed on an ABI 7500, an ABI 7900HT and a Roche LC480 real-time platform under repeatability conditions and following the protocol provided by the applicant with minor adjustments. The calibration curve was modified by increasing the DNA content for S1 from 200 ng to 250 ng per reaction. Test samples with GM levels 0.50 %, 1.0 %, 5.0 % and 10 % 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 0.05 % was tested in 15 replicates in an additional run for each platform. 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. On Roche LC480 platform the method was run at 45 cycles as described in the validated method published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1, and analysed with the second derivative maximum method.

4.4 International collaborative study (step 4)

The international collaborative trial involved twelve randomly selected laboratories, all being "national reference laboratories, assisting the EURL GMFF for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 120/2014[18] who had expressed their interest in participation. 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)[2]
- 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)[3–6]

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.

4.4.1 List of participating laboratories

The twelve laboratories participating in MON 87419 international collaborative study were randomly selected from 28 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories for strictly following the validation protocol that was provided to them. The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the validation of the detection method for maize event MON 87419

Laboratory	Country
Austrian Agency for Health and Food Safety (AGES)	AT
Environment Agency Austria	AT
Walloon Agricultural Research Centre (CRA-W)	BE
Crop Research Institute - Reference Laboratory for GMO Detection and DNA fingerprint	CZ
Center for Agricultural Technology Augustenberg	DE
Federal Office of Consumer Protection and Food Safety - Berlin	DE
Institute for Hygiene and Environment - Hamburg	DE
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE
Departamento de OGM/Técnicas Biomoleculares Laboratorio Arbitral Agroalimentario (LAA)	ES
CREA-DC Sede di Tavazzano	IT
The Netherlands Food and Consumer Product Safety Authority	NL
Institute for Diagnosis and Animal Health, Molecular Biology and GMOs Unit - National Reference Laboratory for GMOs in food and feed	RO

4.4.2 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 QuantStudio 5, three used ABI 7500, two used ABI 7900HT, one used ABI StepOnePLus, one used ABI 7300, one used Roche LC 480II and one used Stratagene Mx3005P.

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

4.4.3 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.

The test samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant (see 4.2 for details) in accordance to Regulation (EC) No 1829/2003[1], Art 2.11[a].

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 87419 maize DNA and non-GM maize DNA.

The calibration sample S1 was prepared by mixing the appropriate amount of MON 87419 DNA with control non-GM maize DNA to obtain a 10 % (in copy number ratio related to haploid genome copies) GM sample. Calibration samples S2-S5 were prepared by 4-fold serial dilutions from the S1 sample.

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

- ✓ Five calibration samples with known concentrations of GM-event (180 µL of DNA solution each) labelled from S1 to S5 (Table 3).
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution, each at 45 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 5)

Table 5. MON 87419 blinded samples GM % contents

MON 87419 GM % GM copy number/maize haploid genome copy number x 100
10
5.0
1.0
0.50
0.05

- ✓ 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:

[a] 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).

hmg taxon-specific assay

- hmg primer 1 (10 µM): 240 µL
- hmg primer 2 (10 µM): 240 µL
- hmg probe (10 µM): 130 µL

MON 87419 assay

- 87419 QF (10 µM): 240 µL
- 87419 QR (10 µM): 240 µL
- 87419 QP (10 µM): 160 µL

4.4.4 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 87419 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 87419 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.

4.4.5 Deviations reported from the protocol

Ten laboratories reported no deviations from the validation protocol. One laboratory reported the exclusion of results from 3 wells on both plates for a repeated failure of the PCR device.

One laboratory reported having performed one plate by setting 40 PCR cycles instead of 45.

5. Results

5.1 EURL GMFF experimental testing (Step 3)

5.1.1 Zygosity ratio in the positive control sample

The results of the digital PCR analysis conducted by the EURL GMFF on the MON 87419 and *hmg* targets to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Zygosity ratio of the MON 87419 and *hmg* targets in the positive control sample.

Mean ratio (MON 87419/*hmg*)	0.49
Standard deviation	0.03
RSD_r (%)	6.8
Standard error of the mean	0.01
Upper 95 % CI of the mean	0.51
Lower 95 % CI of the mean	0.47

The mean ratio (MON 87419/*hmg*) equals 0.49. The 95 % confidence interval (CI) spans around 0.50, the expected ratio for a maize control sample, heterozygous for the GM-locus and assuming single - copy endogenous gene target. Therefore, the measured mean ratio is not significantly different from the expected ratio, for an alpha = 0.05.

The samples of the standard curve and test samples have been prepared as ratio of GM- DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes.

Therefore, results obtained with the control sample used in the present validation study and expressed as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers can be translated into mass fraction by means of the following relationship:

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

5.1.2 In-house verification of method performance against ENGL method acceptance criteria

Samples with GM levels from 0.50 % to 10 % 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 sample at 0.05 % GM-level was tested for its precision in quantification in 15 replicates in separate runs.

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

The standard curve parameters and the results of efficiency, linearity, trueness and precision obtained in the three real-time PCR runs with the test samples are shown in Tables 7A, 7B, 8, 9 and 10.

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R^2 coefficient shall be $\geq$ 0.98. Table 7A and 7B 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 7A. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, ABI 7900HT and Roche LC480 to quantify GM-levels in the range of 0.50 % to 10 % in four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	MON 87419 system			***hmg* system**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.32	100	1.00	-3.34	99	1.00
Run B	-3.38	98	1.00	-3.37	98	1.00
Run C	-3.43	96	1.00	-3.38	98	1.00
Run D	-3.39	97	1.00	-3.40	97	1.00
Run E	-3.29	101	1.00	-3.38	98	1.00
Run F	-3.38	98	1.00	-3.40	97	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 7B. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, ABI 7900HT, and Roche LC480 to quantify the GM-level 0.05 % in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	MON 87419 system			***hmg* system**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run G	-3.46	95	0.99	-3.43	96	1.00
Run H	-3.52	92	0.99	-3.39	97	1.00
Run I	-3.39	97	1.00	-3.34	99	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 and the precision, expressed as RSD_r % (relative standard deviation of repeatability), should be ≤ 25 %, also over the entire dynamic range.

Tables 8, 9 and 10 show that trueness and precision of quantification were within the limits established by the ENGL for the PCR machines used.

Table 8. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI 7500. GM % in copy/copy haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
10	9.9	-1.2	3.8
5.0	4.8	-4.2	5.6
1.0	0.91	-8.7	3.3
0.50	0.44	-11	5.6
0.05	0.05	-0.66	8.7

Table 9. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI 7900HT. GM % in copy/copy haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
10	10	1.5	1.8
5.0	4.8	-3.4	6.4
1.0	1.0	3.1	3.0
0.50	0.50	-0.65	4.7
0.05	0.06	19	15

Table 10. Values of trueness and precision as established by the EURL GMFF in its in-house verification using a Roche LC480. GM % in copy/copy haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
10	10	4.4	3.8
5.0	5.1	2.4	4.5
1.0	1.0	3.7	1.7
0.50	0.51	2.5	4.4
0.05	0.05	-0.60	15

5.2 International collaborative study (Step 4)

5.2.1 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 displayed in Table 11. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

$$\text{Efficiency [\%]} = \left(10^{\frac{-1}{slope}} - 1\right) \times 100$$

Table 11 indicates that the efficiency of amplification for the MON 87419 system ranges from 91 % to 106 % and the linearity from 0.96 to 1.00; the amplification efficiency for the maize-specific system ranges from 90 % to 98 % and the linearity is 1.00 in all runs. The mean PCR efficiency was 97 % for MON 87419 assay and 95 % for the *hmg* one. The average R^2 of the methods was 1.00 and 1.00 for the MON 87419 and *hmg* assays, respectively. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

Table 11. 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 87419			***hmg***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.43	96	1.00	-3.37	98	1.00
	B	-3.36	99	1.00	-3.38	98	1.00
2	A	-3.57	91	1.00	-3.58	90	1.00
	B	-3.52	92	1.00	-3.54	92	1.00
3	A	-3.42	96	0.99	-3.47	94	1.00
	B	-3.29	101	0.99	-3.47	94	1.00
4	A	-3.45	95	0.99	-3.44	95	1.00
	B	-3.37	98	1.00	-3.44	95	1.00
5	A	-3.41	96	1.00	-3.45	95	1.00
	B	-3.40	97	1.00	-3.44	95	1.00
6	A	-3.28	102	1.00	-3.37	98	1.00
	B	-3.19	106	1.00	-3.37	98	1.00
7	A	-3.24	104	1.00	-3.42	96	1.00
	B	-3.40	97	0.96	-3.41	96	1.00
8	A	-3.53	92	1.00	-3.47	94	1.00
	B	-3.45	95	1.00	-3.48	94	1.00
9	A	-3.50	93	1.00	-3.43	96	1.00
	B	-3.37	98	1.00	-3.37	98	1.00
10	A	-3.41	96	1.00	-3.43	96	1.00
	B	-3.42	96	1.00	-3.43	96	1.00
11	A	-3.46	95	1.00	-3.48	94	1.00
	B	-3.40	97	1.00	-3.44	95	1.00
12	A	-3.39	97	1.00	-3.41	96	1.00
	B	-3.40	97	1.00	-3.39	97	1.00
	Mean	**-3.40**	**97**	**1.00**	**-3.44**	**95**	**1.00**

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

5.2.2 GMO quantification

Table 12 reports the values of quantification for the four replicates of each GM level as reported by each of the twelve participating laboratories.

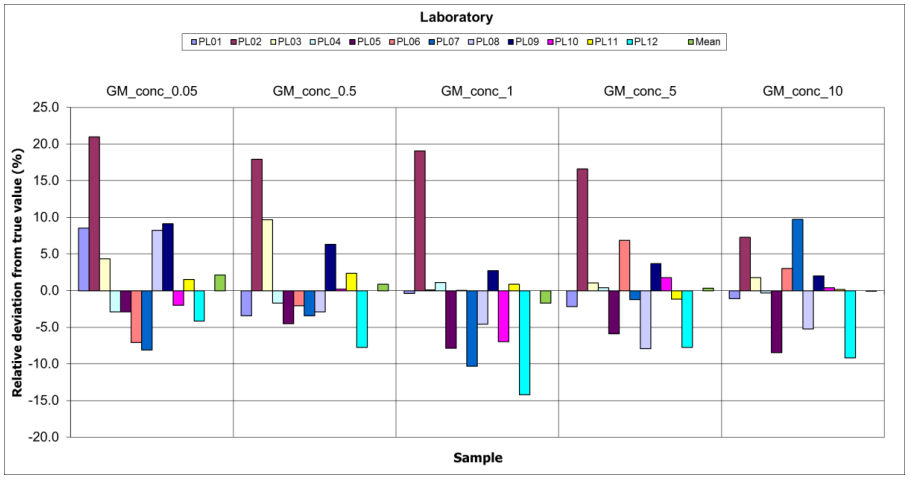
Table 12. GM % values determined by laboratories for test samples

	GMO content (%) *																			
	0.05				0.50				1				5				10			
LAB	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	0.05	0.06	0.06	0.06	0.50	0.49	0.46	0.48	1.0	1.0	0.95	0.98	4.9	5.1	4.9	4.7	10.2	9.7	9.8	9.9
2	0.05	0.06	0.07	0.06	0.64	0.52	0.61	0.59	1.2	1.2	1.1	1.2	5.8	5.9	6.1	5.6	10.5	11.6	10.2	10.5
3	0.05	0.05	0.06	0.05	0.53	0.47	0.66	0.54	0.90	1.1	0.99	1.0	5.0	4.9	5.0	5.2	10.2	10.9	9.5	10.1
4	0.05	0.04	0.05	0.05	0.58	0.41	0.47	0.51	0.78	0.89	1.2	1.2	4.5	5.4	4.8	5.3	11.4	8.8	10.0	9.6
5	0.05	0.05	0.05	0.05	0.47	0.47	0.46	0.51	0.95	0.90	0.95	0.88	4.8	4.7	4.8	4.5	9.0	8.6	9.4	9.6
6	0.04	0.04	0.05	0.05	0.42	0.53	0.54	0.47	1.1	1.1	0.90	0.91	5.8	5.8	4.7	5.1	11.7	11.4	8.7	9.4
7	0.05	0.06	0.04	0.04	0.48	0.49	0.57	0.40	1.1	1.1	0.86	0.56	4.9	5.5	4.3	5.0	11.3	11.5	10.8	10.2
8	0.05	0.05	0.06	0.06	0.48	0.49	0.45	0.52	1.0	0.92	0.96	0.91	4.8	4.5	4.7	4.4	9.4	9.1	8.9	10.5
9	0.05	0.05	0.05	0.06	0.54	0.49	0.54	0.56	0.96	1.0	1.1	1.0	5.0	5.1	4.8	5.9	10.6	10.8	10.1	9.3
10	0.05	0.05	0.05	0.05	0.51	0.51	0.50	0.49	0.93	0.96	0.92	0.90	5.1	5.1	5.1	5.1	10.0	10.2	10.3	9.6
11	0.05	0.05	0.05	0.06	0.51	0.47	0.51	0.55	0.97	1.0	1.0	1.0	4.9	5.0	4.6	5.3	10.3	10.5	9.9	9.3
12	0.05	0.05	0.04	0.05	0.46	0.44	0.46	0.49	0.85	0.88	0.85	0.85	4.5	4.2	4.7	5.0	9.6	9.0	9.1	8.7

* GMO % = (GMO copy number/maize haploid genome copy number) x 100

A graphical representation of the data reported in Table 12 is provided in Figure 1 showing the relative deviation from the true value for each GM level tested for the participating laboratory. The coloured bars represent the deviation of the GM level measured in % of the true GM level; the green bar on the right represents the mean relative deviation over all data before removing outliers for the concerned GM level.

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



PL = participating laboratory

Overall, laboratories' mean relative deviations from the true values were within a maximum of ± 25 %.

5.2.3 Method performance requirements

Among the 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 13 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study (see Table 4 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 13, the method satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R % is 12 % at the 0.05 % GM level, thus within the acceptance criterion.

Table 13. Summary of validation results for the MON 87419 method, expressed as GM copy numbers in relation to target taxon haploid genome copy numbers.

	Test Sample Expected GMO %				
	0.05	**0.50**	**1.0**	**5.0**	**10**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	2	0	0
Reason for exclusion	-	-	2C*	-	-
Mean value	0.05	0.50	0.99	5.0	10
Relative repeatability standard deviation, RSD_r (%)	10	9.2	5.6	6.5	7.1
Repeatability standard deviation	0.01	0.05	0.06	0.32	0.71
Relative reproducibility standard deviation, RSD_R (%)	12	11	10	8.8	8.3
Reproducibility standard deviation	0.01	0.05	0.10	0.44	0.83
Bias** (absolute value)	0.00	0.00	-0.01	0.02	0.00
Bias (%)	2.1	0.89	-1.1	0.35	-0.01

* 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 13 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 through in-house experiments, is below 25 % (see ENGL document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing"[9] http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). As it can be observed from the values reported, the relative repeatability standard deviation is below 25 % at all GM levels, with the highest value of 10 % at the 0.05 % 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 2.1 % at the 0.05 % GM level.

6. Compliance of the method for detection and quantification of event MON 87419 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 14:

- 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 fraction of copies of GM-material) was 13.38 %, based on 15 replicates (Table 2), hence below the maximum value of 25 % required by the ENGL. 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 0.05 % expressed in terms of copy number ratio to haploid genome copy numbers). The experiments were carried out under repeatability conditions on fifteen replicates. The RSD_r resulted to range between 8.7 % and 15 % (Table 8, 9 and 10) 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 10 %, therefore also below 25 % and well in line with the previous data.

The outcome of the different steps is summarised Table 14.

Table 14. Precision of the event-specific method for quantitative detection of MON 87419 at or around 0.1 % level related to mass fractions of GM material.

Source	**RSD_r %**	**GM %**
Applicant's method optimisation	13.38 %	0.085 %
EURL GMFF tests	8.7 - 15 %	0.10 %
Collaborative study	10 %	0.10 %

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[8].

7. 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 confirmed that the method is applicable to the control samples provided by the applicant (see paragraph 4.4.3), in accordance with the requirements of Annex I-3.C.2 to Commission Regulation (EU) No 503/2013[7] and (EU) No 619/2011[8] 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 level presence of 0.1 % (m/m) 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.

8. References

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Annex 1: Event-specific Method for the Quantification of maize MON 87419 by Real-time PCR

Validated Method

Method development:

Monsanto Company

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 87419 DNA to total maize 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. 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 detection of GM event MON 87419, a 97 bp fragment of the region spanning the 5' insert-to-plant junction in maize MON 87419 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 87419, a maize taxon-specific 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 (6-carboxyfluorescein) as reporter dye at its 5' end and TAMRA (6-carboxytetramethylrhodamine) 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 87419 DNA in a test sample, Cq values for the MON 87419 and the *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON 87419 DNA to total maize DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from genetically modified and conventional maize seeds and grains. 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 November 2018.

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 5 copies of MON 87419 per reaction. 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 fraction of copies 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 0.10 % (mass fraction of GM-material).

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' insert-to-plant junction in maize MON 87419 and is therefore event-specific for the event. This was confirmed in the validation study.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- 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 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 87419

3.2.1 General

The real-time PCR set-up for the taxon (*hmg*) and the GMO (event MON 87419) 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 87419) and the taxon (*hmg*) assay with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curves have to be established on at least five samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % maize MON 87419 DNA in a total of 250 ng of maize DNA (corresponding to 91575 maize haploid genome copies with one haploid genome assumed to correspond to 2.73 pg of maize genomic DNA)[1]. Standards S2 to S5 are to be prepared by serial dilutions (dilution factor 4 for samples S2-S5) 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)*	250	63	16	3.9	0.98
Maize haploid genome copies	91575	22894	5723	1431	358
MON 87419 copies	9158	2289	572	143	36

* Total nanograms are rounded to two digits for values ≤ 1, one digit for values between 1 and 10 and no digit for values ≥ 10

A calibration curve is to be 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 software.

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 reaction mixes for the MON 87419 maize specific system (Table 2) and the *hmg* reference gene system (Table 3). Please note that additional volume is included in the total to cover pipetting variability due to the viscosity of the solution.

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 87419 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
87419 QF (10 µM)	300 nM	0.75
87419 QR (10 µM)	300 nM	0.75
87419 QP* (10 µM)	200 nM	0.50
Nuclease free water	-	5.5
DNA	-	5.0
Total reaction volume:		25 µL

*TaqMan® probe labelled with 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* 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	-	5.6
DNA	-	5.0
Total reaction volume:		25 µL

*TaqMan® probe is labelled with 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 the maize MON 87419 and one for the *hmg* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).

5. Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (70 µL for the MON 87419 maize system and 70 µL for the *hmg* system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (17.5 µL DNA). The volume for the additional 0.5 repetition will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.

6. Spin down the tubes. Aliquot 25 µL for MON 87419 system and for the *hmg* reference system in each well.

7. Place an optical cover on the reaction plate and briefly centrifuge the plate.

8. 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 and start the run.

9. Select FAM as reporter dye for the MON 87419 and FAM for the *hmg* reference system. Define TAMRA as quencher dye for MON 87419 specific system and TAMRA for *hmg* reference system. Select ROX as the passive reference dye. Enter the correct reaction volume (25 µL).

10. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for MON 87419/*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	45
		Annealing & Extension	60	60	Yes	

*UNG: Uracil-N-glycosylase

3.3 Data analysis

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

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

a) Set the threshold following the automatic or the manual mode. In the manual mode display the amplification curves of the event specific assay in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the "update" button to ensure changes affect Cq values (only needed for some analysis software). Switch to the linear view mode by clicking on the Y axis of the amplification plot and check that the threshold previously set falls within the exponential phase of the curves.

b) Set the baseline following the automatic or the manual mode. In the manual mode: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Cq = 25, set the baseline crossing at Cq = 25 – 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a), b) and c) on the amplification plots of the taxon specific system.

e) 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 as described above, the instrument's software calculates the Cq values for each reaction.

The standard curves are generated both for the *hmg* and the MON 87419 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 87419 DNA in the unknown sample, the MON 87419 copy number is divided by the copy number of the maize endogenous gene *hmg* and multiplied by 100 (GM% = [MON 87419/*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
- Microcentrifuge
- 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 4304437.

4.3 Primers and Probes

Table 5. Primers and probes for the MON 87419 and *hmg* methods

	MON 87419	**DNA Sequence (5' to 3')**	**Length (nt)**
MON 87419			
Forward primer	87419 QF	CGG TCG CTG CCA GGT ATT G	19
Reverse primer	87419 QR	CAG ACC TCA ATT GCG AGC TTT CT	23
Probe	87419 QP	FAM-TGT GCG CCA GTC AGC ATC ATC ACA CC-TAMRA	26
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	FAM-CAA TCC ACA CAA ACG CAC GCG TA-TAMRA	23

FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.

5. References

1. Bennett MD, Leitch IJ. Plant DNA C-values database (release 6.0, Dec. 2012). http://www.kew.org/cvalues/.

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