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Event-specific Method for the Quantification of Maize MON 87429 Using Real-time PCR

Validation Report

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EUROPEAN COMMISSION
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Directorate F - Health, Consumers and Reference Materials (Geel)
Food and Feed Compliance

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Validation Report

11 August 2021

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 87429 (unique identifier MON-87429-9). 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], Bayer Agriculture BVBA provided the detection method and the positive and negative control samples (genomic DNA from seeds of MON 87429 maize as positive control DNA, and genomic DNA from 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 17025:2017 accredited [certificate number: Belac 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) in food and feed by DNA extraction, DNA identification and Real-time PCR and for determination of Genetically Modified content in % (cp/cp) in food and feed by DNA extraction and digital PCR)].

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], Bayer Agriculture BVBA provided the EURL GMFF with an event-specific method for detection and quantification of maize event MON 87429 (unique identifier MON-87429-9) 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 suitable for quantifying genomic DNA of GM maize MON 87429, 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)

Bayer Agriculture BVBA submitted the identification and quantification method, data demonstrating its adequate performance when applied to genomic DNA extracted from maize MON 87429 GM event and from non GM DNA, and the corresponding positive and negative control DNA samples.

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

3. Scientific assessment and bioinformatics analysis (step 2)

Documentation and data supplied by the applicant were evaluated by the EURL GMFF for compliance with the ENGL method acceptance criteria.

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.

3.1. Specificity assessment conducted 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 in Annex 1 (Tables 1, 2, 3 and 4), using at least 2500 haploid

genome copies per reaction: alfalfa J101, J163, KK179; oilseed rape MON 88302, RT73, 73496, Ms1, Ms8, Rf1, Rf2, Rf3, T45, Topas 19/2; cotton MON 531, MON 1445, MON 15985, MON 88701, MON 88702, MON 88913, GHB119, T304-40, LLCotton25, COT102, 281-24-236 x 3006-210-23, GHB614; maize MON 87429, MON 810, MON 863, MON 87403, MON 87411, MON 87419, MON 87427, MON 87460, MON 88017, MON 89034, NK603, 3272, 98140, Bt11, Bt176, TC1507, DAS-59122, DAS-40278-9, VCO-01981-5, DP-004114-3, GA21, T25, MIR162, MIR604; potato EH92-527-1, AM04-1020, AV43-6-G7; rice LL62; soybean 40-3-2, MON 87701, MON 87705, MON 87708, MON 87751, MON 87769, MON 89788, DAS-81419-2, DAS-68416-4, 356043, 305423, DAS-444406-6, FG72, A2704-12, A5547-127, BPS-CV-127-9; sugar beet H7-1 and conventional alfalfa, cotton, maize, oilseed rape, potato, rice, soybean, sugar beet, wheat.

According to the method developer the MON 87429 assay did not react with any sample except the positive control. All samples reacted with their taxon-specific reference systems.

In addition, the applicant performed an in-silico specificity analysis by using the amplicon sequence as a query for [BLASTN 2.2.21+] algorithm search against a local copy of the GenBank NT database_2018, posted by NCBI on December 3, 2017. The applicant did not observe alignments that fully contained both MON 87429 primers. Additionally, no alignment spanned the full length of the probe sequence.

3.2. Specificity assessment conducted by the EURL GMFF

The detection method spans the 5' insert-to-plant junction in maize MON 87429. The forward primer "MON 87429 primer 1" binds to the maize flanking region. The reverse primer "MON 87429 primer 2" binds to the left border region of the T-DNA from *Agrobacterium tumefaciens*. The "MON 87429 probe" binds to the plant-to-insert junction. According to the applicant's annotation, the probe is designed between the 5'-flanking genomic DNA, on DNA co-inserted during the transformation, and the 5'-end of the T-DNA.

The amplicon size is expected to be 116 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.

3.3. Verification of the ENGL acceptance parameters

The applicant prepared the calibration curve from a DNA solution (S1) of 10 % maize event MON 87429 genomic DNA (expressed as copy number ratio, considering a 0.5:1 event-to-taxon specific zigosity ratio and assuming a 2.73 pg weight for the maize genome) in 200 ng of total maize DNA which was serially diluted (1:4) to obtain solutions S2, S3, S4 and S5. The tests were conducted on an ABI 7500 instrument. The parameters (slope, R2 coefficient) of five runs of the calibration curve are reported as provided by the applicant (Table 1).

Table 1. Summary of the slope and R^2 values obtained by the applicant

MON 87429		***hmg***	
Slope	**R^2**	**Slope**	**R^2**
-3.38	0.9952	-3.40	0.9994
-3.36	0.9967	-3.41	0.9997
-3.42	0.9977	-3.44	0.9994
-3.33	0.9958	-3.41	0.9993
-3.31	0.9984	-3.37	0.9995

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 87429) and the taxon-specific *high mobility group* (*hmg*) system, as established by the applicant, were within the ENGL acceptance criteria.

The applicant established the precision of the method using four GM levels (expressed as copy number ratio). Three independent DNA extractions were used to generate three DNA test samples for each of the 10%, 1.0 % and 0.085 % (copy number ratio) levels of MON 87429 in a total amount of 160 ng of maize DNA. The nine DNA test samples were analysed in five replicates each. An additional level at 0.05 % (copy number ratio, corresponding to 0.1 % in mass fraction according to the applicant's information) was prepared in a total amount of 180 ng of maize DNA in three preparations and each preparation was tested in five replicates by three runs. Table 2A reports values for the four GM-levels as provided by the applicant for the precision assay. Both precision and trueness were within the ENGL acceptance criteria (trueness ± 25 %, RSD_r ≤ 25 % across the entire dynamic range).

Table 2A. Mean %, precision and trueness values provided by the applicant estimated for combined PCR modules expressed in relative concentrations

	Test results			
Expected GM %	**10**	**1**	**0.085**	**0.05**
Measured mean GM %	9.50	1.07	0.084	0.049
Precision (RSD_r %)	5.26	6.82	13.50	18.16
Trueness (bias %)	-4.95	7.03	-1.13	-1.94

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

The method met the ENGL acceptance criteria for trueness and precision at all the GM levels. The data provided confirm the applicant's statement that the 0.085 % sample is in line with the requirements for testing the limit of quantification (LOQ, below or equal to 0.09 % or 50 copies). Additionally, it is noted that also the sample at 0.05 % GM-content meets the same requirements.

The absolute limit of detection (LOD_{abs}) of the MON 87429 event specific real-time PCR method was assessed by the applicant in 60 PCR replicates at 10, 5 and 1 haploid genome copies per reaction of MON 87429 event. The LOD_{abs} was found to be at least 5 haploid genome copies for MON 87429 event-specific method. The LOD_{abs} is in line with the ENGL acceptance criteria (below 0.045 % or 25 copies with a level of confidence of 95 %).

The robustness of the method was assessed in 8 combinations of the following variations to the method: exact/+5 %/-5 % master mix concentration, exact/+10 %/-10 % primer concentration, exact/+10 %/-10 % probe concentration, exact/+1 µL/-1 µL master mix volume, +1 °C/-1 °C in annealing temperature. The RSD_r and the trueness calculated for each combination of variations on a sample at the 0.085 % level did not exceed 30 %, thus meeting the ENGL acceptance criteria.

Precision and trueness of the method were tested in a transferability study performed at a second laboratory with an ABI 7300 instrument: 15 values for each of the three GM levels (expressed as copies GM/total haploid genome copies) were provided. Table 2B reports precision and trueness values for the three GM-levels as provided by a laboratory different from the method developer. Both parameters were within the ENGL acceptance criteria (trueness ≤ 25 %, RSDr ≤ 25 % across the entire dynamic range).

Table 2B. Mean %, precision and trueness values obtained by the applicant in the transferability study

	Test results		
Expected GM %	**10**	**1**	**0.085**
Measured mean GM %	8.84	1.02	0.082
Precision (RSD_r %)	7.60	8.87	14.73
Trueness (bias %)	-11.63	2.29	-3.60

3.4. DNA extraction

Genomic DNA was isolated from ground maize seeds, using a method that was already validated in-house by the EURL GMFF. The protocol for DNA extraction and a report on testing are published at https://gmo-crl.jrc.ec.europa.eu/summaries/CRL-VL-16-05-XP-Corrected-version-2.pdf. According to the experimental data submitted by the applicant, the protocol for DNA extraction produced DNA of suitable quantity and quality for PCR based applications when applied to ground seeds from the maize event MON 87429.

In agreement with the ENGL position, endorsing the modularity principle (see also Annex III to Regulation (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 87429.

Annex III to Regulation (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) expected to be placed on the market. To this regard, the applicant stated that the applicability of the real-time quantitative TaqMan® PCR developed for MON 87429 depends on the isolation of sufficient quantity and quality of purified DNA. This method has been tested on DNA extracted from ground seed material. The detection method for MON 87429 should work as far as good quality and intact DNA can be extracted from processed food and feed materials. 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 processing of maize grain involves varying degrees of mechanical, enzymatic, solvent, heat, acid, pressure treatment, or combinations of these steps. These steps can influence the quality and intactness of DNA contained in the final processed maize products. After extraction of DNA from certain of these processed matrices, the DNA may need additional rounds of processing in order to clean up the DNA and eliminate PCR inhibitors, in order to achieve a quality of genomic DNA that is suitable for PCR [10, 11]. 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 [12, 13]. Random DNA

fragmentation is known to lead to variability in quantitating DNA by qPCR [14], thus affecting the ability to accurately quantify the presence of a GM event in processed fractions".

Whenever DNA is extracted from more complex and difficult 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 method

4.1. Samples

The following positive and negative control samples were provided by the applicant to the EURL GMFF in accordance to Regulation (EC) No 1829/2003 Art 2.11[a]:

- genomic DNA extracted by the applicant from hemizygous maize seeds for the MON 87429 event, and
- genomic DNA extracted by the applicant from conventional maize seeds genetically similar to those harbouring the MON 87429 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 87429 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 87429 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 87429, a 116 bp fragment of the region spanning the 5' insert-to-plant junction in maize MON 87429 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 (tetramethylrhodamine) as a quencher dye at its 3' end.

For the relative quantification of GM event MON 87429, 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 a *hmg* gene-specific probe labelled with FAM as reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

Standard curves are generated for both the MON 87429 and the *hmg* systems by plotting the Cq values measured for the calibration points against the logarithm of the DNA copy numbers and by

[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).

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 87429 DNA in a test sample, the MON 87429 copy number is divided by the copy number of the maize haploid genome and multiplied by 100 to obtain the percentage value (GM % = MON 87429/ maize haploid genome x 100).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (picograms) by the published average 1C value for the maize genome (2.73 pg) [15]. 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 indicated. 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 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)	200	50	12.5	3.1	0.78
Target taxon [haploid genome copies]	73260	18315	4579	1145	286
Target MON 87429 copies	7326	1832	458	114	29

4.3. EURL GMFF experimental testing (step 3)

4.3.1. Determination of the zygosity ratio in the positive control sample

The EURL GMFF verified the zygosity ratio (GM-target to reference target ratio) in the positive control sample to assess the method performance at 0.1% GM level -expressed as mass fraction of GM material- in relation to the provisions of Regulation (EU) No 619/2011 [8].

The ratio between the copy number of MON 87429 and *hmg* targets in the positive control sample was determined by digital droplet PCR (ddPCR) performed with the Bio Rad QX200 Droplet Reader.

The zygosity was tested in simplex in eighteen data sets for the GM target and eighteen for the reference target.

Reaction mixes were prepared in order to obtain a final volume of 22 µL and contained 1X ddPCR Super Mix (Bio Rad, Cat. number 1863024), primers and probes at concentrations indicated in the

corresponding validated method (MON 87429 primer 1 and MON 87429 primer 2 at 400 nM each, MON 87429 probe at 200 nM; hmg primers at 300 nM each, hmg probe at 160 nM), and 5 µL of DNA at a concentration of 25 ng/µL.

Reaction mixes were loaded into a semi skirt 96-well plate. 'No template controls' were included. After sealing with a sealing aluminium foil using the PX1™ PCR Plate Sealer, the plate was briefly centrifuged (1 min at 1000 rpm) and placed on Bio Rad Automated Droplet Generator (AutoDG).

The instrument added the Automated Droplet Generation oil for Probes (Bio Rad, Cat. number 1864110), generated the droplets in a final volume of 40 µL of the emulsion containing droplets. The AutoDG then transferred all the emulsions into a new semi skirt 96-well plate. The new plate was sealed with a sealing foil with the PX1™ PCR Plate Sealer and run in a Bio Rad C1000 TouchThermal Cycler. The thermal cycling conditions in a final volume of 40 µL were as indicated below:

Step	**Temperature (°C)**	**Time (mm:ss)**	**Ramp rate**	**Number of cycles**
Polymerase activation	95	10:00	2°C/sec	1
DNA denaturation	94	00:30		40
Annealing/extension	60	01:00		
Enzyme deactivation	98	10:00		1

The sealed 96-well plate was then placed in the QX200 Droplet Reader to determine through cytofluorimetry the fraction of fluorescent PCR-positive droplets with respect of the total number of droplets in the original sample by selecting the proper fluorescent dye used. Data analysis and copy number calculations were performed using the Bio-Rad QX200 Droplet Reader Analysis software (QuantaSoft version 1.7.4).

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' [16].

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 (0.054 % - 10 %, see Table 4). The blind test samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant (see 4.1 for details) by mixing MON 87429 maize DNA and non-GM maize DNA.

Table 4. MON 87429 blinded samples GM % contents

MON 87429 GM % GM copy number/maize haploid genome copy number x 100
10
5.0
0.90
0.40
0.054

The calibration sample S1 was prepared from the genomic DNA provided by the applicant by mixing the appropriate amount of MON 87429 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 (see Table 3).

The experiments were performed on an ABI 7500, an ABI 7900HT, QuantStudio 7 Flex and a Roche LC480 real-time platform under repeatability conditions and followed the protocol provided by the applicant.

Test samples with GM levels 10 %, 5.0 %, 0.90 % and 0.40 % 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 0.054 % 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.

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 [17] 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 87429 international collaborative study were randomly selected from 29 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 5.

Table 5. Laboratories participating in the validation of the detection method for maize event MON 87429

Laboratory	**Country**
BioGEVES – (Groupement d'Intérêt Public – Groupe d'Etude et de contrôle des Variétés et des Semences)	FR
Center for Agricultural Technology Augustenberg	DE
GMO department of LAA (Laboratorio Arbitral Agroalimentario), Ministry of Agriculture	ES
Federal State Agency of Analysis and Diagnosis for Rhineland-Palatinate	DE
Institute for National Investigation for the Health and Veterinarian Nature Saxonia	DE
National Centre for Food, Spanish Agency for Food Safety and Nutrition (AESAN)	ES
National Health Laboratory	LU
National Institute of Biology	SI
National Research Institute of Animal Production, National Feed Laboratory	PL
Laboratory Service of the Ministry of Economy and Finance - Strasbourg	FR
Veterinary Public Health Institute for Lazio and Toscana Regions	IT
Walloon Agricultural Research Centre	BE

4.4.2. Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: four laboratories used QuantStudio 5, three laboratories used QuantStudio 7 Flex, three others used ABI 7500 (one Fast instrument), one laboratory used Roche LC480 and another used BioRad CFX96.

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.

4.4.3. Materials used in the international collaborative study

For the validation of the quantitative event-specific method, calibration samples (of known GMO content) and blind test samples (of undisclosed GM content = blind samples) were provided by the EURL GMFF to the participating laboratories (for test samples preparation see 4.3.2).

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

- ✓ Five calibration samples with known concentrations of GM-event (140 µL of DNA solution each) labelled from S1 to S5 (Table 3).

- ✓ Twenty blinded test DNA samples (70 µL of DNA solution, each at 45 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 4)

- ✓ Reaction reagents:
  - TaqMan® Universal PCR Master Mix (2x), one vial: 8 mL
  - distilled sterile water, one vial: 5 mL

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

  hmg taxon-specific assay
  - MaiJ-F (10 µM): 240 µL
  - mhmg-R (10 µM): 240 µL
  - mhmg-P (10 µM): 130 µL

  MON 87429 assay
  - MON 87429 primer 1 (10 µM): 320 µL
  - MON 87429 primer 2 (10 µM): 320 µL
  - MON 87429 probe (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 87429 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 87429 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 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

None of the participating laboratories reported deviations from the validation protocol.

5. Results

5.1. EURL GMFF experimental testing

5.1.1. Zygosity ratio in the positive control sample

The results of the digital PCR analysis conducted by the EURL GMFF to determine the zygosity ratio in the positive control samples, by targeting the MON 87429 and *hmg* targets, are shown in Table 6. The average of accepted droplets was above 18,000. All NTCs were negative (6 NTCs for each amplification system).

Table 6. Zygosity ratio of the MON 87429 and *hmg* targets in the positive control sample.

Mean ratio (MON 87429/(*hmg*)	0.538
Standard deviation	0.015
RSD_r (%)	2.8
Standard error of the mean	0.004
Upper 95 % CI of the mean	0.545
Lower 95 % CI of the mean	0.531

The mean ratio (MON 87429/*hmg*) equals 0.538. The 95 % confidence interval (CI) spans around 0.54; this is in agreement with the expected ratio for a maize control sample, hemizygous for the GM-locus, with a GM parental contribution of female origin, as indicated by the applicant's information on the positive control sample, and assuming a single - copy endogenous gene target.

Hence,

0.054 % GM in haploid genome copy numbers = 0.1 % in mass fraction of GM DNA

Note: the zygosity ratio herein reported is valid for the positive control sample DNA in the context of the present validation study. It is used to assess the method performance at 0.1% GM level, expressed as mass fraction of GM material in relation to the provisions of Regulation (EU) No 619/2011.

When analytical results of official laboratories are primarily expressed as ratio of GM DNA copy numbers, they shall be translated into mass fraction results by means of the specific conversion factor published in the document "Conversion factors (CF) for certified references materials (CRM)" (https://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

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

Test samples with GM levels from 10 % to 0.40 % 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 0.054 % GM-level was tested for its precision in quantification in 15 replicates in separate runs.

Tests were conducted on ABI 7500, ABI 7900HT, QuantStudio 7 Flex and Roche LC480 for robustness.

The standard curve parameters and the results of efficiency, linearity, trueness and precision obtained in real-time PCR runs with the test samples are shown in Tables from 7A, 7B to 11.

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, QuantStudio 7 Flex and Roche LC480 to quantify GM-levels in the range 10 % to 0.40 % in four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	MON 87429 system			***hmg* system**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.42	96	1.00	-3.36	98	1.00
Run B	-3.49	94	1.00	-3.35	99	1.00
Run C	-3.41	97	1.00	-3.39	97	1.00
Run D	-3.35	99	0.99	-3.35	99	1.00
Run E	-3.40	97	0.99	-3.37	98	1.00
Run F	-3.39	97	1.00	-3.39	97	1.00
Run G	-3.34	99	1.00	-3.39	97	1.00
Run H	-3.38	98	1.00	-3.34	99	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$
Runs A and B were carried out on ABI 7500; runs C and D were carried out on ABI 7900HT; runs E and F were carried out on QuantStudio 7Flex; runs G and H were carried out on Roche LC480.

Table 7B. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, ABI 7900HT, QuantStudio 7 Flex and Roche LC480 to quantify the GM-level 0.054 % (corresponding to 0.1 % in mass fraction of GM DNA) in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	MON 87429 system			***hmg* system**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run I	-3.45	95	1.00	-3.36	99	1.00
Run J	-3.52	92	1.00	-3.37	98	1.00
Run K	-3.45	95	1.00	-3.36	99	1.00
Run L	-3.37	98	1.00	-3.35	99	1.00
Run M	-3.46	95	1.00	-3.35	99	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$
Run I and J were carried out on ABI 7500; run K was carried out on ABI 7900HT; run L was carried out on QuantStudio 7 Flex; run M 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, 10 and 11 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	9.4	-5.9	2.3
5.0	4.5	-10	2.4
0.90	0.81	-10	2.4
0.40	0.34	-14	2.6
0.054	0.042/0.051**	-22/-5.5**	16/19**

** results from respectively runs I and J § Table 7B

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	9.4	-5.8	1.9
5.0	4.4	-13	5.8
0.90	0.77	-15	2.4
0.40	0.33	-18	8.6
0.054	0.046	-15	12

Table 10. Values of trueness and precision as established by the EURL GMFF in its in-house verification using a QuantStudio 7Flex. GM % in copy/copy haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
10	9.8	-2.4	3.0
5.0	5.1	1.9	2.5
0.90	0.85	-5.9	3.0
0.40	0.38	-5.2	4.3
0.054	0.047	-14	12

Table 11. 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 (RSDr %)
10	11	6.7	1.8
5.0	5.0	-0.91	3.8
0.90	0.86	-4.0	1.0
0.40	0.35	-13	4.6
0.054	0.048	-12	20

5.2. Results of the international collaborative study

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 12. 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 12 indicates that the efficiency of amplification for the MON 87429 system ranges from 86 % to 101 % and the linearity from 0.98 to 1.00; the amplification efficiency for the *hmg* species-specific system ranges from 90 % to 100 % and the linearity is 1.00.

The mean PCR efficiency was 95 % for the MON 87429 assay and 96 % for the *hmg* one. The mean R^2 of the methods was 1.00 for both the MON 87429 and *hmg* assays. Both PCR efficiency and linearity mean values are within the ENGL acceptance criteria.

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 87429			***hmg***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.45	95	1.00	-3.45	95	1.00
	B	-3.51	93	1.00	-3.40	97	1.00
2	A	-3.72	86	0.99	-3.58	90	1.00
	B	-3.68	87	1.00	-3.55	91	1.00
3	A	-3.38	98	1.00	-3.42	96	1.00
	B	-3.65	88	0.98	-3.37	98	1.00
4	A	-3.34	99	1.00	-3.38	98	1.00
	B	-3.50	93	1.00	-3.41	97	1.00
5	A	-3.43	96	1.00	-3.37	98	1.00
	B	-3.32	100	1.00	-3.40	97	1.00
6	A	-3.47	94	1.00	-3.45	95	1.00
	B	-3.47	94	1.00	-3.45	95	1.00
7	A	-3.45	95	1.00	-3.38	97	1.00
	B	-3.42	96	1.00	-3.33	100	1.00
8	A	-3.30	101	1.00	-3.35	99	1.00
	B	-3.44	95	1.00	-3.33	100	1.00
9	A	-3.35	99	1.00	-3.39	97	1.00
	B	-3.40	97	1.00	-3.34	99	1.00
10	A	-3.42	96	1.00	-3.43	96	1.00
	B	-3.34	99	1.00	-3.40	97	1.00
11	A	-3.44	95	1.00	-3.42	96	1.00
	B	-3.40	97	0.99	-3.37	98	1.00
12	A	-3.48	94	1.00	-3.48	94	1.00
	B	-3.45	95	0.99	-3.56	91	1.00
	Mean	-3.45	95	1.00	-3.42	96	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 13 reports the values of quantification for the four replicates of each GM level as reported by each of the twelve participating laboratories.

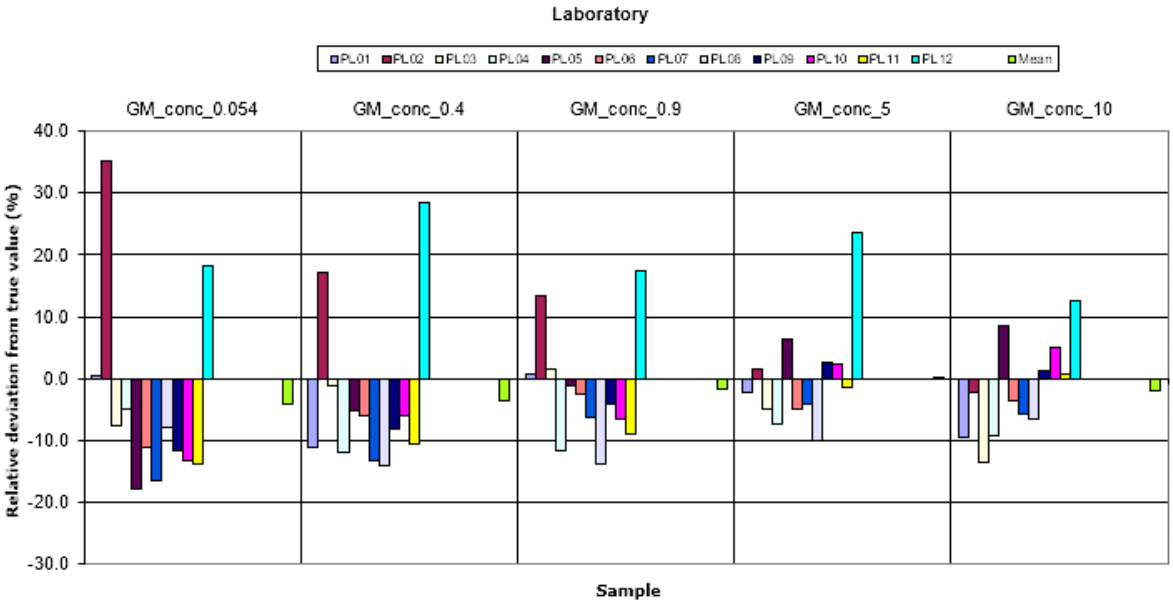
Table 13. GM % values determined by laboratories for test samples

	GMO content (%) *																			
LAB	0.054				0.40				0.90				5.0				10			
	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	0.05	0.06	0.06	0.32	0.36	0.41	0.32	0.76	0.90	0.93	1.0	6.0	4.5	4.3	4.8	9.2	8.2	9.4	9.3
2	0.08	0.06	0.07	0.08	0.46	0.48	0.45	0.48	1.0	1.0	1.0	1.0	5.1	5.0	5.2	5.1	9.8	10	9.6	9.8
3	0.04	0.04	0.05	0.06	0.43	0.42	0.37	0.36	1.0	0.77	0.93	0.91	6.1	3.7	4.0	5.3	7.6	7.0	9.9	10
4	0.06	0.05	0.05	0.05	0.36	0.36	0.35	0.34	0.86	0.77	0.79	0.75	4.4	4.8	4.8	4.6	9.7	9.1	8.4	9.1
5	0.04	0.04	0.05	0.05	0.38	0.40	0.37	0.38	0.86	0.89	0.92	0.89	5.5	5.0	5.4	5.4	10	12	10	11
6	0.05	0.05	0.05	0.05	0.41	0.38	0.35	0.36	0.94	0.91	0.81	0.85	4.9	4.8	4.7	4.6	10	9.9	9.4	9.3
7	0.04	0.05	0.04	0.05	0.31	0.35	0.37	0.36	0.87	0.84	0.83	0.83	4.7	5.1	4.9	4.4	9.6	9.4	9.4	9.2
8	0.05	0.06	0.05	0.04	0.34	0.34	0.37	0.33	0.69	0.85	0.80	0.76	4.4	4.9	4.3	4.4	9.4	9.7	8.8	9.4
9	0.05	0.05	0.05	0.04	0.36	0.40	0.38	0.32	0.78	0.90	0.87	0.91	5.2	5.2	5.1	5.1	10	9.9	10	9.9
10	0.05	0.04	0.04	0.05	0.35	0.37	0.40	0.39	0.87	0.85	0.83	0.82	4.7	5.4	5.2	5.1	11	11	10	10
11	0.04	0.05	0.05	0.05	0.35	0.36	0.34	0.37	0.86	0.88	0.78	0.76	5.0	4.5	5.5	4.8	9.6	10	10	10
12	0.07	0.05	0.08	0.05	0.52	0.60	0.49	0.44	1.1	1.2	0.99	1.0	5.4	5.6	7.1	6.6	9.9	12	13	10

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

A graphical representation of the data reported in Table 13 is provided in Figure 1 that shows 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 eliminating outliers for each GM level.

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



PL = participating laboratory.

Overall, most laboratories' mean relative deviations from the true values were within a maximum of ± 25 %. At GM-level 0.054 % and 0.40 % eleven laboratories were within the limit; at GM-levels 0.90 %, 5.0 % and 10 % twelve laboratories were within the limits. At GM-levels 0.054 % and 0.40 % one laboratory overestimated by more than 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 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 14 % at the 0.054 % GM level, thus within the acceptance criterion.

Table 14. Summary of validation results for the MON 87429 method, expressed as GM copy numbers in relation to target taxon haploid genome copy numbers.

	Test Sample Expected GMO %				
	0.054	**0.40**	**0.90**	**5.0**	**10**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	2	2	0	1	2
Reason for exclusion*	2 DG	2 DG	-	1 C	2 C
Mean value	0.048	0.36	0.88	5.0	9.8
Relative repeatability standard deviation, RSD_r (%)	14	7.3	7.3	7.9	4.6
Repeatability standard deviation	0.007	0.027	0.064	0.40	0.45
Relative reproducibility standard deviation, RSD_R (%)	14	7.8	11	11	7.3
Reproducibility standard deviation	0.007	0.028	0.10	0.57	0.72
Bias** (absolute value)	-0.006	-0.035	-0.016	0.029	-0.22
Bias (%)	-10	-8.8	-1.8	0.59	-2.2

* C= Cochran's test; DG = Double Grubbs' 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 through 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 relative repeatability standard deviation is below 25 % at all GM levels, with the highest value of 14 % at the 0.054 % 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 lowest value of bias (%) of -10 % at the 0.054 % GM level.

6. Compliance of the method for detection and quantification of event MON 87429 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 0.05 % in copy number ratio (approximately corresponding to a 0.1 % in mass fraction of GM-DNA) level shown by the applicant's dossier was 18.16 %, based on 15 replicates (Table 2A), 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 0.054 % related to MON 87429 copy numbers in relation to *hmg* specific DNA copy numbers calculated in terms of haploid genomes). The experiments were carried out under repeatability conditions on fifteen replicates. The RSD_r resulted to range between 12 % and 20 % (Table 8, 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 at the level of 0.1 % related to mass fraction of GM-material the RSD_r of the method was 14 %, therefore also below 25 % and well in line with the previous data.

The outcome of the different steps is summarised Table 15.

Table 15. Precision of the event-specific method for quantitative detection of MON 87429 at or around 0.1 % level related to mass fractions of GM material.

Source	**RSD_r %**	**GM %**
Applicant's method optimisation	18.16 %	~ 0.10 %
EURL GMFF tests	12 - 20 %	0.10 %
Collaborative study	14 %	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.

7. Conclusion

The method provided by the applicant was 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 4.1), in accordance with the requirements of Annex I-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-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.

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Annex 1 - Event-specific Method for the Quantification of maize MON 87429 by Real-time PCR

Validated Method

Method development:

Bayer Agriculture BVBA

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 87429 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 87429, a 116 bp fragment of the region spanning the 5' insert-to-plant junction in maize MON 87429 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 (tetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of GM event MON 87429, a maize taxon-specific system amplifies a 79 bp fragment of a maize *high mobility group* (*hmg*) endogenous gene (Accession number: GenBank 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 87429 DNA in a test sample, Cq values for the MON 87429 and the *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON 87429 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 grain. 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-December 2020.

A detailed validation report is available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

2.3 Limit of detection (LOD)

According to the method developer, the absolute LOD of the method is at least 5 copies of MON 87429 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 copy number ratio) in 160 ng of total suitable maize DNA. The method developer also provided data showing that a sample at 0.05 % in copy number ratio in 180 ng of maize DNA could be quantified in 15 replicates within the ENGL acceptance criteria for the LOQ. The lowest relative GM content of the target sequence included in the collaborative trial was 0.054 % in copy number ratio, corresponding to 0.1 % in mass fraction of GM-DNA.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' insert-to-plant junction in maize MON 87429 and is therefore event-specific for the event MON 87429.

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 and amendment 1 (2013-04-15).
- 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, e.g. 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 87429

3.2.1 General

The real-time PCR set-up for the taxon (*hmg*) and the GMO (event MON 87429) 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 87429) 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 87429 DNA in a total of 200 ng of maize DNA (corresponding to 73260 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)*	200	50	13	3.1	0.78
Maize haploid genome copies	73260	18315	4579	1145	286
MON 87429 copies	7326	1832	458	114	29

* Total nanograms are rounded to the integral value

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 87429 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 87429 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.50
MON 87429 primer 1 (10 µM)	400 nM	1.0
MON 87429 primer 2 (10 µM)	400 nM	1.0
MON 87429 probe* (10 µM)	200 nM	0.5
Nuclease free water	-	6.0
DNA	-	4.0
Total reaction volume:		25.0 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* assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.50
MaiJ-F (10 µM)	300 nM	0.75
mhmg-R (10 µM)	300 nM	0.75
mhmg-P* (10 µM)	160 nM	0.40
Nuclease free water	-	6.60
DNA	-	4.0
Total reaction volume:		25 µL

*TaqMan® probe 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 the maize MON 87429 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 (73.5 µL for the MON 87429 maize system and 73.5 µL for the *hmg* system). Add to each tube the correct

amount of DNA for 3.5 PCR repetitions (14 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 the MON 87429 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 87429 and for the *hmg* reference system. Define TAMRA as quencher dye for the MON 87429 specific system and for the *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 the MON 87429/*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:

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 for the MON 87429 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 87429 DNA in the unknown sample, the MON 87429 copy number is divided by the copy number of the maize endogenous gene *hmg* and multiplied by 100 (GM% = MON 87429/*hmg* x 100).

3. 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 mL, 1.5 mL and 5 mL or 15 mL DNAse free reaction tubes

4.2 Reagents

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

4.3 Primers and Probes

Table 5. Primers and probes for the MON 87429 and *hmg* methods

	MON 87429	**DNA Sequence (5' to 3')**	**Length (nt)**
MON 87429			
Forward primer	MON 87429 primer 1	CgA gAC AgA CTC AAT gTA TCC gAg ATA CTC	30
Reverse primer	MON 87429 primer 2	CCA TCA TAC TCA TTg CTg ATC CAT gTA	27
Probe	MON 87429 probe	6- FAM-TCC Cgg ACA TgA AAC CAA ACA AgA gTg gTC-TAMRA	30
hmg			
Forward primer	MaiJ-F	TTg gAC TAg AAA TCT CgT gCT gA	23
Reverse primer	mhmg-R	gCT ACA TAg ggA gCC TTg TCC T	22
Probe	mhmg-P	6-FAM-CAA TCC ACA CAA ACg CAC gCg TA-TAMRA	23

FAM: 6-carboxyfluorescein; TAMRA: tetramethylrhodamine

5. References

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

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