



JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Event-specific Method for the Quantification of Maize Event MON 94804 Using Real-time PCR

Validation Report

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Abstract

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 94804 (unique identifier MON-94804-4). The validation study was conducted according to the EURL GMFF validation procedure <https://gmo-crl.jrc.ec.europa.eu/guidance-documents> and the relevant internationally accepted guidelines (2-6).

In accordance with current EU legislation (1), Bayer CropScience LP, represented by Bayer Agriculture BV, provided the detection method and the positive and negative control samples (genomic DNA from seeds of MON 94804 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 as 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 (5), and it fulfils the analytical requirements of Regulation (EU) No 619/2011 (6). This validation report is published at <https://gmo-crl.jrc.ec.europa.eu/method-validations>.

Quality assurance

The EURL GMFF is ISO/IEC 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.

Event-specific Method for the Quantification of Maize MON 94804 Using Real-time PCR

Validation Report

7 March 2024

European Union Reference Laboratory for GM Food and Feed

1 Introduction

In line with Regulation (EC) No 1829/2003 (1), Bayer CropScience LP, represented by Bayer Agriculture BV, provided the EURL GMFF with an event-specific method for detection and quantification of maize event MON 94804 (unique identifier MON-94804-4) 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 (7), 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 94804, 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 CropScience LP, represented by Bayer Agriculture BV, submitted the identification and quantification method, data demonstrating its adequate performance when applied to genomic DNA extracted from maize MON 94804 GM event and from non GM maize, 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 method 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 method was assessed by the applicant in duplicate real-time PCR reactions, according to the method described in Annex 1 (Tables 1, 2, 3, 4 and 5), using at least 2500 copies of non- target GM DNA or at least 2500 conventional genomic DNA extracted from:

alfalfa J101, J163, KK179; 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 94804, MON 87429, MON 810, MON 87403, MON 87411, MON 87419, MON 87427, MON 87460, MON 88017, MON 89034, NK 603, MON 95379, 3272, 5307, 98140, Bt11, Bt176, MZHGOJG, TC1507, DAS 59122, DAS 40278-9, VCO-01981-5, DP-004114-3, GA21, T25, MIR162, MIR604, MZIRO98; oilseed rape MON 88302, RT73, MON 94100, 73496, Ms1, Ms8, Rf1, Rf2, Rf3, Topas19/2, T45, MS11; potato EH92-527-1, AM04-1020, AV43-6-G7; rice LLRice62; 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-44406, FG72, A2704-12, A5547-127, BPS-CV 127, SYHT0H2; sugarbeet H7-1 and conventional alfalfa, cotton, maize, oilseed rape, potato, rice, soybean, sugarbeet and wheat. According to the method developer, the MON 94804 method did not react with any sample, except the positive control.

In addition, the applicant performed an *in-silico* specificity analysis by using the amplicon sequence as a query for BLASTN 2.11.0+ algorithm search against public sequence (updated January 6, 2021) of the Genbank NT databases. No sequence showed alignments that fully contained both MON 94804 primer 1 and MON 94804 primer 2, no alignment spanned the full length of the probe sequence.

A previously validated maize-specific PCR method (<https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-22-VM.pdf>), which amplifies a 79 base pair (bp) fragment of the *high mobility group (hmg)* of *Zea mays*, was used as a reference method.

3.2 Specificity assessment conducted by the EURL GMFF

The detection method spans the 5' insert-to-plant junction in maize MON 94804. The forward primer "MON 94804 primer 1" binds to the plant genome *Zea mays* border adjacent to the insertion. The reverse primer "MON 94804 primer 2" binding site was found in the insert. The probe "MON 94804 probe" binds to the junction between the insert and the 5' genomic region of maize.

The amplicon size is expected to be 103 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. A perfect match of the amplicon and of the primers was identified with the sequence deposited for MON 94804. The tool "Perfect match GMO methods matrix" and "ePCR matrix" did not find any perfect match of the event-specific methods of the GMO Method database (<https://gmo-crl.jrc.ec.europa.eu/gmomethods/>) with the MON 94804 insert.

3.3 Verification of the ENGL acceptance parameters

The applicant prepared the calibration curve from a DNA solution (S1) of 10% maize event MON 94804 genomic DNA (expressed as copy number ratio) which was serially diluted (1:4) to obtain samples S2, S3, S4 and further diluted 1:5 to obtain sample S5. The parameters (slope, R^2 coefficient) of five runs of the calibration curve obtained in BioRad CFX Touch real-time PCR are reported as provided by the applicant (Table 1).

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

MON 94804		<i>hmg</i>	
Slope	R^2	Slope	R^2
-3.46	0.9984	-3.42	0.9988
-3.45	0.9968	-3.43	0.9981
-3.41	0.9979	-3.45	0.9993
-3.50	0.9972	-3.45	0.9982
-3.50	0.9985	-3.49	0.9986

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 MON 94804 and the maize-specific *high mobility group (hmg)*, as established by the applicant, were within the ENGL acceptance criteria.

Precision and trueness of the method were established by the applicant and 15 values for each of three GM levels (expressed as copy number ratio) per each three experiments were provided (total of 45 replicates per GM-level, precision experiments). Table 2A reports precision and trueness values for the three GM-levels as provided by the applicant. Both parameters were within the ENGL acceptance criteria (trueness $\pm 25\%$, $RSD_r \leq 25\%$ across the entire dynamic range).

Table 2A. Mean%, precision and trueness values (*) provided by the applicant

Expected GM%	Test results			
	10	1	0.085	0.05
Measured mean GM%**	9.13	1.00	0.096	0.059
Precision (RSD _r %)	6.18	7.29	16.35	16.44
Trueness (bias%)	-8.70	-0.14	13.07	18.22

Samples in the range 10% to 0.085% were prepared in a total of 160 ng maize DNA; sample at 0.05% was prepared in a total of 220 ng maize DNA.

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

** copy number ratio

The method met the ENGL acceptance criteria for trueness and precision at the lowest GM level (i.e. 0.085% (expressed as copy number ratio), which contains 50 copies of MON 94804 in 160 ng of total DNA per reaction. The GM content of this sample is in line with the requirements for testing the Limit of Quantification (LOQ, below or equal to 0.09% or 50 copies). The copy number was based on the assumption of a 2.73 pg weight for the maize genome. The applicant also verified that the method precision and trueness on a sample containing 0.05% (copy number ratio, equivalent to 0.10% in mass fraction, according to the applicant) in a total of 220 ng of total maize DNA per reaction, containing 40 GM genome copies, meets the ENGL requirements.

The limit of detection (LOD) of the MON 94804 event-specific method was assessed by the applicant in 60 PCR replicates in a total amount of 36630 maize genome copies. The LOD was found to be below 5 haploid genome copies for MON 94804 event-specific method. The relative LOD (LOD_{rel}) was not determined by the applicant; however it can be estimated as at least 0.014% (related to copies GM/total haploid genome copies in 100 ng of total maize DNA per reaction, i.e. 36630 maize haploid genome copies). The LOD_{abs} and LOD_{rel} are 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 eight combinations of the following variations to the method: per protocol/+5%/-5% Master Mix concentration, per protocol/+10/-10% primer concentration, per protocol/+10%/-10% probe concentration, per protocol/+1 µL/-1 µL Master Mix volume, +/-1 °C in annealing temperature. The RSD_r and the trueness calculated for each combination of variations on a sample at the LOQ level (0.085%) did not exceed 30%, thus meeting the ENGL acceptance criteria.

Precision and trueness of the method were tested in a transferability study: 45 values for each of the four GM levels (expressed as copies GM/total haploid genome copies), prepared as previously described (Table 2A), were provided. Table 2B reports precision and trueness values for the GM-levels as provided by a laboratory using a QuantStudio 6 ProSystem and different from the method developer. Both parameters were within the ENGL acceptance criteria (trueness ≤ 25%, RSD_r ≤ 25% across the entire dynamic range).

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

Expected GM%	Test results			
	10	1.0	0.085	0.05
Measured mean GM%	8.97	0.87	0.084	0.053
Precision (RSD _r %)	4.52	5.88	13.72	15.38
Trueness (bias%)	-10.34	-13.05	-1.72	6.59

3.4 DNA extraction

Genomic DNA was isolated from ground maize seeds, using a modified extraction method from Rogers and Bendich (1985)¹ 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 94804.

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 94804.

Annex III to Reg. (EU) No 503/2013 (5) 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: "Seeds have been chosen as the appropriate samples rather than grain since seeds are more homogenous. The applicability of the Real-Time Quantitative TaqMan® PCR developed for MON 94804 depends on the isolation of sufficient quantity and quality of purified DNA. This method has been tested on DNA extracted from ground seed material. Conceptually, the detection method for MON 94804 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 (Alexander, 1987²; May, 1987³; Pollak and White, 1995⁴; Watson, 1988⁵; White and Pollak, 1995⁶). These steps can influence the quality and intactness of DNA contained in the final processed maize products (Bauer et al., 2003⁷; Murray et al., 2007⁸). 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 (Demeke and Jenkins, 2010⁹; Peano *et al.*, 2004¹⁰). 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

¹ Rogers, S.O. and A.J. Bendich, 1985, Extraction of DNA from Milligram Amounts of Fresh Herbarium and Mummified Plant Tissue, *Plant Mol Biol Sci*, 69-76

² Alexander RJ, 1987. Corn dry milling: processes, products and applications. *Corn: Chemistry and Technology*, Chapter 11, 351-375.

³ May JB, 1987. Wet milling: process and products. *Corn: Chemistry and Technology*, Chapter 12, 377-397.

⁴ Pollak LM and White PJ, 1995. Corn as a food source in the United States: Part I. Historical and current perspectives. *Cereal Foods World*, 40, 1-6.

⁵ Watson SA, 1988. Corn marketing, processing and utilisation. In: *Corn and corn improvement - Agronomy Monograph*. GF Sprague, JW Dudley. American Society of Agronomy, Crop Science Society of America and Soil Science Society of America, Madison, Wisconsin, 881-940.

⁶ White PJ and Pollak LM, 1995. Corn as a food source in the United States: Part II. Processes, products, composition, and nutritive values. *Cereal Foods World*, 40, 756-762.

⁷ Bauer T, Weller P, Hammes WP and Hertel C, 2003. The effect of processing parameters on DNA degradation in food. *European Food Research and Technology*, 217, 338-343

⁸ Murray SR, Butler RC, Hardacre AK and Timmerman-Vaughan GM, 2007. Use of quantitative real-time PCR to estimate maize endogenous DNA degradation after cooking and extrusion or in food products. *Journal of Agricultural and Food Chemistry*, 55, 2231-2239

⁹ Demeke T and Jenkins GR, 2010. Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. *Analytical and Bioanalytical Chemistry*, 396, 1977-1990

¹⁰ Peano C, Samson MC, Palmieri L, Gulli M and Marmiroli N, 2004. Qualitative and quantitative evaluation of the genomic DNA extracted from GMO and Non-GMO foodstuffs with four different extraction methods. *Journal of Agricultural and Food Chemistry*, 52, 6962-6968

pairs (Bauer *et al.*, 2003⁷; Murray *et al.*, 2007⁸). Random DNA fragmentation is known to lead to variability in quantitating DNA by qPCR (Sedlackova *et al.*, 2013¹¹), 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.

¹¹ Sedlackova T, Repiska G, Celec P, Szemes T and Minarik G, 2013. Fragmentation of DNA affects the accuracy of the DNA quantitation by the commonly used methods. *Biological Procedures Online*, 15, 1-9

4 Materials and method

4.1 Samples

The following positive and negative control samples were provided and described by the applicant to the EURL GMFF in accordance to Regulation (EC) No 1829/2003 Art 2.11 ⁽¹²⁾:

- genomic DNA extracted by the applicant from hemizygous maize seeds harbouring the MON 94804 event, the maternal inbred parent was the GM line; the paternal inbred parent was non GM maize.
- genomic DNA extracted by the applicant from conventional maize seeds genetically similar to those harbouring the MON 94804 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 94804 DNA to total maize DNA. The procedure is a simplex system, in which a maize-specific method targeting the endogenous gene *high mobility group (hmg)*, and the GM target method for MON 94804 are performed in separate wells. The validated method protocol is published by the EURL GMFF at <https://gmo-crl.jrc.ec.europa.eu/method-validations> and can be found in Annex 1 to this report.

For the detection of GM event MON 94804, a 103 bp fragment of the region spanning the 5' insert-to-plant junction in maize MON 94804 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 as quencher dye at its 3' end.

For the relative quantification of GM event MON 94804, a maize taxon-specific method amplifies a 79 bp fragment of a maize *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 quencher dye at its 3' end.

Standard curves are generated for both MON 94804 and *hmg* 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 94804 DNA in a test sample, the MON 94804 copy number is divided by the copy number of the maize haploid genome and multiplied by 100 to obtain the percentage value (GM% = MON 94804 / 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) (8). 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. With agreement of the applicant, the calibration curves were produced at the EURL GMFF by means of a 1:4 dilution factor from sample S1 to sample S5.

¹² 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).

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 reported. 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 94804 copies	9158	2289	572	143	36

4.3 EURL GMFF experimental testing (step 3)

4.3.1 Determination of the zygosity ratio in the positive control sample

The EURL GMFF experimentally 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 (6).

The copy number of the MON 94804 and of the *hmg* targets in the positive control sample were determined by digital PCR (dPCR) performed on the QIAcuity Digital PCR System (QIAGEN).

Reaction mixes were prepared in order to test the zygosity in sixteen replicates (eight replicates per test) in a final volume of 40 μL , containing 1X Master Mix (QIAcuity Probe PCR kit) (QIAGEN, Cat. Number 250102), primers and probes at concentrations indicated (MON 94804 primer 1 and MON 94804 primer 2 at 400 nM each, MON 94804 probe at 200 nM; *hmg* primer 1 and *hmg* primer 2 at 300 nM each, *hmg* probe at 180 nM), and 5 μL of DNA at a concentration of 12.5 ng/ μL .

Reaction mixes were loaded into 24-well nanoplates with 26000 partitions (QIAGEN QIAcuity Nanoplates 26k 24-well Cat. Number 250001). 'No template controls' were included. After sealing with the appropriate rubber film, the plates were transferred to the QIAcuity One instrument. The reaction mixture with the sample is transferred automatically and partitioned. The subsequent amplification reaction generates fluorescence in the partitions containing the target DNA.

Box 1: Thermal cycling conditions			
Step	Temperature (°C)	Time (sec)	Number of cycles
Polymerase activation	95	600	1
DNA denaturation	95	15	40
Annealing/extension	60	60	

The plate is then read to count the number of PCR-positive fluorescent partitions.

The Software Suite evaluates the fraction of positive and negative partitions. Poisson statistics determines the absolute concentration of target DNA molecules in the reaction mix; consequently the concentration in the sample, expressing it in copies/ μ l, is calculated.

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' (9).

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 (10% - 0.056%, 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 94804 maize DNA and non-GM maize DNA in a total amount of 220 ng maize DNA

Table 4. MON 94804 blinded samples GM% contents

MON 94804 GM%
GM copy number/maize haploid genome copy number x 100
10
5.0
0.90
0.52
0.056

The calibration sample S1 was prepared from the genomic DNA provided by the applicant by mixing the appropriate amount of MON 94804 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 (see Table 3).

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

Test samples from GM level 10%, to 0.52%, 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 0.056% (in copy number ratio, equal to 0.1% mass ratio), containing 45 copies of MON 94804 in 220 ng of total DNA per reaction, 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 (10) 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)
- 5725-1: 2023 "Accuracy (trueness and precision) of measurement methods and results. General principles and definitions." (3)
- 5725-2: 2019. "Accuracy (trueness and precision) of measurement methods and results — Basic method for the determination of repeatability and reproducibility of a standard measurement method" (4)

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 94804 international collaborative study were randomly selected from 25 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 94804

Laboratory	Country
Center for Agricultural Technology Augustenberg	DE
Crop Research Institute - Reference Laboratory for GMO Detection	CZ
Departamento de OGM/Técnicas Biomoleculares Laboratorio Arbitral Agroalimentario	ES
Environment Agency Austria	AT
Hellenic Agricultural Organisation_Dimitra	GR
Institute of Food Safety, Animal Health and Environment "BIOR"	LV
LUFA Speyer	DE
National Centre for Food, Spanish Agency for Food Safety and Nutrition (AESAN)	ES
National Institute of Biology	SI
National Research Institute of Animal Production, National Feed Laboratory	PL
Sciensano	BE
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:

Three laboratories used the QuantStudio 5.

Two laboratories used the QuantStudio 6 and two others the ABI 7500.

The BioRad CFX 96, Roche LC 480 II, PCRmax Eco48, QuantStudio 7, and StepOne Plus were each used by one laboratory.

Participants in the ring trial were instructed not to use the second derivative analysis when using the Roche LC 480 real-time PCR instrument, as this analysis mode did not produce consistent results, according to analyses conducted by the EURL GMFF.

Overall, the 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 μ L of DNA solution each) labelled from S1 to S5 (Table 3).
- ✓ Twenty blinded test DNA samples (70 μ L of DNA solution, each at 55 ng/ μ L) 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
 - *hmg* primer 1 (10 μ M): 240 μ L
 - *hmg* primer 2 (10 μ M): 240 μ L
 - *hmg* probe (10 μ M): 130 μ L
 - MON 94804
 - MON 94804 primer 1 (10 μ M): 320 μ L
 - MON 94804 primer 2 (10 μ M): 320 μ L
 - MON 94804 probe (10 μ M): 160 μ L

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 MON 94804 and for the *hmg*. In total, two plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for MON 94804 and *hmg* 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

No deviations from the validation protocol were declared. One laboratory reported the use of a PCR system for 48 wells-format instead of 96. However, the results were grouped and analysed by the software as 96 samples.

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 on the MON 94804 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 94804 and *hmg* targets in the positive control sample.

Mean ratio (MON 94804/ <i>hmg</i>)	0.56
Standard deviation	0.020
RSD _r (%)	3.5
Standard error of the mean	0.005
Upper 95% CI of the mean	0.57
Lower 95% CI of the mean	0.55

The mean ratio (MON 94804/*hmg*) equals 0.56. The 95% confidence interval (CI) spans around 0.56, in agreement with the expected ratio for a maize control sample, hemizygous for the GM-locus, with a GM parental contribution of female origin and assuming single - copy endogenous gene target.

Hence, 0.056 GM% in haploid genome copy numbers corresponds to 0.1 GM% in mass fraction of GM DNA.

Box 2: Note on reporting of analytical results

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 0.52% (expressed in copy number) 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 0.056% GM-level (copy number, corresponding to 0.1% mass fraction) was tested for its precision in quantification in 15 replicates in separate runs.

Tests were conducted on ABI 7500, a QuantStudio 7 Flex System (QS7) and a Roche LC480 II for robustness. The data generated with the Roche LC 480 II were processed using the two analysis modes: "fit-point" and "second derivative". However, the results obtained at the EURL GMFF with the second derivative analysis were

inconsistent. Therefore, only the results obtained in the "fit-point" analysis were accepted and are shown in the following tables.

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 ≥ 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, QS7 and Roche LC480 II to quantify GM-levels in the range 10% to 0.52% in four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	MON 94804			<i>hmg</i>		
	Slope	PCR efficiency (*)	R^2	Slope	PCR efficiency (*)	R^2
Run A	-3.38	98	1.00	-3.33	100	1.00
Run B	-3.34	99	1.00	-3.37	98	1.00
Run C	-3.32	100	1.00	-3.30	101	1.00
Run D	-3.34	99	1.00	-3.38	98	1.00
Run E	-3.21	105	1.00	-3.26	103	1.00
Run F	-3.29	101	1.00	-3.42	96	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/\text{slope})} - 1) \times 100$

Runs A-B were carried out on ABI 7500; runs C-D were carried out on QS7; runs E and F were carried out on Roche LC480 II with the fit-point method.

Table 7B. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, QS7, and Roche LC480 II to quantify the GM-level 0.56% (copy number, equal to 0.1% in mass fraction) in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	MON 94804			<i>hmg</i>		
	Slope	PCR efficiency (*)	R^2	Slope	PCR efficiency (*)	R^2
Run G	-3.36	98	1.00	-3.37	98	1.00
Run H	-3.28	102	1.00	-3.40	97	1.00
Run I	-3.36	98	0.99	-3.33	100	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/\text{slope})} - 1) \times 100$

Run G was carried out on ABI 7500; run H was carried out on QS7; run I was carried out on Roche LC480 II.

According to the ENGL method acceptance criteria the method trueness (measured as bias in% of the target GM level) should be within $\pm 25\%$ of the accepted reference value over the entire dynamic range and the precision, expressed as $RSD_r\%$ (relative standard deviation of repeatability), should be $\leq 25\%$, 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	9.5	-5.3	0.52
5.0	4.5	-11	2.6
0.9	0.84	-7.2	1.1
0.52	0.47	-9.8	6.1
0.056	0.059	5.8	21

Table 9. Values of trueness and precision as established by the EURL GMFF in its in-house verification using a QS7. GM% in copy/copy haploid genomes.

Target GM-levels%	Measured GM-level%	Bias% of the target GM-level	Precision (RSD _r %)
10	9.8	-2.5	1.5
5.0	4.9	-1.5	4.2
0.9	0.89	-1.2	0.85
0.52	0.54	2.9	1.2
0.056	0.057	1.6	15

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

Target GM-levels%	Measured GM-level%	Bias% of the target GM-level	Precision (RSD _r %)
10	11	14	17
5.0	5.3	7.0	11
0.9	0.97	7.7	22
0.52	0.53	1.5	8.1
0.056	0.066	18	22

5.2 Results of the international collaborative study

5.2.1 PCR efficiency and linearity

The PCR efficiency (%) and R² 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:

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

Table 11 indicates that the efficiency of amplification for MON 94804 ranges from 94% to 105% and the linearity from 0.99 to 1.0; the amplification efficiency for the maize-specific method ranges from 93% to 103% and the linearity is 1.00. The mean PCR efficiency was 99% for MON 94804 and 98% for the *hmg* one. The average R² of the methods was 1.00 for both MON 94804 and *hmg*. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

Table 11. Values of slope, PCR efficiency and R² obtained during the international collaborative trial. Slope and R² coefficient values were rounded to two digits.

Lab	Plate	MON 94804			<i>hmg</i>		
		Slope	PCR Efficiency (%)	R ²	Slope	PCR Efficiency (%)	R ²
1	A	-3.39	97	1.00	-3.38	98	1.00
	B	-3.44	95	0.99	-3.36	99	1.00
2	A	-3.36	98	1.00	-3.38	98	1.00
	B	-3.47	94	1.00	-3.25	103	1.00
3	A	-3.42	96	1.00	-3.38	98	1.00
	B	-3.46	94	1.00	-3.39	97	1.00
4	A	-3.25	103	1.00	-3.39	97	1.00
	B	-3.29	102	0.99	-3.42	96	1.00
5	A	-3.41	97	0.99	-3.50	93	1.00
	B	-3.42	96	1.00	-3.48	94	1.00
6	A	-3.36	98	1.00	-3.40	97	1.00
	B	-3.42	96	1.00	-3.37	98	1.00
7	A	-3.28	102	1.00	-3.34	99	1.00
	B	-3.26	103	1.00	-3.36	98	1.00
8	A	-3.40	97	1.00	-3.37	98	1.00
	B	-3.35	99	1.00	-3.37	98	1.00
9	A	-3.29	101	1.00	-3.45	95	1.00
	B	-3.37	98	0.99	-3.36	98	1.00
10	A	-3.32	100	1.00	-3.42	96	1.00
	B	-3.41	97	1.00	-3.42	96	1.00
11	A	-3.28	102	1.00	-3.37	98	1.00
	B	-3.33	100	1.00	-3.36	98	1.00
12	A	-3.29	101	1.00	-3.35	99	1.00
	B	-3.21	105	1.00	-3.32	100	1.00
	Mean	-3.35	99	1.00	-3.38	98	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.

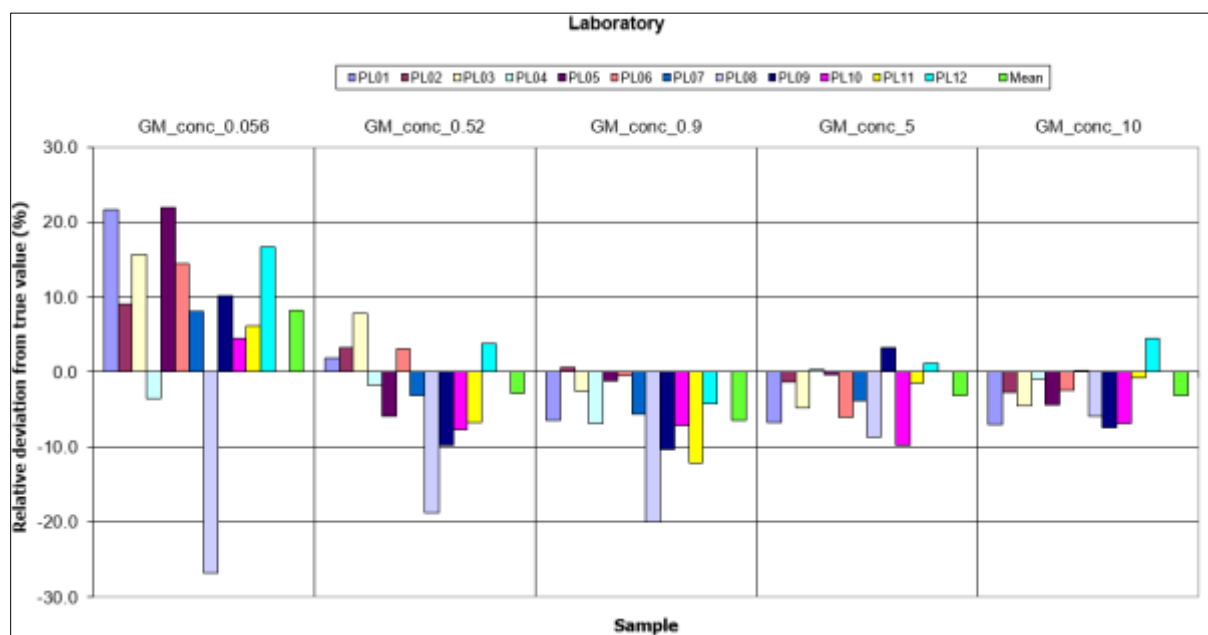
Table 12. GM% values determined by laboratories for test samples

LAB	GMO content (%) (*)																			
	0.056				0.52				0.9				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	0.07	0.07	0.06	0.07	0.55	0.54	0.51	0.51	0.84	0.89	0.80	0.83	4.9	4.7	4.5	4.6	9.5	9.1	9.3	9.3
2	0.07	0.07	0.05	0.05	0.58	0.58	0.47	0.52	0.96	0.98	0.82	0.86	5.0	5.2	4.5	5.1	10	9.4	9.6	9.8
3	0.07	0.06	0.07	0.06	0.54	0.56	0.57	0.57	0.90	0.92	0.85	0.84	4.8	4.6	4.6	5.0	9.3	9.2	10	9.5
4	0.05	0.07	0.05	0.05	0.52	0.54	0.48	0.50	0.84	0.85	0.82	0.85	4.9	4.9	5.0	5.2	10	9.7	10	9.7
5	0.06	0.07	0.07	0.07	0.48	0.48	0.49	0.51	0.86	0.79	0.94	0.97	4.9	5.2	4.9	5.0	8.9	9.0	10	10
6	0.07	0.06	0.06	0.07	0.52	0.56	0.54	0.52	0.93	0.86	0.90	0.89	4.6	4.7	4.8	4.7	10	9.1	9.5	10
7	0.06	0.06	0.05	0.07	0.50	0.47	0.53	0.51	0.78	0.84	0.87	0.90	4.6	4.9	4.7	5.0	10	8.8	10	11
8	0.04	0.04	0.04	0.04	0.31	0.39	0.48	0.51	0.65	0.78	0.72	0.73	4.4	5.1	4.2	4.6	8.7	9.4	10	9.3
9	0.06	0.07	0.06	0.05	0.53	0.35	0.54	0.46	0.90	0.73	0.78	0.82	4.9	5.3	5.1	5.4	9.5	9.9	8.0	9.8
10	0.06	0.06	0.05	0.06	0.48	0.44	0.50	0.49	0.78	0.80	0.84	0.93	4.3	4.3	4.9	4.6	10	8.3	8.9	9.8
11	0.05	0.06	0.07	0.06	0.45	0.49	0.49	0.51	0.82	0.82	0.79	0.73	4.7	4.8	5.1	5.1	9.9	9.5	10	10
12	0.06	0.06	0.06	0.08	0.49	0.50	0.54	0.63	0.79	0.84	0.86	0.96	5.0	5.1	4.8	5.3	11	9.3	11	10

* 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 that shows the relative deviation from the true value for each GM level tested by 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.

Over the entire range of concentrations, the laboratories' mean deviation from the true value was well within the maximum acceptable limit of $\pm 25\%$. Only one laboratory reported a deviation exceeding this limit, with an underestimation of more than 25% at a copy number ratio of 0.056% (equivalent to a mass fraction of 0.10% GM DNA). No clear trend for over- or underestimation was observed.

5.2.3 Method performance requirements

Among the performance requirements established by ENGL and adopted by the EURL GMFF (<https://gmo-crl.jrc.ec.europa.eu/guidance-documents>), 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 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 13, the method satisfies this requirement at all GM levels tested. Indeed, the highest value of RSD_R is 12% at the 0.056% GM level, thus within the acceptance criterion.

Table 13. Summary of validation results for the MON 94804 method, expressed as GM copy numbers in relation to target taxon haploid genome copy numbers.

	Test Sample Expected GMO%				
	0.056	0.52	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	1	0	0	0	0
Reason for exclusion (*)	G	-	-	-	-
Mean value	0.062	0.51	0.84	4.8	9.7
Relative repeatability standard deviation, RSD_r (%)	11	9.4	6.6	4.5	6.5
Repeatability standard deviation	0.007	0.048	0.056	0.22	0.63
Relative reproducibility standard deviation, RSD_R (%)	12	11	8.4	5.7	6.7
Reproducibility standard deviation	0.007	0.056	0.071	0.28	0.65
Bias (**) (absolute value)	0.006	-0.015	-0.057	-0.16	-0.32
Bias (%)	11	-2.8	-6.4	-3.2	-3.2

* G = 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 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" <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>). As it can be observed from the values reported, the repeatability standard deviation is below 25% at all GM levels, with the highest value of 11% at the 0.056% 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 $\pm 25\%$ across the entire dynamic range. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 11% at the 0.056% GM level.

6 Compliance of the method for detection and quantification of event MON 94804 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 0.05% level shown by the applicant's dossier (expressed as copy number ratio) was 16.44%, based on 45 replicates (Table 2A), and 15.38% based on 45 replicates in the transferability study (Table 2B), 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 0.056% 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 15% and 22% (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 11%, therefore also below 25% and well in line with the previous data.

The outcome of the different steps is summarised in Table 14.

Table 14. Precision of the event-specific method for quantitative detection of MON 94804 at or around 0.1% level related to mass fractions of GM material.

Source	$RSD_r\%$	GM%
Applicant's method optimisation	16.44%	0.05%*
Applicant's transferability study	15.38%	0.05%*
EURL GMFF tests	15 - 22%	0.1%
Collaborative study	11%	0.1%

*Expressed in copy number ratio

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 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 4.1), 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 <https://gmo-crl.jrc.ec.europa.eu/method-validations> and in Annex 1.

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List of abbreviations and definitions

EURL GMFF	European Union Reference Laboratory for GM Food and Feed
PCR	Polymerase chain reaction
RT-PCR	Real-time PCR
dPCR	digital Polymerase chain reaction
ENGL	European Network of GMO Laboratories
LOD	Limit of Detection
LOQ	Limit of Quantification

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Annex 1. Event-specific Method for the Quantification of maize MON 94804 by Real-time PCR

Validated Method

Method development:

Bayer CropScience LP represented by Bayer Agriculture BV

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 94804 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. 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 94804, a 103 bp fragment of the region spanning the 5' insert-to-plant junction in maize MON 94804 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 quencher dye at its 3' end.

For the relative quantification of GM event MON 94804, a maize taxon-specific method amplifies a 79 bp fragment of a maize high mobility group (*hmg*) endogenous gene (Accession number, GeneBank: 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 94804 DNA in a test sample, Cq values for MON 94804 and *hmg* are determined for the sample. Standard curves are then used to estimate the relative amount of MON 94804 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 December 2023.

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 in a total of 36630 maize genome copies, corresponding to 0.014 % (copy number ratio) in 100 ng of total 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 % (copy number ratio) in 160 ng of total suitable maize DNA, containing 50 GM-maize genome copies. The applicant also verified that the method precision and trueness on a sample containing 0.05% of GM target (copy number ratio) in a total of 220 ng total maize DNA per reaction (containing 40 GM-genome copies) meets the ENGL requirements. The lowest relative GM content of the target sequence included in the collaborative trial was 0.056 % in copy number ratio, corresponding to 0.1 % in 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 94804 and is therefore event-specific for the event MON 94804. 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 94804

3.2.1 General

The real-time PCR set-up for the taxon (*hmg*) and the GMO (event MON 94804) 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 94804) and the taxon (*hmg*) targets 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 94804 DNA in a total of 250 ng of maize DNA (corresponding to MON 94804 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 94804 copies	9158	2289	572	143	36

* Total nanograms are rounded to the integral value

A calibration curve is produced by plotting the C_q values against the logarithm of the target copy number for the calibration points. This may 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 MON 94804 (Table 2) and *hmg* (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 MON 94804

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
MON 94804 primer 1 (10 µM)	400 nM	1.0
MON 94804 primer 2 (10 µM)	400 nM	1.0
MON 94804 probe (*) (10 µM)	200 nM	0.5
Nuclease free water	-	6.0
DNA	-	4.0
Total reaction volume:		25 µL

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

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for *hmg*

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

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

- Mix well and centrifuge briefly.
- Prepare two 0.5 mL reaction tubes (one for the maize MON 94804 and one for *hmg*) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (73.5 µL for MON 94804 and 73.5 µL for *hmg*). 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.
- Spin down the tubes. Aliquot 25 µL for MON 94804 and for *hmg* in each well.
- Place an optical cover on the reaction plate and briefly centrifuge the plate.
- 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 94804 and for *hmg*. Define TAMRA as quencher dye for MON 94804 and for *hmg*. Select ROX, if requested in your real-time PCR equipment, 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 94804/*hmg*

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 method 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 method.
- 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 C_q values for each reaction.

The standard curves are generated both for *hmg* and MON 94804 by plotting the C_q 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 94804 DNA in the unknown sample, the MON 94804 copy number is divided by the copy number of the maize endogenous gene *hmg* and multiplied by 100 (GM% = $\text{MON 94804}/\text{hmg} \times 100$).

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 4318157.

4.3 Primers and Probes

Table 5. Primers and probes for the MON 94804 and *hmg* methods

	MON 94804	DNA Sequence (5' to 3')	Length (nt)
	MON 94804		
Forward primer	MON 94804 primer 1	CTC TTC TAA TCC GGG CCA TCG	21
Reverse primer	MON 94804 primer 2	AGT TAG TCG CGC CAA ATC GTG	21
Probe	MON 94804 probe	6-FAM-CTG GAT CCG AAG GAC GTG TCT ACA TTC AC-TAMRA	29
	<i>hmg</i>		
Forward primer	<i>hmg</i> primer 1	TTG GAC TAG AAA TCT CGT GCT GA	23
Reverse primer	<i>hmg</i> primer 2	GCT ACA TAG GGA GCC TTG TCC T	22
Probe	<i>hmg</i> probe	6-FAM-CAA TCC ACA CAA ACG CAC GCG TA-TAMRA	23

FAM: 6-carboxyfluorescein

5 References

1. Plant DNA C-values Database. Royal Botanic Gardens, Kew, <https://cvalues.science.kew.org/>

List of abbreviations and definitions

EURL GMFF	European Union Reference Laboratory for GM Food and Feed
PCR	Polymerase chain reaction
RT-PCR	Real-time PCR
ENGL	European Network of GMO Laboratories
LOD	Limit of Detection
LOQ	Limit of Quantification

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