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JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Event-specific Method for the Quantification of Maize DP23211 Using Real-time PCR

Validation Report

Vasileva, V., Sacco M.G., Maretti M., Savini C., Mazzara M., Vincent U.

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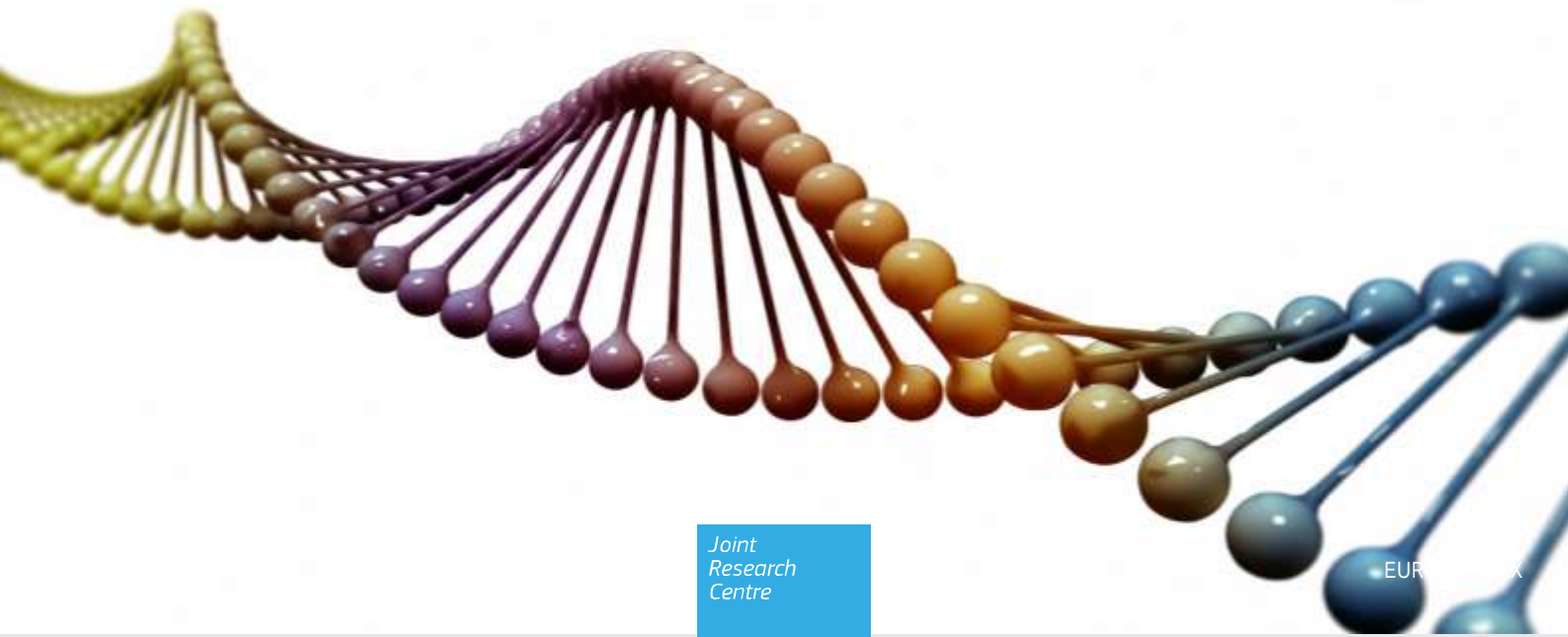

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Abstract

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 DP23211 (unique identifier DP-Ø23211-2). 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), Corteva Agriscience Belgium B.V. on behalf of Corteva Agrisciences LLC provided the detection method and the positive and negative control samples (genomic DNA from seeds of DP23211 maize as positive control DNA, and genomic DNA from seeds of conventional maize as negative control DNA). The EURL GMFF verified the method performance data provided by the applicant, where necessary experimentally, prepared the validation samples (calibration samples and blind samples at different GM percentage ([copies GM/total maize haploid genome copies]), organised an international collaborative study and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL, in line with the provisions of Annex III-3.C.2 to Regulation (EU) No 503/2013 (7), and it fulfils the analytical requirements of Regulation (EU) No 619/2011 (8). This validation report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

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 DP23211 Using Real-time PCR

Validation Report

23/11/2023

European Union Reference Laboratory for GM Food and Feed

1 Introduction

In line with Regulation (EC) No 1829/2003 (1), Corteva Agriscience Belgium B.V. on behalf of Corteva Agrisciences LLC provided the EURL GMFF with an event-specific method for detection and quantification of maize event DP23211 (unique identifier DP-Ø23211-2) 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 DP23211, 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)

Corteva Agriscience Belgium B.V. on behalf of Corteva Agrisciences LLC submitted the identification and quantification method, data demonstrating its adequate performance when applied to genomic DNA extracted from maize DP23211 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 triplicate real-time PCR reactions, according to the method described in Annex 1, using a minimum of 50 ng genomic DNA extracted from at least 4% in mass fraction: maize DP-023211-2, DAS-Ø1131-3, DP-915635-4, DP-91Ø521-2, DP-Ø56113-9, DP-2Ø2216-6, DP-Ø51291-2, ACS-ZMØØ3-2, DAS-Ø15Ø7-1, MON-ØØ6Ø3-6, SYN-IR162-4, SYN-IR6Ø4-5, MON-ØØ81Ø-6, SYN-E3272-5, DP-Ø9814Ø-6, DAS-59122-7, SYN-Ø53Ø7-1, MON-88Ø17-3, MON-89Ø34-3, MON-ØØ863-5, MON-ØØØ21-9, SYN-BTØ11-1, SYN-EV176-9, MON-87427-7, MON-8746Ø-4, DAS-4Ø278-9, DP-ØØ4114-3, VCO-Ø1981-5, MON-874Ø3-1, MON-87411-9, SYN-ØØØJG-2, SYN-ØØØ98-3; soybean DAS-444Ø6-6, ACS-GMØØ5-3, ACS-GMØØ6-4, BPS-CV127-9, DAS-81419-2, MON-877Ø5-6, MON-87751-7, MST-FGØ72-2, MON-87769-7, DP-356Ø43-5, DP-3Ø5423-1, MON-Ø4Ø32-6, MON-877Ø1-2, DAS-68416-4, MON-89788-1, MON-877Ø8-9, SYN-ØØØH2-5, BCS-GM151-6; cotton ACS-GHØØ1-3, BCS-GHØØ2-5, MON-ØØ531-6, MON-15985-7, MON-Ø1445-2, DAS-21Ø23-6 x DAS-24236-5, BCS-GHØØ5-8, BCS-GHØØ4-7, MON-88913-8, MON-887Ø1-3, DAS-8191Ø-7, SYN-IR1Ø2-7, BCS-GH811-4; canola ACS-BNØØ3-6, MON-ØØØ73-7, MON-883Ø2-9, ACS-BNØØ1-4, ACS-BNØØ4-7, ACS-BNØØ8-2, ACS-BNØØ2-5, ACS-BNØØ5-8, ACS-BNØØ7-1, DP-Ø73496-4, BCS-BNØ12-7; rice ACS-OSØØ2-5; potato BPS-25271-9, BPS-A1Ø2Ø-5, , AVE-436G7-1, BPS-PHØ48-1; sugar beet KM-ØØØH71-4; and conventional canola, conventional soybean, conventional maize, conventional cotton, conventional sugar beet, conventional rice, conventional potato, conventional wheat. According to the method developer the DP23211 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 2.13.0+ algorithm search against public sequence of National Center for Biotechnology Information (NCBI) and Patent Genbank databases. No sequence showed alignment of both forward and reverse primers or with the full length of the probe, besides relevant similarity hits to DP23211 patents.

A previously validated maize-specific PCR method (https://gmo-crl.jrc.ec.europa.eu/summaries/2012-08-15_EURL-VL-10-10%20VM_JRC76621.pdf), which amplifies a 79 base pair (bp) fragment of the *Zea mays* High Mobility Group (HMG) Protein A gene (*hmg*), was used as a reference method.

3.2 Specificity assessment conducted by the EURL GMFF

The detection method spans the 3' insert-to-plant junction in maize DP23211. The forward primer PHN175787_f binds to the insert. The reverse primer PHN175788_r binding site was found in the maize genomic border adjacent to the insertion. The PHN175789_p:FAM:MGB probe binds to the intervening sequence of the insert for its first 10 nucleotides and for the remaining 9 nucleotides of its length to the maize genomic sequence.

The amplicon size is expected to be 75 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, except a very unlikely one in the chromosome 14 of *Olea europaea var. sylvestris* (wild olive), with two reverse primers; an additional analysis showed that the probe could not anneal in proximity. A perfect match of the amplicon and of the primers was identified with the sequence deposited for DP23211.

3.3 Verification of the ENGL acceptance parameters

The applicant prepared the calibration curve from a DNA solution (S1) of 10% maize event DP23211 genomic DNA (expressed as copy number ratio) which was serially diluted ([1:6]) to obtain samples S2 and S3 followed by one 1:8 dilution rate to produce the S4. The parameters (slope, R2 coefficient) of eight runs of the calibration curve are reported as provided by the applicant (Table 1).

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

DP23211		*hmg*	
Slope	R^2	Slope	R^2
-3.4	1.00	-3.3	1.00
-3.3	1.00	-3.4	1.00
-3.3	1.00	-3.3	1.00
-3.3	1.00	-3.3	1.00
-3.3	1.00	-3.3	1.00
-3.3	1.00	-3.3	1.00
-3.4	1.00	-3.4	1.00
-3.3	1.00	-3.4	1.00

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 (DP23211) and the maize-specific *hmg*, as established by the applicant, were within the ENGL acceptance criteria.

Precision and trueness of the method were established by the applicant on an ABI7500 (fast) Real-Time PCR and sixteen values for each of the four GM levels (expressed as copies GM/total haploid genome copies) were provided. 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 estimated for single measurements

	Test results			
Expected GM** %	0.04	0.09	0.90	5.00
Measured mean GM %	0.04	0.08	0.90	4.9
Precision (RSD_r %)	12	7.8	9.0	7.1
Trueness (bias %)	-4.8	-10	-0.2	-1.6

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

** Expressed as copy number ratio

The method met the ENGL acceptance criteria for trueness and precision at the lowest GM level [i.e. 0.04% (expressed as copy number ratio)], which contains 50 copies of DP23211 in 325 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 absolute limit of detection (LOD_{abs}) of the DP23211 event-specific and the maize reference real-time PCR methods was assessed by the applicant in 60 PCR replicates. The LOD_{abs} was found to be below 10 haploid genome copies for DP23211 event-specific method and below 20 haploid genome copies for *hmg* reference method. The relative LOD (LOD_{rel}) of the combined method was found to be at least 0.0084% (related to copies GM/total haploid genome copies) in 325 ng of total maize DNA per reaction. 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 16 combinations of the following variations to the method: exact/+10%/-10% enzyme mix concentration, exact/+30%/-30% primer concentration, exact/+30%/-30% probe concentration, exact/+1 µL/-1 µL master mix volume, +/-1 °C annealing temperature. The RSD_r and the trueness calculated for each combination of variations on a sample at the LOQ level (0.04%) did not exceed 30%, thus meeting the ENGL acceptance criteria.

Precision and trueness of the method were tested in a transferability study involving a QuantStudio5 and a LC480II PCR cyclers: the values provided for each of the three GM levels (expressed as copies GM/total haploid genome copies) were the mean of two sets of triplicates. Table 2B reports precision and trueness values for the three GM-levels as provided by two 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 in the transferability study of the applicant

	Test results		
Expected GM %	0.04	0.09	0.90
Measured mean GM* %	0.04 [1] 0.05[2]	0.09 [1] 0.10 [2]	0.89 [1] 0.92 [2]
Precision* (RSD_r %)	15 [1] 0.0 [2]	3.9 [1] 4.2 [2]	10 [1] 4.9 [2]
Trueness* (bias %)	0.0 [1] 9.5 [2]	0.0 [1] 10 [2]	-1.0 [1] 1.8 [2]

*Data from experiments run on two different machines QuantStudio5 (1) and LC480II (2)

3.4 DNA extraction

Genomic DNA was isolated from ground maize seeds, using a cetyltrimethylammonium bromide (CTAB)-based method previously submitted for detection of maize event DP4114 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/EURL-VL-02-14-XP.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 DP23211.

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 *DP23211*.

Annex III to Reg. (EU) No 503/2013 (7) requires the applicant to discuss the validity and limitations of the detection methods in the various types of foods and feeds (matrices) that are expected to be placed on the market. To this regard the applicant stated that *''the 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. 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 [1,8,6,7,14]. These steps influence the quality and intactness of DNA contained in the final processed maize products [2,10,11,19] which may result in significant degradation of high molecular weight DNA and failure to PCR amplify products greater than a few 100 base pairs [2,10]. Random DNA fragmentation is known to lead to variability in quantifying DNA by qPCR [15], 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 [3,13]. These extraction methods are widely used for plant-based materials, are economical and can be easily scaled [16]. The DP23211 detection method has been developed and was pre-validated on maize seed and tissues. The DP23211 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."*

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.

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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[2]:

- genomic DNA extracted by the applicant from hemizygous maize segment harbouring the DP23211 event, and
- genomic DNA extracted by the applicant from conventional maize segment genetically similar to those harbouring the DP23211 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 DP23211 DNA to total maize DNA. The procedure is a simplex system, in which a maize-specific method targeting the endogenous gene (*hmg*), and the GM target method for DP23211 are performed in separate wells. The validated method protocol is published by the EURL GMFF at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and can be found in Annex 1 to this report.

For the detection of GM event DP23211, a 75 bp fragment of the region spanning the 3' insert-to-plant junction in maize DP23211 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 MBG (minor groove binder) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event DP23211, an maize taxon-specific system amplifies a 79 bp fragment of an maize (*hmg*) endogenous gene, using *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and BHQ-1 (Black Hole Quencher® 1) as non-fluorescent quencher dye at its 3' end.

Standard curves are generated for both the DP23211 and the *hmg* systems by plotting the Cq values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves.

For the relative quantification of event DP23211 DNA in a test sample, the DP23211 copy number is divided by the copy number of the maize [haploid genome] and multiplied by 100 to obtain the percentage value (GM % = DP23211/ 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) (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.

[2] 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 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 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 *DP23211* 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 copy number of the DP23211 and of the *hmg* targets in the positive control sample were determined by digital PCR (dPCR) performed on the Bio Rad QX200 Droplet Reader.

Reaction mixes were prepared in order to test the zygosity in 18 replicates to a final volume of 22 µL and contained 1X ddPCR Super Mix no dUTP (Bio Rad, Cat. number 1063024), primers and probes at concentrations indicated in the corresponding validated method (PHN175787_f and PHN175788_r primers at 600 nM each, PHN175789_p:FAM:MGB probe at 120 nM; MaiJ-F2 and mhmg-rev primers at 300 nM each, mhmg-probe probe at 180 nM), and 2 µL of DNA at a concentration of 65 ng/µL; the DNA concentration was chosen in order to avoid panel saturation (optimal between 200<positive partitions<700).

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

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

Box 1: Thermal cycling conditions in a final volume of 40 µL.

Step	Temperature (°C)	Time (mm:ss)	Ramp rate	Number of cycles
Polymerase activation	95	10:00	2°C/sec	1
DNA denaturation	94	00:30		40
Annealing/extension	60	01:00		
Enzyme deactivation	98	10:00		1
Hold	4	infinite		1

The sealed 96-well plate was then placed in the QX200 Droplet Reader to determine through cytofluorimetry the fraction of fluorescent PCR-positive droplets with respect of the total number of droplets in the original sample by selecting the proper fluorescent dye used. Data analysis and copy number calculations were performed using the Bio-Rad QX200 Droplet Reader Analysis software (QuantaSoft version 1.7.4.0917).

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 (0.04% - 5%, 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 DP23211 maize DNA and non-GM maize DNA.

Table 4. DP23211 blinded samples GM % contents

DP23211 GM % GM copy number/maize haploid genome copy number x 100
5.0
2.0
0.9
0.09
0.04

The calibration sample S1 was prepared from the genomic DNA provided by the applicant by mixing the appropriate amount of DP23211 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, a QuantStudio 7 Flex System and a Roche LC480 real-time platform under repeatability conditions and followed the protocol provided by the applicant.

Test samples with GM levels 5%, 2%, 0.9%, 0.09% and 0.04% were tested in two real-time PCR runs with three replicates for each GM-level on each plate. The test sample with GM level 0.04% (in copy number ratio, equal to 0.10% mass ratio), containing 48 copies of DP23211 in 325 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. On Roche LC480 platform the method was run at 45 cycles as described in the validated method published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1, and analysed with the second derivative maximum method.

4.4 International collaborative study (step 4)

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

- 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 DP23211 international collaborative study were randomly selected from 21 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 DP23211

Laboratory	Country
Institute for Diagnosis and Animal Health	Romania
LUFA Speyer – Referat II/2 (Molecular Biology)	Germany
National Center of Public Health and Analyses	Bulgaria
WFSR	Netherlands
Institut für Hygiene und Umwelt	Germany
Laboratoire de la Santé des Végétaux – ANSES	France
Departamento de Tecnicas Biomoleculares	Spain
Thüringer Landesamt für Verbraucherschutz (TLV)	Germany
Umweltbundesamt GmbH	Austria
CREA DC, sede di Tavazzano	Italy
Crop Research Institute (Výzkumný ústav rostlinné výroby, v.v.i.), PGI	Czech Republic
National Institute of Biology	Slovenia

4.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: two laboratories used ABI 7500, one ABI 7300, two ABI 7500 Fast, one Quant Studio 7 Flex, one QuantStudio 3, one QuantStudio 5, one QuantStudio Q6 Pro, one Quant Studio Q7 Pro and two used Biorad CFX96 TOUCH.

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

4.4.3 Materials used in the international collaborative study

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

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

- ✓ 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 65 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 4)
- ✓ Reaction reagents:
  - JumpStart™ Taq ReadyMix (2x), one vial: 8 mL
  - distilled sterile water, one vial : 1.2 mL
  - ROX (50X), one tube 60 µL
  - MgCl2 (25 mM), one vial 2.7 mL
- ✓ Primers and probes (1 tube each) as follows:

  Hmg taxon-specific
  - MaiJ-F2 (10 µM): 250 µL
  - mhmg-rev (10 µM): 250 µL
  - mhmg-probe:FAM:BHQ (10 µM): 150 µL

  DP23211
  - PHN175787_f (10 µM): 500 µL
  - PHN175788_r (10 µM): 500 µL
  - PHN175789_p:FAM:MGB (10 µM): 100 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 DP23211 and for *hmg*. In total, two plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for DP23211 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

Ten laboratories reported no deviations from the validation protocol. Two laboratories reported protocol deviations (PL03 and PL07). Laboratory PL03 reported severe issues with running the protocol. New reagents were shipped to PL03 to repeat the experiments. In both runs PL03 laboratory reported very inconsistent results, unsuitable for data analysis. Therefore the PL03 data were not included in the data analysis. Laboratory PL07 reported several issues with running the protocol such as (i) the amount of ROX reagent not being adjusted for 7900 equipment, (ii) exchange in plate set-up between the reference and GM samples, (iii) a missing DNA sample not added to the plate and (iv), an abnormal Ct value that had been eliminated. The data of PL07 were retained for data analysis.

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 DP23211 and *hmg* targets to determine the zygosity ratio in the positive control samples are shown in Table 6. For reference, the zygosity ratio reported by the applicant was 0.42.

Table 6. Zygosity ratio of the DP23211 and *hmg* targets in the positive control sample.

Mean ratio (DP23211/*hmg*)	0.41
Standard deviation	0.01
RSD_r (%)	3.3
Standard error of the mean	0.003
Upper 95% CI of the mean	0.41
Lower 95% CI of the mean	0.40

The mean ratio (DP23211/*hmg*) equals 0.41. The 95% confidence interval (CI) spans around 0.42, 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 not significantly different from the expected ratio, for an alpha = 0.05.

Hence, the relative (%) GM content of the samples expressed in haploid genome copy numbers corresponds to the GM content expressed in mass fraction. For example, the 0.04 GM% in DNA copy number ratio corresponds to a 0.1 GM % in mass fraction.

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 0.04% to 5.00% (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 0.04% 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 Q7 (QuantStudio 7 Flex System) and a Roche LC480 for robustness.

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

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R^2 coefficient shall be ≥ 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, Q7 and Roche LC480 to quantify GM-levels in the range 0.09% to 5.00% in four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	DP23211			*hmg*		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.4	99	1.00	-3.3	100	1.00
Run B	-3.3	100	1.00	-3.4	98	1.00
Run C	-3.3	100	1.00	-3.4	100	1.00
Run D	-3.4	97	1.00	-3.3	100	1.00
Run E	-3.5	93	1.00	-3.4	97	1.00
Run F	-3.5	92	1.00	-3.4	97	1.00

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

Runs A-B were carried out on ABI 7500; runs C-D were carried out on Q7 runs E and F were carried out on Roche LC480.

Table 7B. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, Q7, and Roche LC480 to quantify the GM-level 0.04% (copy number, equal to 0.1% in mass fraction) in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	DP23211			*hmg*		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run G	-3.4	99	1.00	-3.4	98	1.00
Run H	-3.4	97	1.00	-3.4	99	1.00
Run I	-3.5	92	1.00	-3.4	97	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 Q7; run I was carried out on Roche LC480.

According to the ENGL method acceptance criteria the method trueness (measured as bias in % of the target GM level) should be within ± 25% of the accepted reference value over the entire dynamic range and the

precision, expressed as RSD_r % (relative standard deviation of repeatability), should be ≤ 25%, also over the entire dynamic range.

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

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

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	5.0	0.47	3.2
2.0	2.1	5.5	8.9
0.90	0.9	1.2	6.5
0.09	0.09	-1.6	9.1
0.04	0.04	-0.38	13.18

Table 9. Values of trueness and precision as established by the EURL GMFF in its in-house verification using a Q7. GM % in copy/copy haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	4.9	-1.1	2.5
2.0	2.0	1.4	1.7
0.90	0.9	1.1	1.3
0.09	0.09	3.9	1.6
0.04	0.04	7.3	11.59

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

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	5.2	4.3	1.23
2.0	2.1	7.0	1.6
0.90	1.0	13	0.73
0.09	0.11	19	4.9
0.04	0.05	15.74	17.66

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 DP23211 ranges from 93% to 103% and the linearity is equal to 1.00; the amplification efficiency for *hmg* ranges from 93% to 103.07% and the linearity is equal to 1.00. The mean PCR efficiency was 98% for DP23211 and 98% for *hmg*. The average R^2 of the methods was 1.00 and 1.00 for DP23211 and *hmg*, respectively. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

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

		DP23211			*hmg*		
Lab	Plate	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.3	100	1.00	-3.4	97	1.00
	B	-3.3	100	1.00	-3.3	100	1.00
2	A	-3.4	99	1.00	-3.3	103	1.00
	B	-3.5	95	1.00	-3.3	101	1.00
3	A	N/A	N/A	N/A	N/A	N/A	N/A
	B	N/A	N/A	N/A	N/A	N/A	N/A
4	A	-3.4	96	1.00	-3.35	99	1.00
	B	-3.4	97	1.00	-3.42	96	1.00
5	A	-3.5	94	1.00	-3.51	93	1.00
	B	-3.5	93	1.00	-3.49	93	1.00
6	A	-3.3	103	1.00	-3.33	100	1.00
	B	-3.3	103	1.00	-3.33	100	1.00
7	A	-3.3	99	1.00	-3.39	97	1.00
	B	-3.3	100	1.00	-3.4	99	1.00
8	A	-3.3	100	1.00	-3.4	96	1.00
	B	-3.4	96	1.00	-3.4	99	1.00
9	A	-3.4	99	1.00	-3.4	98	1.00
	B	-3.4	96	1.00	-3.4	98	1.00
10	A	-3.4	97	1.00	-3.3	100	1.00
	B	-3.4	98	1.00	-3.3	100	1.00
11	A	-3.3	100	1.00	-3.4	99	1.00
	B	-3.4	98	1.00	-3.4	97	1.00
12	A	-3.4	97	1.00	-3.4	98	1.00
	B	-3.2	103	1.00	-3.4	98	1.00
	Mean	-3.4	98	1.00	-3.4	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

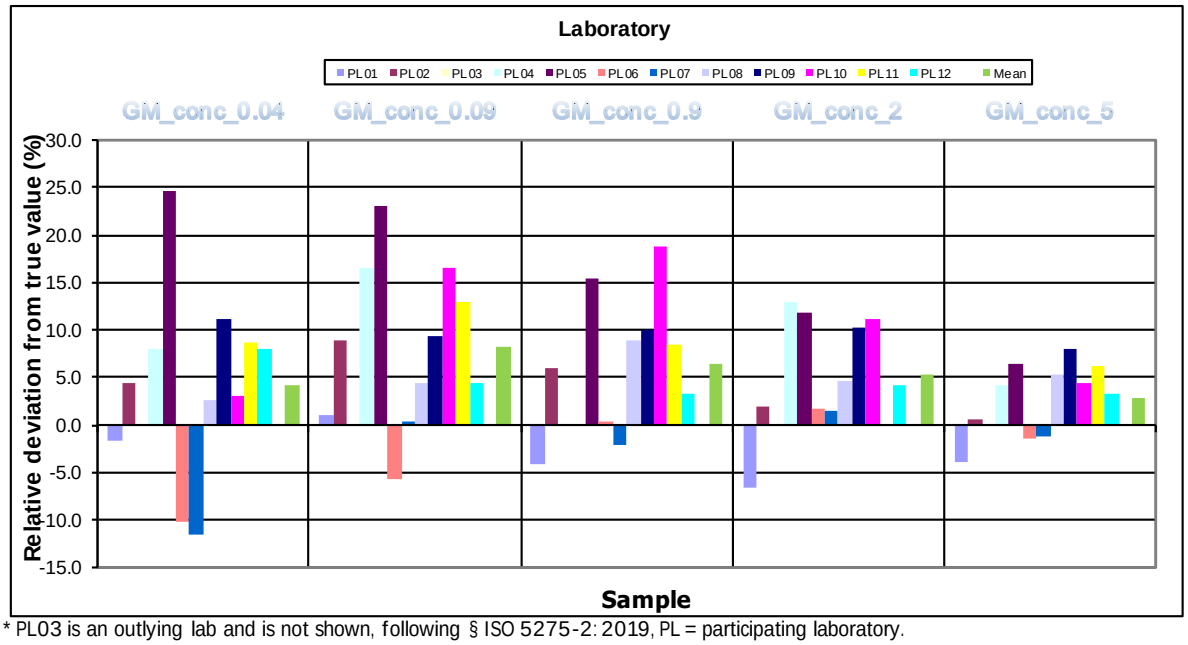
	GMO content (%) *																			
LAB	0.04				0.09				0.90				2.00				5.00			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.04	0.04	0.04	0.04	0.09	0.09	0.09	0.09	0.83	0.83	0.86	0.93	1.87	1.84	1.90	1.87	4.46	4.96	4.87	4.92
2	0.05	0.05	0.03	0.04	0.09	0.11	0.10	0.09	0.99	0.92	0.97	0.94	2.10	2.16	1.96	1.95	4.94	5.07	4.75	5.33
3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4	0.05	0.04	0.04	0.04	0.11	0.11	0.10	0.10	1.02	1.08	0.83	0.92	2.24	2.41	2.18	2.20	5.31	5.07	5.38	5.07
5	0.05	0.05	0.05	0.05	0.11	0.11	0.11	0.11	1.00	1.01	1.09	1.05	2.15	2.29	2.29	2.22	5.54	4.87	5.52	5.34
6	0.04	0.04	0.03	0.04	0.08	0.09	0.09	0.07	0.89	0.93	0.89	0.91	2.12	1.96	2.04	2.02	5.00	4.96	4.84	4.92
7	0.04	0.04	0.04	0.03	0.10	0.08	0.09	0.09	0.95	0.92	0.83	0.82	0.00	2.04	2.05	2.00	5.16	4.84	5.03	4.75
8	0.04	0.05	0.04	0.04	0.10	0.09	0.09	0.09	1.02	0.94	1.00	0.96	2.08	2.15	2.06	2.08	5.14	5.39	4.92	5.61
9	0.05	0.04	0.04	0.04	0.10	0.09	0.11	0.10	1.01	1.02	0.98	0.96	2.25	2.09	2.21	2.26	5.23	5.60	5.26	5.52
10	0.04	0.04	0.04	0.04	0.10	0.10	0.11	0.11	1.05	1.06	1.02	1.14	2.12	2.30	2.22	2.25	5.25	5.16	5.32	5.15
11	0.05	0.05	0.04	0.04	0.11	0.10	0.10	0.10	0.96	1.00	0.95	0.99	2.25	1.86	2.24	2.34	5.98	4.89	5.50	4.85
12	0.04	0.04	0.05	0.05	0.09	0.09	0.10	0.10	0.92	0.93	0.95	0.92	2.06	2.05	2.12	2.11	5.37	5.05	5.19	5.04

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

n.a. not available

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 data of PL03 laboratory was removed from the data analysis due to failure of the laboratory to run the method. 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 *



* PL03 is an outlying lab and is not shown, following § ISO 5275-2: 2019, PL = participating laboratory.

Overall, all laboratories' mean relative deviations from the true values were within a maximum of ± 25% (with the exception of PL03 which was not included in the analysis). At all GM-levels, eleven laboratories were within the limit.

5.2.3 Method performance requirements

Among the performance requirements established by ENGL and adopted by the EURL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), repeatability and reproducibility are to be assessed through an international collaborative trial. Table 13 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study (see Table 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 15.45% at the 2.00% GM level, thus within the acceptance criterion.

Table 13. Summary of validation results for the *DP23211* method, expressed as GM copy numbers in relation to target taxon haploid genome copy numbers

	Test Sample Expected GMO %				
	0.04	0.09	0.90	2.0	5.0
Laboratories having returned valid results	11	11	11	11	11
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	1	1	0
Reason for exclusion *	N/A	N/A	C	C	N/A
Mean value	0.04	0.10	0.96	2.1	5.1
Relative repeatability standard deviation, RSD_r (%)	9.2	6.8	4.1	3.3	5.0
Repeatability standard deviation	0.004	0.007	0.040	0.069	0.256
Relative reproducibility standard deviation, RSD_R (%)	12	9.8	7.8	6.5	5.7
Reproducibility standard deviation	0.005	0.010	0.075	0.138	0.292
Bias** (absolute value)	0.002	0.007	0.059	0.108	0.144
Bias (%)	4.3	8.3	6.5	5.4	2.9

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

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

Table 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 9.2% at the 0.04 % GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 8.3% at the 0.09% GM level.

6 Compliance of the method for detection and quantification of event DP23211 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 0.1% level shown by the applicant's dossier (expressed as mass fraction of GM-material) was 11.8%, based on 16 replicates (Table 2A), and 15.2% based on two sets of triplicates 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 0.04% 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 12% and 18% (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 9.20%, 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 DP23211 at or around 0.1% level related to mass fractions of GM material.

Source	RSD_r %	GM %
Applicant's method optimisation	12%	0.1%
Applicant's transferability study	15%	0.1%
EURL GMFF tests	12 – 18 %	0.1%
Collaborative study	9.2%	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 http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1.

8 References

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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 DP23211 using Real-time PCR

Validated Method

Method development:

Corteva Agriscience Belgium B.V.

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 DP23211 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 DP23211, a 75 bp fragment of the region spanning the 3' insert-to-plant junction in maize DP23211 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 MBG (minor groove binder) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event DP23211, a maize taxon-specific PCR amplifies a 79 bp fragment of a maize *high-mobility group A* (*hmgA*) endogenous gene (Accession number, GeneBank: AJ131373), using *hmgA* gene-specific primers and a *hmgA* gene-specific probe labelled with 6-FAM as reporter dye at its 5' end and BHQ-1 (Black Hole Quencher® 1) 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 DP23211 DNA in a test sample, Cq values for DP23211 and *hmgA* are determined for the sample. Standard curves are then used to estimate the relative amount of DP23211 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 July 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 0.0084% (related to mass fraction of GM material) in 325 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% (related to mass fraction of GM material) in 325 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 DP23211 and is therefore event-specific for the event DP23211. 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 species event name

3.2.1 General

The real-time PCR set-up for the taxon (*hmgA*) and the GMO (event DP23211) 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 DP23211) and *hmgA* 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 DP23211 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) [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 below.

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	1.13
Maize haploid genome copies	119048	19841	3307	413
DP23211 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 DP23211 (Table 2) and *hmgA* (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 DP23211.

Component	Final concentration	µL/reaction
JumpStart™ Taq ReadyMix (2x)	1x	12.50
ROX (50X)	0.1X	0.05
$MgCL_2$ (25 mM)	4 mM	4.00
PHN175787_f (10 µM)	600 nM	1.50
PHN175788_r (10 µM)	600 nM	1.50
PHN175789_p:FAM:MGB (10 µM)	120 nM	0.30
Nuclease free water	-	0.15
DNA	-	5.00
Total reaction volume:		25.00 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 *hmgA*

Component	Final concentration	µL/reaction
JumpStart™ Taq ReadyMix (2x)	1x	12.50
ROX (50X)	0.1X	0.05
$MgCL_2$ (25 mM)	4 mM	4.00
MaiJ-F2 (10 µM)	300 nM	0.75
mhmg-rev (10 µM)	300 nM	0.75
mhmg-probe (10 µM)	180 nM	0.45
Nuclease free water	-	1.50
DNA	-	5.00
Total reaction volume:		25.00 µL

*TaqMan® probe is labelled with 6-FAM at its 5'-end and BHQ1™ at its 3'-end

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for the DP23211 and one for *hmgA*) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (70 µL for DP23211 and 70 µL for *hmgA*). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (17.5 µL DNA). The volume for the additional 0.5 repetition will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
6. Spin down the tubes. Aliquot 25 µL for DP23211 and for *hmgA* 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 DP23211 and for *hmgA*. Define MGBNFQ™ or non-fluorescent as quencher dye for DP23211 and BHQ1™ or non-fluorescent for *hmgA*. Select ROX as the passive reference dye. Enter the correct reaction volume (25 µL).
10. Run the PCR with the cycling program described in Table 4. Users who plan to use the second derivative maximum analysis method (an option e.g. on Roche LC480 instruments) are advised to program 45 cycles instead of 40, in order to be able to quantify down to Cq 40.

Table 4. PCR cycling program for DP23211/*hmgA*.

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

*UNG: Uracil-N-glycosylase

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

3.3 Data analysis

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

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 Cq values for each reaction.

The standard curves are generated both for *hmgA* and DP23211 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 DP23211 DNA in the unknown sample, the DP23211 copy number is divided by the copy number of the maize endogenous gene *hmgA* and multiplied by 100 (GM% = DP23211/*hmgA* x 100).

4. Equipment and Materials

4.1 Equipment

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

4.2 Reagents

- JumpStart Taq Ready Master Mix. Sigma Cat No D7440-100RXN
- ROX (100X) Cat No R4526
- $MgCL_2$ Cat No M8787

4.3 Primers and Probes

Table 5. Primers and probes for DP23211 and *hmgA*

	DP23211	**DNA Sequence (5' to 3')**	Length (nt)
DP23211			
Forward primer	PHN175787_f	TTA CGG CAT CTA GGA CCG ACT AG	23
Reverse primer	PHN175788_r	GAA GCA CTT GTT TTT CAA TTC CAA	24
Probe	PHN175789_p: FAM:MGB	6-FAM-CTA GTA CGT AGT GAA TCT G-MGBNFQ™	19
hmgA			
Forward primer	MaiJ-F2	TTG GAC TAG AAA TCT CGT GCT GA	23
Reverse primer	mhmg-rev	GCT ACA TAG GGA GCC TTG TCC T	22
Probe	mhmg-probe	6-FAM-CAA TCC ACA CAA ACG CAC GCG TA-BHQ1™	23

[FAM: 6-carboxyfluorescein; MGB: minor groove binder; JOE: 4,5-dichloro-dimethoxy-fluorescein; BHQ1: black hole quencher].

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
qPCR	Real-time PCR
Cq	Threshold cycle
ENGL	European Network of GMO Laboratories
LOD	Limit of Detection
LOQ	Limit of Quantification

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