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Event-specific Method for the Quantification of Maize DP915635 Using Real-time PCR

Validation Report

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European Union Reference Laboratory for Genetically Modified Food and Feed

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EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F – Health and Food
Food and Feed Compliance

Event-specific Method for the Quantification of Maize DP915635 Using Real-time PCR

Validation Report

20/02/2023

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 DP915635 (unique identifier DP-915635-4). 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], Pioneer Overseas Corporation provided the detection method and the positive and negative control samples (genomic DNA from seeds of DP915635 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 https://gmo-crl.jrc.ec.europa.eu/method-validations.

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], Pioneer Overseas Corporation provided the EURL GMFF with an event-specific method for detection and quantification of maize event DP915635 (unique identifier DP-915635-4) 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 [9], allowed moving the method forward to step 3 of the procedure (experimental testing), where the EURL GMFF verified the purity of the control samples provided and conducted an in-house testing of samples and method.

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

Step 3 was completed with the conclusion that the method could be submitted to the collaborative study (step 4). This study confirmed that the method is well suited for quantifying genomic DNA of GM maize DP915635, 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)

Pioneer Overseas Corporation submitted the identification and quantification method, data demonstrating its adequate performance when applied to genomic DNA extracted from maize DP915635 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 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 triplicate real-time PCR reactions, according to the method described in Annex 1 (Tables 1 to 5), using 100 ng per reaction of target DNA DP-915635-4, at least a 4% GM mass fraction of transgenic non-target DNA: maize, DP-202216-6, DP-56113-9, DP-023211-2, T25, TC1507, NK603, MIR162, MIR604, MON810, 3272, 98140, 59122, 5307, MON88017, MON89034, MON863, GA21, Bt11, Bt176, MON87427, MON87460, DAS-40278-9, DP4114, VCO-01981-5, MON87403, MON87411, MZHG0JG; soybean DAS-44406-6; A2704-12, A5547-127, CV127, DAS-81419-2, MON87705, MON87751, FG72, MON87769, 356043, 305423, GTS-40-3-2, MON87701, DAS-68416-4, MON89788, MON87708, SYHT0H2; cotton LLCotton25, GHB614, MON531, MON15985, MON1445, 281-24-236 x 3006-210-23, GHB119, T304-40, MON88913, MON88701, DAS-81910-7, COT102; oilseed rape Rf3, GT73/RT73, MON88302, Rf1, Ms1, T45, Rf2, Ms8, Topas 19/2, 73496; rice LLRice62; potato EH92-527-1, AM04-1020, AV43-6-G7, PH05-026-0048; sugar beet H7-1. At least 50 ng per reaction were used for non-transgenic non-target DNA: conventional oilseed rape, cotton, rice, potato, sugar beet, maize, soybean, and wheat. According to the method developer the DP915635 assay did not react with any sample except the positive control. All the 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 2.10.1 algorithm search against the nucleotide (nt) databases of the National Center for Biotechnology Information (NCBI) (July 2020). No contiguous DNA sequence contained both the forward and reverse primer binding site.

3.2. Specificity assessment conducted by the EURL GMFF

The detection method spans the 3' insert-to-plant junction in maize DP915635. According to the annotation provided, the forward primer binding site was found in the insert. The probe binds on the insert-to-plant junction. The reverse primer binds to the maize genomic flanking region. The amplicon size is expected to be 74 bp, consistent to what is 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 for the relative quantification of event DP915635 from a DNA solution (S1) of 10 % maize event DP915635 genomic DNA (expressed as copy number

ratio) at a concentration of 65 ng/µL. This solution was serially diluted (1:6) to produce standard S2 and S3 and then diluted an additional time at a 1:8 dilution rate to produce standard S4. The parameters (slope, R2 coefficient) of eight runs of the calibration curve are reported as provided by the applicant (Table 1). All runs were performed on Bio-Rad CFX96 Touch™ instrument following the protocol provided in Annex 1.

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

	DP915635		***hmg***	
Run	**Slope**	**R^2**	**Slope**	**R^2**
1	-3.33	1.000	-3.21	0.999
2	-3.28	1.000	-3.25	0.999
3	-3.33	1.000	-3.26	1.000
4	-3.26	1.000	-3.24	0.998
5	-3.23	1.000	-3.23	0.999
6	-3.32	1.000	-3.23	0.999
7	-3.40	1.000	-3.14	0.999
8	-3.26	0.998	-3.26	0.999
Mean	**-3.30**	**1.000**	**-3.23**	**0.999**

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 (DP915635) and the maize-specific (*hmg*) system, as established by the applicant, were within the ENGL acceptance criteria.

Precision and trueness of the method were established by the applicant and 16 values for each of four GM levels (expressed as DP915635 maize DNA copy numbers relative to haploid maize genome copy numbers) were provided. Each reaction contained 300 ng of maize DNA which corresponds to 109890 *hmg* copies, assuming a maize haploid genome weights 2.73 pg. Table 2A reports precision and trueness values for the four GM-levels as provided by the applicant. Both parameters were within the ENGL acceptance criteria (trueness ± 25 %, RSD_r ≤ 25 % across the entire dynamic range).

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

	Test results			
Expected GM %**	**5.00**	**0.900**	**0.090**	**0.042**
Measured mean GM %	4.78	0.736	0.077	0.035
Precision (RSD_r %)	8.2	7.1	8.6	10.6
Trueness (bias %)	-4.4	-18.2	-14.4	-16.7

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

** percentage values are expressed as copy number ratio

The method met the ENGL acceptance criteria for trueness and precision over the dynamic range, including the lowest GM level (i.e. 0.042 % cp/cp corresponding to a 0.1 % in mass fraction according to the applicant). 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 relative LOD (LOD_{rel}) of the combined method was assessed by the applicant in 60 PCR replicates and it was found to be at least 0.009 % (related to copies GM/total haploid genome copies) in 300 ng of total maize DNA per reaction. This sample contains about 10 copies of the DP915635 event, assuming a weight of 2.73 pg per maize haploid genome. The LOD_{rel} 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 16 combinations of the following variations to the method: *per protocol* /+10 %/-10 % enzyme mix concentration, *per protocol* /+30 %/-30 % primer concentration, *per protocol*/+30 %/-30 % probe concentration, *per protocol*/+1 µL/-1 µL master mix volume, +/-1 °C in annealing temperature, ABI7500/MX3005P real-time PCR equipment. The RSD_r and the trueness calculated for each combination of variations on a sample at the LOQ level (0.042 %) did not exceed 30 %, thus meeting the ENGL acceptance criteria.

Precision and trueness of the method were tested in a transferability study in two independent laboratories. The alternate laboratory 2 used a Bio-Rad CFX96 Touch™ and the alternate laboratory 3 used an Applied Biosystems™ 7500 real-time PCR platform. Each laboratory provided two results (average of triplicates) for each of the four GM levels (expressed as copies GM/total haploid genome copies). Table 2B reports precision and trueness values for the four GM-levels as provided by the laboratories 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 %**	**5.00**	**0.900**	**0.090**	**0.042**	
Measured mean GM %	4.23	0.770	0.069	0.037	Alternate lab 2
Precision (RSD_r %)	8.4	12.9	1.0	0.0	
Trueness (bias %)	-15.4	-14.4	-23.3	-11.9	
Measured mean GM %	5.24	0.950	0.103	0.046	Alternate lab 3
Precision (RSD_r %)	0.1	5.1	4.8	1.5	
Trueness (bias %)	4.7	5.6	14.4	9.5	

* Numbers are not rounded but are presented as reported by the applicant
** Percentage values are expressed as copy number ratio

3.4. DNA extraction

Genomic DNA was isolated from ground maize seeds, using a protocol previously submitted for detection of maize event TC1507 that has already been 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/TC1507-DNAextrc.pdf. According to the experimental data submitted by the applicant, the protocol for DNA extraction generated DNA of suitable quantity and quality for PCR based applications when applied to ground seeds from the maize event DP915635.

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 DP915635.

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) that are expected to be placed on the market. To this regard, the applicant stated that "*The foundation to detect the presence of transgenes in seed, food and feed matrices is primarily based on the quality of genomic DNA template that is utilized. Pure DNA extractions such as the cetyltrimethyl ammonium bromide (CTAB) method or the Wizard (Promega) method are suitable for the isolation of pure genomic DNA from a wide variety of cereal based matrices (Nguyen et al., 2009; Sönmezoğlu et al., 2015; Stefanova et al., 2013). Exceptionally pure DNA, applicable for molecular biology procedures, such as Polymerase Chain Reaction (PCR) amplification, is imperative to provide adequate source template suitable for use for a wide variety of agricultural products, including maize grain and derived matrices supporting food and feed products. The processing of maize grain involves varying degrees of mechanical, enzymatic, solvent, heat, acid, pressure treatment, or combinations of these steps (Alexander, 1987; May, 1987; Gwirtz and Garcia-Casal,*

2014; Khalsa, 1994; Pollak and White, 1995; Watson, 1988; White and Pollak, 1995). These steps influence the quality and intactness of DNA contained in the final processed maize products (Bauer et al., 2003; Murray et al., 2007; Nguyen et al., 2009; Terry et al., 2002) which may result in significant degradation of high molecular weight DNA and failure to PCR amplify products greater than a few 100 base pairs (Bauer et al., 2003; Murray et al., 2007). Random DNA fragmentation is known to lead to variability in quantifying DNA by qPCR (Sedlackova et al., 2013), thus affecting the ability to accurately quantify the presence of a GM event and taxon-specific target in processed fractions. Moreover, the DNA extraction procedure necessary for some of these processed matrices may need additional rounds of processing to clean-up the DNA, to eliminate PCR inhibitors in order to achieve quality genomic DNA suitable for PCR testing (Demeke and Jenkins, 2010; Peano et al., 2004). These extraction methods are widely used for plant-based materials, are economical and can be easily scaled (Smith et al., 2005)[#].

Each PCR detection method has been developed and was pre-validated on maize seed and tissues. This method can, in principle, be applied to any sample from which sufficient quantities of maize DNA, free of PCR inhibitors, can be purified. This DNA extraction method will be specifically applicable to the certified reference materials that have been produced for quality control and calibration of the detection method."

[#] *Alexander RJ, 1987. Corn dry milling: processes, products and applications. Corn: Chemistry and Technology, Chapter 11, 351-375.*
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.
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.
EU-RL-GMFF, 2018. Event-specific Method for the Quantification of Maize DP-ØØ4114-3 using Real-Time PCR, version EURL-VL-02/14VP, European Union Reference Laboratory for Genetically Modified Food and Feed, http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-02-14-VP.pdf.
Gwirtz JA, Garcia-Casal MN (2014) Processing maize flour and corn meal food products. Annals of the New York Academy of Sciences 1312: 66-75
Khalsa N (1994) Methods for making tortilla chips and tortilla chips produced thereby. US Patent 5298274 A
May JB, 1987. Wet milling: process and products. Corn: Chemistry and Technology, Chapter 12, 377-397.
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.
Nguyen T, Son CT, Raha AR, Lai OM, Clemente M (2009) Comparison of DNA extraction efficiencies using various methods for the detection of genetically modified organisms (GMOs). International Food Research Journal. 16: 21-30
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.
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.
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.
Smith DS, Maxwell PW, Solke HD (2005) Comparison of Several Methods for the Extraction of DNA from Potatoes and Potato-Derived Products. Journal of Agricultural and Food Chemistry. 53: 9848-9859
Sönmezoğlu ÖA, Keskin H (2015) Determination of Genetically Modified Corn and Soy in Processed Food Products. Journal of Applied Biology and Biotechnology. Vol. 3 (03): 032-037
Stefanova P, Taseva M, Georgieva T, Gotcheva V, Angelov A (2013) A Modified CTAB Method for DNA Extraction from Soybean and Meat Products. Biotechnology and Biotechnological Equipment. 27:3: 3803-3810
Terry C, Harris N, Parkes H (2002) Detection of Genetically Modified Crops and Their Derivatives: Critical Steps in Sample Preparation and Extraction. Journal of AOAC International. 85:3: 768-774
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.
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.

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[*]:

- genomic DNA extracted by the applicant from maize seeds hemizygous for the DP915635 event donated by the male parent of the crossing , and
- genomic DNA extracted by the applicant from conventional maize seeds genetically similar to those harbouring the DP915635 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 DP915635 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 DP915635 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 DP915635, a 74 bp fragment of the region spanning the 3' insert-to-plant junction in maize DP915635 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonudeotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and MGB (minor groove binder) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event DP915635, a maize taxon-specific system amplifies a 79 bp fragment of a maize high mobility group *hmg* endogenous gene, using *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM as reporter dye at its 5' end and MGB as non-fluorescent quencher dye at its 3' end.

Standard curves are generated for both the DP915635 and the *hmg* systems by plotting the Cq values measured for the calibration points against the logarithm of the DNA copy numbers and by

[*] 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 DP915635 DNA in a test sample, the DP915635 copy number is divided by the copy number of the maize haploid genome and multiplied by 100 to obtain the percentage value (GM % = DP915635/ 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) in agreement with the published value [10]. 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 final results.

Table 3. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4
Total amount of maize DNA in the reaction (ng)	325	54	9.0	1.1
Target taxon haploid genome copies	119048	19841	3307	413
Target DP915635 copies	11905	1984	331	41

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 [8].

The ratio between the copy number of the DP915635 event and of the *hmg* targets in the positive control sample was determined by digital droplet PCR (ddPCR) performed on the Bio Rad QX200 Droplet Reader.

The zygosity was tested in simplex resulting in eighteen data sets.

Reaction mixes were prepared in order to obtain a final volume of 22 µL and contained 1X ddPCR Super Mix for Probes no dUTP (Bio Rad, Cat. number 1863024), primers and probes at the concentrations indicated in the corresponding methods and 5.5 µL of genomic DNA from the positive control sample at a concentration of 14 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 a 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 40µL 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	95	00:30		40
Annealing/extension	60	01:00		
Enzyme deactivation	98	10:00		1
Hold	12	Infinite		1

The sealed 96-well plate was then placed in the QX200 Droplet Reader to determine, by 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'[(11)].

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 (5.0 % - 0.039 %, 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 DP915635 maize DNA and non-GM maize DNA.

Table 4. DP915635 blinded samples GM % contents

DP915635 GM % GM copy number/maize haploid genome copy number x 100
5.0
2.0
0.90
0.38
0.039

The calibration sample S1 was prepared from the genomic DNA provided by the applicant by mixing the appropriate amount of DP915635 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-S3 were prepared by 6-fold serial dilutions from the S1 sample and sample S4 by an 8-fold serial dilution from the S3 sample (see Table 3).

The experiments were performed on an ABI 7500, QuantStudio™ 7 and a Roche LC480 II real-time platform under repeatability conditions and followed the protocol provided by the applicant (Annex 1).

The test samples over the dynamic range were tested in two real-time PCR runs with two replicates for each GM-level each plate (total of four replicates per GM-level). The test sample with GM level 0.039 % was also tested in 15 replicates in an additional run for each platform. Average values of the slope and of the R^2 coefficient of the standard curves and method trueness and precision over the dynamic range were evaluated against the ENGL method acceptance criteria. On Roche LC480 II platform the method was run at 45 cycles as described in the validated method published at https://gmo-crl.jrc.ec.europa.eu/method-validations and in Annex 1, and analysed with the second derivative maximum method.

4.4. International collaborative study (step 4)

The international collaborative trial involved twelve randomly selected laboratories, all being "national reference laboratories, assisting the EURL GMFF for testing and validation of methods for detection", as listed in Annex to Regulation (EU) No 120/2014 [12] 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 DP915635 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 DP915635

Laboratory	**Country**
AGES-Austrian Agency for Health and Food Safety	AT
BioGEVES - Groupement d'Intérêt Public – Groupe d'Etude et de contrôle des Variétés	FR
Center for Agricultural Technology Augustenberg	DE
Environment Agency Austria	AT
Hellenic Agricultural Organisation	GR
National Food and Veterinary Risk Assessment Institute	LT
National Research Institute of Animal Production, National Feed Laboratory	PL
Plant Health Laboratory	FR
Sciensano	BE
Service commun des laboratoires du ministere de l'economie et des finances	FR
Voivodeship Sanitary and Epidemiological Station in Rzeszów	PL
Wageningen Food Safety Research (WFSR)	NL

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 Bio-Rad CFX96, three used ABI 7500, two used ABI QuantStudio 5, one Roche LC 480 II, one used ABI QuantStudio 7 and one used PCRmax Eco48.

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) 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:

- ✓ Four calibration samples with known concentrations of GM-event (175 µL of DNA solution each) labelled from S1 to S4 (Table 3).

- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution, each at 60 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 4).

- ✓ Reaction reagents:
  - 2x SSOAdvancedTM Universal Probes Supermix, one vial: 6.4 mL
  - distilled sterile water, one vial: 1.5 mL
  - Bovine Serum Albumin (BSA), one tube: 40 µL

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

  hmg taxon-specific assay
  - hmg primer 1 (10 µM): 384 µL
  - hmg primer 2 (10 µM): 384 µL
  - PHN149436 (10 µM): 80 µL

  DP915635 assay
  - DP-915635-4 F (10 µM): 384 µL
  - DP-915635-4 R (10 µM): 384 µL
  - 2059 probe (10 µM): 80 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 DP915635 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 DP915635 and the *hmg* assay in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per pre-determined plate layout.

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

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

4.4.5. Deviations reported from the protocol

Eleven laboratories reported no deviations from the validation protocol. One laboratory used a 48 wells format instead of 96 wells; however, the results were grouped and analyzed 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 DP915635 and *hmg* targets to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Zygosity ratio of the DP915635 and *hmg* targets in the positive control sample.

Mean ratio (DP915635/*hmg*)	0.39
Standard deviation	0.01
RSD_r (%)	2.7
Standard error of the mean	0.002
Upper 95 % CI of the mean	0.39
Lower 95 % CI of the mean	0.38

The mean ratio (DP915635/*hmg*) is 0.39. The 95 % confidence interval (CI) spans around 0.39, the expected ratio for a maize control sample, hemizygous for the GM-locus, with a GM parental contribution of male origin and assuming single-copy endogenous gene target. Therefore, the measured mean ratio is in line with the expected ratio.

Hence, a sample at 0.039 GM % in DNA copy number ratio corresponds to a 0.1 GM % in mass fraction.

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/method-validations).

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

The test samples from 5.0 % to 0.039 % 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 0.039 % GM-level was tested for its precision in quantification in 15 replicates in separate runs.

All the assays were conducted on both ABI 7500, ABI QuantStudio 7 and Roche LC480 II instruments for testing robustness.

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

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R^2 coefficient shall be $\geq$ 0.98. Table 7A and 7B document that the slopes of the standard curves and the R^2 coefficients were within the limits established by the ENGL. The EURL GMFF in-house results confirm the data provided by the applicant.

Table 7A. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, ABI QuantStudio 7 and Roche LC480 II to quantify GM-levels in the range 5.0 % to 0.38 % in four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	DP915635			***hmg***		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.41	97	1.00	-3.36	98	1.00
Run B	-3.35	99	1.00	-3.39	97	1.00
Run C	-3.05	113	0.99	-3.18	106	1.00
Run D	-3.23	104	0.99	-3.16	107	1.00
Run E	-3.26	103	1.00	-3.19	106	1.00
Run F	-3.44	95	1.00	-3.33	100	1.00

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

Table 7B. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, ABI QuantStudio 7 and Roche LC480 II to quantify the GM-level 0.039 % in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	DP915635			***hmg***		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run G	-3.37	98	1.00	-3.34	99	1.00
Run H	-3.35	99	1.00	-3.29	101	1.00
Run I	-3.40	97	1.00	-3.34	99	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$
Run G was carried out on ABI 7500; run H was carried out on ABI QuantStudio 7; 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 ± 25 % of the accepted reference value over the entire dynamic range and the precision, expressed as RSD_r % (relative standard deviation of repeatability), should be ≤ 25 %, also over the entire dynamic range.

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

Table 8. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI 7500 instrument.

Target GM-levels %*	Measured GM-level %*	Bias % of the target GM-level	Precision (RSD_r %)
5.0	5.3	5.0	2.8
2.0	2.1	6.4	2.1
0.9	0.97	8.2	1.3
0.38	0.43	13	3.4
0.039	0.04	15	17

* percentage values are expressed as copy number ratio

Table 9. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI QuantStudio 7 instrument.

Target GM-levels %*	Measured GM-level %**	Bias % of the target GM-level	Precision (RSD_r %)
5.0	5.3	6.5	4.7
2.0	2.0	1.7	2.7
0.9	0.92	1.9	5.4
0.38	0.37	-3.1	7.1
0.039	0.04	8.9	15

* percentage values are expressed as copy number ratio

Table 10. Values of trueness and precision as established by the EURL GMFF in its in-house verification using a Roche LC480 II instrument.

Target GM-levels %*	Measured GM-level %*	Bias % of the target GM-level	Precision (RSD_r %)
5.0	5.1	1.3	2.7
2.0	2.0	-2.1	6.2
0.9	0.95	5.1	8.9
0.38	0.40	6.6	10
0.039	0.05	24	15

* percentage values are expressed as copy number ratio

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

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

Table 11 indicates that the efficiency of amplification for the DP915635 system ranges from 90 % to 108 % and the linearity was always equal to 1.00; the amplification efficiency for the maize-specific reference system ranges from 93 % to 102 % and the linearity ranges from 0.98 to 1.00. The mean PCR efficiency was 97 % for DP915635 assay and 98 % for the *hmg* one. The average R^2 of the methods was 1.00 for both the DP915635 and *hmg* assays. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

Table 11. Values of slope, PCR efficiency and R^2 obtained during the international collaborative trial. Slope and R^2 coefficient values were rounded to two digits.

		DP915635			***hmg***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.40	97	1.00	-3.37	98	1.00
	B	-3.28	102	1.00	-3.39	97	1.00
2	A	-3.45	95	1.00	-3.39	97	1.00
	B	-3.43	96	1.00	-3.50	93	1.00
3	A	-3.48	94	1.00	-3.35	99	1.00
	B	-3.37	98	1.00	-3.35	99	1.00
4	A	-3.36	99	1.00	-3.38	98	1.00
	B	-3.31	100	1.00	-3.35	99	1.00
5	A	-3.34	99	1.00	-3.36	99	1.00
	B	-3.37	98	1.00	-3.37	98	1.00
6	A	-3.54	92	1.00	-3.33	100	0.98
	B	-3.58	90	1.00	-3.31	100	0.99
7	A	-3.39	97	1.00	-3.29	101	1.00
	B	-3.45	95	1.00	-3.37	98	1.00
8	A	-3.39	97	1.00	-3.49	94	1.00
	B	-3.43	96	1.00	-3.41	96	1.00
9	A	-3.52	92	1.00	-3.46	94	1.00
	B	-3.45	95	1.00	-3.46	94	1.00
10	A	-3.39	97	1.00	-3.40	97	1.00
	B	-3.43	96	1.00	-3.41	97	1.00

11	A	-3.46	95	1.00	-3.43	96	1.00
	B	-3.39	97	1.00	-3.28	102	1.00
12	A	-3.14	108	1.00	-3.34	99	1.00
	B	-3.50	93	1.00	-3.34	99	1.00
	Mean	**-3.41**	**97**	**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.

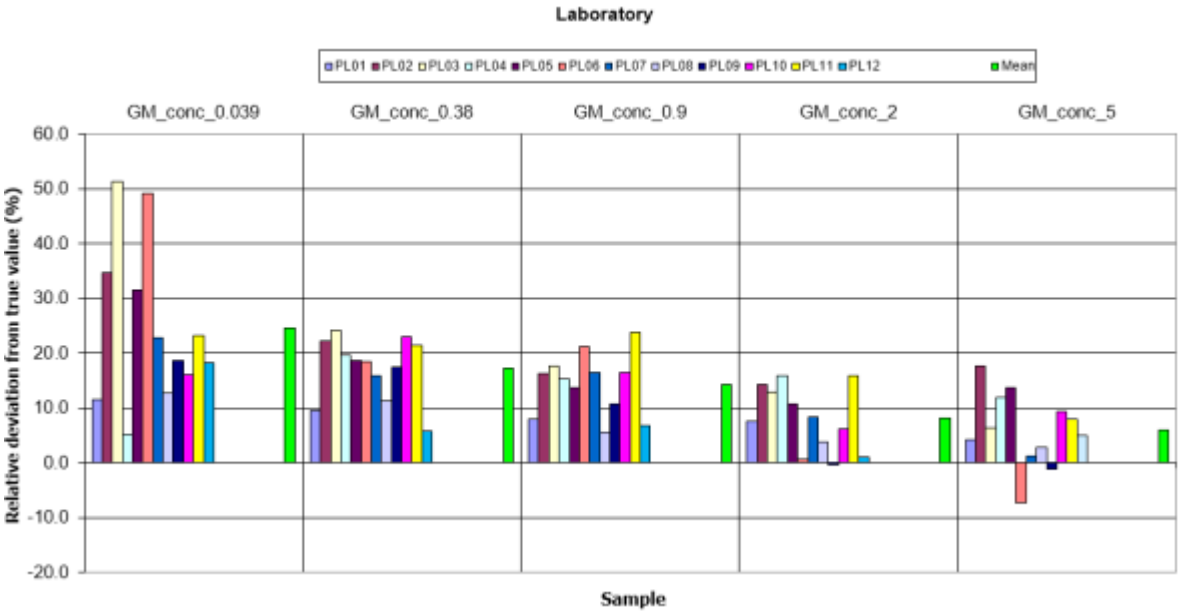
Table 12. GM % values determined by laboratories for test samples

	GMO content (%) *																			
LAB	**0.039**				**0.38**				**0.90**				**2.0**				**5.0**			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.039	0.040	0.049	0.046	0.43	0.40	0.41	0.42	1.0	0.92	1.0	0.97	2.2	2.3	2.0	2.1	5.2	5.4	5.3	4.9
2	0.047	0.047	0.049	0.067	0.50	0.43	0.48	0.45	1.1	1.0	1.1	0.99	2.2	2.2	2.3	2.5	5.9	6.3	5.9	5.4
3	0.060	0.059	0.061	0.055	0.52	0.44	0.43	0.51	1.1	1.0	1.0	1.1	2.3	2.3	2.1	2.3	5.5	5.5	5.2	5.0
4	0.036	0.051	0.035	0.042	0.48	0.42	0.48	0.44	1.0	1.0	1.0	1.1	2.3	2.4	2.3	2.3	5.5	5.9	5.5	5.5
5	0.047	0.056	0.053	0.049	0.46	0.44	0.47	0.44	1.0	0.97	1.1	0.99	2.1	2.1	2.2	2.4	5.5	5.4	5.9	6.0
6	0.047	0.057	0.055	0.073	0.49	0.43	0.36	0.52	1.1	1.1	1.1	1.1	2.1	1.6	2.1	2.2	4.7	4.7	4.3	4.9
7	0.048	0.052	0.045	0.047	0.42	0.47	0.47	0.40	1.2	1.1	0.95	0.99	2.2	2.4	2.0	2.1	5.2	5.4	4.9	4.8
8	0.044	0.044	0.044	0.043	0.41	0.41	0.46	0.41	1.0	0.99	0.93	0.93	2.1	2.0	2.1	2.1	5.2	5.1	5.0	5.2
9	0.046	0.043	0.043	0.053	0.43	0.43	0.45	0.48	1.0	0.97	0.99	1.0	1.8	2.2	1.9	2.1	4.9	4.8	5.0	5.1
10	0.047	0.047	0.042	0.044	0.51	0.44	0.46	0.47	1.0	1.0	1.0	1.1	2.1	2.1	2.2	2.2	5.8	6.0	4.9	5.3
11	0.040	0.039	0.060	0.053	0.50	0.43	0.41	0.51	1.0	1.0	1.2	1.2	2.1	2.2	2.4	2.5	4.9	5.1	5.7	5.9
12	0.064	0.051	0.037	0.032	0.34	0.46	0.42	0.38	1.0	0.99	0.93	0.89	2.1	2.1	1.9	1.9	5.2	5.3	5.3	5.3

* 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 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.

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 0.38 %, 0.9 %, 2.0 % and 5.0 % all the twelve laboratories were within the limit; at GM-level 0.039 % eight laboratories were within the limit and four laboratories overestimated the assigned value by more than 25 %.

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 18 % at the 0.039 % GM level, thus within the acceptance criterion.

Table 13. Summary of validation results for the DP915635 method, expressed as GM copy numbers in relation to target taxon haploid genome copy numbers.

	Test Sample Expected GMO %				
	0.039	**0.38**	**0.90**	**2.0**	**5.0**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers*	0	0	0	0	0
Reason for exclusion	-	-	-	-	-
Mean value	0.049	0.45	1.0	2.2	5.3
Relative repeatability standard deviation, RSD_r (%)	15	8.6	5.5	6.8	5.4
Repeatability standard deviation	0.007	0.04	0.06	0.15	0.28
Relative reproducibility standard deviation, RSD_R (%)	18	8.9	6.8	8.1	7.9
Reproducibility standard deviation	0.009	0.04	0.07	0.17	0.42
Bias** (absolute value)	0.010	0.07	0.13	0.16	0.30
Bias (%)	25	17	14	8.0	5.9

*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" https://gmo-crl.jrc.ec.europa.eu/guidance-documents). 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 15 % at the 0.039 % 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 within ± 25 % across the entire dynamic range. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 25 % (actually, 24.6 %) at the 0.039 % GM level.

6. Compliance of the method for detection and quantification of event DP915635 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 0.042 % level shown by the applicant's dossier (expressed in copy number ratio and corresponding approximately to 0.1 % in mass fraction of GM-material) was 10.6 %, based on 16 replicates (Table 2A), and ≤ 1.5 % 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 0.039 % 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 17 % (Tables 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 the RSD_r of the method at the level of 0.1 % related to mass fraction of GM-material (corresponding to 0.039 % in terms of copy numbers ratio) was 15 %, 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 DP915635 at or around 0.1 % level related to mass fractions of GM material.

Source	**RSD_r %**	**GM %**
Applicant's method optimisation	10.6 %	~ 0.1 %
Applicant's transferability study	< 1.5 %	~ 0.1 %
EURL GMFF tests	15 - 17 %	0.1 %
Collaborative study	15 %	0.1 %

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

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.

8. References

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Annex 1: Event-specific Method for the Quantification of Maize DP915635 by Real-time PCR

Validated Method

Method development:

Pioneer Overseas Corporation

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 DP915635 (unique identifier DP-915635-4) 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 DP915635, a 74 bp fragment of the region spanning the 3' insert-to-plant junction in maize DP915635 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonudeotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and MGB (minor groove binder) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event DP915635, a maize taxon-specific system amplifies a 79 bp fragment of a maize high mobility group (*hmg*) endogenous gene (Accession number, GeneBank: AJ131373), using *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM as reporter dye at its 5' end and MGB as non-fluorescent 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 DP915635 DNA in a test sample, Cq values for the DP915635 and the *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DP915635 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 May 2022.

A detailed validation report can be found at https://gmo-crl.jrc.ec.europa.eu/method-validations

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.009 % (GM copy numbers relative to haploid maize genome copy numbers) in 300 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 0.042 % (GM copy numbers relative to haploid maize genome copy numbers), equivalent to 0.1 % in mass fraction in 300 ng of total suitable maize DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1 % (mass fraction of GM-material).

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 3' insert-to-plant junction in maize DP915635 and is therefore event-specific for the event DP915635. This was confirmed by the applicant's specificity studies and by *in silico* analysis performed by the applicants and the EURL GMFF.

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 Amd.1:2013(E).
- 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 DP915635

3.2.1 General

The real-time PCR set-up for the taxon (*hmg*) and the GMO (event DP915635) 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 20 µL per reaction mixture for the GM (event DP915635) 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 four samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % maize DP915635 DNA in a total of 325 ng of maize DNA (corresponding to 119048 maize haploid genome copies with one haploid genome assumed to correspond to 2.73 pg of maize genomic DNA), in good agreement with the "Plant DNA C-values Database" [(1)]. Standards S2 to S4 are to be prepared by serial dilutions (dilution factor 6 for samples S2-S3 and dilution factor 8 for standard S4) according to Table 1.

Table 1. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4
Total amount of maize DNA in reaction (ng)*	325	54	9.0	1.1
Maize haploid genome copies	119048	19841	3307	413
DP915635 copies	11905	1984	331	41

* 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 DP915635 maize (Table 2) and the *hmg* reference (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 DP915635 assay.

Component	Final concentration	µL/reaction
SsoAdvanced™ Universal Probes Supermix (2x)	1x	10
DP-915635-4 F (10 µM)	600 nM	1.20
DP-915635-4 R (10 µM)	600 nM	1.20
2059 probe * (10 µM)	120 nM	0.240
Bovine Serum Albumin (BSA, 30 %)	0.081 %	0.054
Nuclease free water	-	2.306
DNA	-	5
Total reaction volume:		20 µL

*TaqMan® probe labelled with 6-FAM at its 5'-end and MGB 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
SsoAdvanced™ Universal Probes Supermix (2x)	1x	10
hmg primer 1 (10 µM)	600 nM	1.20
hmg primer 2 (10 µM)	600 nM	1.20
PHN149436 * (10 µM)	120 nM	0.240
Bovine Serum Albumin (BSA, 30 %)	0.08 %	0.054
Nuclease free water	-	2.306
DNA	-	5
Total reaction volume:		20 µL

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

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for the maize DP915635 and one for the *hmg*) 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 (52.5 µL for the DP915635 maize and 52.5 µL for the *hmg*). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (17.5 µL DNA). The volume for the additional 0.5 repetition will ensure adequate volume when loading the samples. Vortex each tube for approximately 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 20 µL for the DP915635 and for the *hmg* reference 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 DP915635 and for the *hmg* reference. Define MGB or non-fluorescent as quencher dye for DP915635 and for *hmg* reference. Select ROX as the passive reference dye. Enter the correct reaction volume (20 µL).
10. Run the PCR with the cycling program described in Table 4. Users who plan to use the second derivative maximum analysis method (an option e.g. on Roche LC480 II instruments) are advised to program 45 cycles instead of 40, in order to be able to quantify down to Cq 40.

Table 4. Cycling program for the DP915635/*hmg* assays.

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

** see comment above for users of second derivative maximum analysis method

3.3 Data analysis

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

a) 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 DP915635 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 DP915635 DNA in the unknown sample, the DP915635 copy number is divided by the copy number of the maize endogenous gene *hmg* and multiplied by 100 (GM% = DP915635/*hmg* x 100).

4. Equipment and Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition) and appropriate analysis software
- 96-well reaction plates
- Optical caps/adhesion covers

- Microcentrifuge
- Micropipettes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex
- Racks for reaction tubes, also cooled
- 0.5 mL , 1.5 mL and 5 mL or 15 mL DNAse free reaction tubes

4.2 Reagents

™ Universal Probes Supermix, Bio-Rad, Cat. 1725281

4.3 Primers and Probes

Table 5. Primers and probes for the DP915635 and *hmg* methods

	Name	DNA Sequence (5' to 3')	Length (nt)
DP915635			
Forward primer	DP-915635-4 F	gCA TCT Agg ACC gAC TAg CTA ACT AAC	27
Reverse primer	DP-915635-4 R	CTT TgC ATC ATg TCT TgA ACA ATg	24
Probe	2059 probe	6-FAM-CgC CAT gAg gAg CAA-MGB	15
hmg			
Forward primer	hmg primer 1	TTg gAC TAg AAA TCT CgT gCT gA	23
Reverse primer	hmg primer 2	gCT ACA TAg ggA gCC TTg TCC T	22
Probe	PHN149436_FAM	6-FAM-CAA TCC ACA CAA ACg C-MGB	16

FAM: 6-carboxyfluorescein; MGB: minor groove binder.

5. References

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

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