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JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Event-specific Method for the Quantification of Maize DP-ØØ4114-3 Using Real-time PCR

Validation Report

European Union Reference Laboratory for
Genetically Modified Food and Feed

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EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Health, Consumers & Reference Materials
Food & Feed Compliance Unit

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Validation Report

10 April 2018

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate[a] 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 DP4114 (unique identifier DP-ØØ4114-3). 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 [1-5].

In accordance with current EU legislation[b], Pioneer Overseas Corporation provided the detection method and the positive and negative control samples (genomic DNA extracted from ground seed of hybrid DP-ØØ4114-3 maize as positive control DNA, and genomic DNA from extracted from ground seed of hybrid near-isogenic 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 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 ENGL performance parameters of precision (reproducibility and repeatability) and of trueness, 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, and it fulfils the analytical requirements of Regulation (EU) No

[a] Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed".
[b] Regulation (EC) No 503/2013 of 3 April 2013 "on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006".

619/2011[c]. However, the method overestimates the GM content at the 0.1 % in mass fraction of GM DNA.

The EURL GMFF concludes that the method is fit for purpose and can be used for regulatory control of food and feed. Laboratories using the method for the purposes of Regulation (EU) No 619/2011 are asked to provide the EURL GMFF with their experimental data and results in order to allow further verification of the performance of the method in the lower part of the dynamic range.

This validation report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

[c] Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired.

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: Belac 268 TEST (Flexible Scope for DNA extraction, DNA identification and real-time PCR) and ISO 17043:2010 accredited (certificate number: Belac 268 PT, proficiency test provider).

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

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Content

1.

1. Introduction

In line with Regulation (EC) No 1829/2003, Pioneer Overseas Corporation provided the EURL GMFF with an event-specific method for detection and quantification of maize event DP4114 (unique identifier DP-ØØ4114-3) together with genomic DNA as positive and negative control samples in June 2014.

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[d], 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 results of step 3 indicated that the method was underperforming at the lowest end of the dynamic range (corresponding to 0.1 % m/m). An amended version of the method was submitted by the applicant in August 2015 and found similarly underperforming. Therefore, Pioneer Overseas Corporation submitted a new method in May 2016. The scientific assessment concluded that the performance characteristics of the newly submitted method were met and proceeded to step 3 of the validation procedure (experimental testing).

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. In the course of step 3 the EURL GMFF asked for additional negative control DNA, as it was not sufficient. The negative control sample DNAs were pooled before use (as they were of the same origin).

Step 3 was completed with the conclusion that the method could be submitted to the collaborative study (step 4). All experiments described in this validation report refer to the accepted version of the method submitted in 2016, unless otherwise stated.

2. Step 1 (dossier acceptance) and step 2 (scientific dossier assessment and bioinformatics analysis)

Documentation and data supplied by the applicant were evaluated by the EURL GMFF for completeness (step 1) and compliance with the ENGL method acceptance criteria (step 2).

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.

Specificity assessment by the applicant

[d] EURL/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm)

A BLASTN search (BLASTN 2.3.1 algorithm) was performed with the event-specific PCR amplicon of the DP4114 method against the nucleotide (nt) database on the National Center for Biotechnology Information website (NCBI, www.ncbi.nlm.nih.gov/Blast.cgi) to identify sequence alignment with high homology, specifically at the binding sites of primers and probe.

In addition, the specificity of the method was assessed by the applicant in real-time PCR reactions, according to the method described, using 200 ng genomic DNA extracted from the following Certified Reference Materials or seeds: maize 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-8746Ø-4, MON-ØØ863-5, MON-ØØØ21-9, MON-87427-7, SYN-BTØ11-1, SYN-EV176-9; soybean DAS-444Ø6-6, ACS-GMØØ5-3, ACS-GMØØ6-4, MST-FGØ72-3, BPS-CV127-9, DAS-81419-2, MON-877Ø5-6, MON-87769-7, DP-356Ø43-5, DP-3Ø5423-1, MON-Ø4Ø32-6, MON-877Ø1-2, DAS-68416-4, MON-89788-1, MON-87708-9; cotton ACS-GHØØ1-3, BCS-GHØØ2-5, MON-ØØ531-6, MON-15985-7, MON-Ø1445-2, DAS-24236-5, BCS-GHØØ5-8, BCS-GHØØ4-7, MON-88913-8; sugar beet KM-ØØØH71-4; potato BPS-25271-9; canola ACS-BNØØ3-6, MON-ØØØ73-7, MON-88302-9, ACS-BNØØ1-4, ACS-BNØØ4-7, ACS-BNØØ8-2, ACS-BNØØ2-5, ACS-BNØØ5-8, ACS-BNØØ7-1, DP-Ø73496-4; rice ACS-OSØØ2-5; and non-GM rice, potato, sugar beet, cotton, soybean, canola, non-GM 5155-P1 maize, non-GM 25W43 and non-GM 25R25 wheat.

According to the applicant, the *in silico* analysis demonstrated that no contiguous DNA sequence contained both the forward and reverse primer binding sites, indicating that the specific primers and probe were capable of detecting DP4114 maize only. In addition, the method developer demonstrated experimentally that the DP4114 method did not react with any sample except with the positive control.

The taxon-specific method is an already validated maize-specific PCR for High Mobility Group (HMG) Protein A gene (*hmg*) (GenBank Accession No. AJ131373); however, the probe was shortened to 16 nucleotides to allow replacement of the TAMRA quencher with a Minor Groove Binding (MGB) quencher. The method amplifies a 79-bp product.

Bioinformatics specificity assessment by the EURL GMFF

The amplicon produced by the method spans the junction between the 5' genomic region and the transgenic insert DP4114. The forward primer "PHN164689" binds to the genomic region adjacent to the insertion. The reverse primer "PHN1641690" binding site was found in the insert. The probe "PHN164691" binding site spans the junction region between the 5' genomic region of *Zea mays* and the transgenic insert.

The amplicon size of the event specific assay for DP4114 maize is expected to be 80 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 100 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.

Verification of the ENGL acceptance parameters

The applicant prepared the calibration curve from a DNA solution (S1) of 10 % maize event DP4114 genomic DNA (expressed as copy number ratio) which was serially diluted (1:5) in TE 0.1x to obtain solutions S2, S3, S4 and S5. The parameters (slope, R^2 coefficient) of five runs of the calibration curve are reported as provided by the applicant (Table 1).

Table 1. Slope and R^2 values obtained by the applicant

	DP4114		***hmg***	
	Slope	**R^2**	**Slope**	**R^2**
Run 1	-3.46	1.0	-3.40	1.0
Run 2	-3.50	0.99	-3.43	1.0
Run 3	-3.31	0.99	-3.33	1.0
Run 4	-3.13	0.99	-3.28	1.0
Run 5	-3.27	0.98	-3.21	0.99
Mean	**-3.33**	**0.99**	**-3.33**	**1.0**

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

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve should range from -3.6 to -3.1 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 assay (DP4114 maize) and the maize-specific *high mobility group* (*hmg*) assay, as established by the applicant, were within the ENGL acceptance criteria.

Also precision and trueness of the method were established by the applicant. Table 2 is based on three independent real-time PCR runs and averages of 14-15 replicate values for each of three GM levels (expressed as percentage of DP4114 maize DNA mass fractions). Precision and trueness values for the three GM levels are reported as provided by the applicant.

Both parameters were within the ENGL acceptance criteria (trueness ± 25 %, RSD_r ≤ 25 % across the entire dynamic range).

Table 2. Mean percentage (expressed as copy number ratio), precision and trueness values provided by the applicant

	Test results*		
Expected GM %	**0.09**	**2.0**	**4.5**
Measured mean GM %	0.103	1.95	4.49
Precision (RSD_r %)	16	20	17
Trueness (bias %)	14.3	-2.27	-0.254

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

3. Step 3 (experimental testing of the samples and method)

3.1 DNA extraction

A DNA extraction method from ground maize seeds was used by the method developer for extracting genomic DNA from DP4114 and non-GM maize seeds. This DNA extraction method was verified by the EURL GMFF on maize seeds and found to provide DNA of acceptable quality. The verification report of the CTAB-based extraction method coupled with anion-exchange purification columns is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

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.

3.2 Protocol 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 DP4114 DNA to total maize DNA. The procedure is a simplex system, in which a maize specific method targeting the endogenous *high mobility group* protein A (*hmg*) gene, and the GM target method for DP4114 are performed in separate wells. The sequence of the *hmg* probe is different from the previously validated *hmg* assay. The validated 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 DP4114, an 80 bp fragment of the region spanning the 5' maize-to-insert junction in maize DP4114 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 BHQ (Black Hole quencher) at its 3' end.

For the relative quantification of GM event DP4114, a maize taxon-specific system amplifies a 79 bp fragment of a *high mobility group* protein A (*hmg*) gene, using *hmg* gene-specific primers and an

hmg gene-specific probe labelled with FAM as reporter dye at its 5' end and MGB (Minor Groove Binding) quencher at its 3' end.

Standard curves are generated for both the DP4114 and the *hmg* assays 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 DP4114 DNA in a test sample, the DP4114 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value (GM % = DP4114/*hmg* x 100).

The absolute copy numbers of the calibration curve samples are estimated by dividing the sample DNA mass (nanograms) by the published average 1C value for the maize genome (2.73 pg) [(6)]. 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 three digits for values ≤ 0.1, two digits for values between 0.1 and 1, one digit for values between 1 and 10 and no digit for values ≥ 10, unless otherwise stated. The calculations in the MS Excel files however were done over not rounded data. This approach might create small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.

Table 3. Copy number values per 20 µL reaction volume in the standard curve samples

Sample code	**S1**	**S2**	**S3**	**S4**	**S5**
Total amount of maize DNA in the reaction (ng)	325	65	13	2.6	0.52
Target taxon *hmg* copies	119048	23810	4762	952	190
Target DP4114 copies	11905	2381	476	95	19

3.3 EURL GMFF experimental testing

3.3.1 Determination of the zygosity ratio in the positive control sample

Annex II of Regulation (EU) No 619/2011 requires that "when results are primarily expressed as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EURL GMFF." This requires knowledge of the zygosity of the event. In order to satisfy this requirement, the EURL GMFF assessed the zygosity (GM-target to reference target ratio) in the positive control sample submitted by the applicant.

The copy number of the DP4114 and of the *hmg* targets in the positive control sample were determined by digital PCR (dPCR) performed on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Reaction mixes were prepared in order to test the zygosity in five replicates to a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix, without UNG (Applied Biosystems, Cat. number 4324020), 1X GE sample loading reagent (Fluidigm PN 85000746), primers and probes as submitted by the applicant (concentrations of forward DP4114 primer "08-O-2677": 500 nM, reverse DP4114 primer "08-O-2678": 900 nM and "08-QP74" DP4114 probe: 200 nM; *hmg* forward primer: "MaiJ-F2" 400 nM; reverse *hmg* "mhmg-rev": 400 nM and *hmg* probe "mhmg-probe": 150 nM), and 1 µL of digested with *Eco*RI DNA at a concentration of 3.5 ng/uL; the DNA concentration was chosen in order to avoid panel saturation (optimal between 200<positive partitions<700). The result of the zygosity test was obtained on the positive control sample with the first version of the method submitted in 2014 and not repeated with the revised method obtained in 2016. However, as dPCR exploits the end-point amplification of target analyte, the quantification was not affected by the suboptimal efficiency observed for the qPCR. As a consequence, the results of the zygosity test conducted in 2014 were considered as valid.

Loading of the digital chip was performed according to the manufacturer's instructions by using the IFC controller (Fluidigm). Approximately 4.6 µL of the reaction mixes were loaded into each well and distributed into the 765 partitions constituting one panel. The experiment was repeated three times for a total number of fifteen data sets for the GM target and fifteen for the reference target. 'No template controls' were included. Amplification conditions were as reported in Annex 1 or in the Validated Method document at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx. Data analysis and copy number calculations were performed using the BioMark digital PCR Analysis software. The range of Cq retention was from 20 to 40.

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 inter-laboratory validated methods'[e].

3.3.2 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 (0.058 % -to 5.0 %). The experiments were performed on an ABI 7500, an ABI 7900HT and a Roche LC480 real-time platform under repeatability conditions and followed the protocol provided by the applicant. Test samples with GM levels 0.52 %, 0.90 %, 2.6 % and 5.0 % were tested in two real-time PCR runs with two replicates

[e] Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods. European Network of GMO Laboratories (ENGL), 2011.
http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf

for each GM level on each plate (total of four replicates per GM level). The test sample with GM level 0.058 % (corresponding to 0.10 % in mass fractions of GM material) was tested in 15 replicates in an additional run each for ABI 7500 and ABI 7900HT platform. On Roche LC480 platform test samples with GM levels 0.52 %, 0.90 %, 2.6 % and 5.0 % were run at 45 cycles and analysed with the Absolute Quantification/Second Derivative Maximum method with High Confidence algorithm, according to the applicant's suggestions, which were applied and specific to this platform only.

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.

3.4 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 who had expressed their interest in participating. 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) [(1)]
- 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) [(2-5)]

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.

3.4.1 List of participating laboratories

The twelve laboratories participating in DP4114 international collaborative study (see Table 4) were randomly selected from the 29 National Reference Laboratories (NRLs) that offered to participate.

Clear guidance was given to the selected laboratories for strictly following the validation protocol provided.

Table 4. Laboratories participating in the validation of the detection method for maize event DP4114

Laboratory	Country
Environment Agency Austria	AT
Fera Science Ltd	UK
Institute for Hygiene and Environment- Hamburg	DE
Institute for National Investigation for the Health and Veterinarian Nature Saxonia-Dresden	DE
Laboratorio Arbitral Agroalimentario	ES
Laboratory for the Detection of GMO in Food - Bad Langensalza	DE
Landeslabor Schleswig-Holstein - Food, Veterinary and Environmental Diagnostic Institute	DE
National Center of Public Health and Analyses (NCPHA), GMO Unit	BG
National Food Agency, Science Department	SE
National Food and Veterinary Risk Assessment Institute Molecular Biology and GMO	LT
National Health Laboratory, Food Control Department	LU
National Institute of Biology	SL

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: five laboratories used ABI 7500, two ABI 7900HT, one used ABI 7300, one used Roche LC480, one used Biorad CFX, one used ABI ViiA 7™ for one run and ABI 7500 for the other, and one laboratory used Stratagene Mx 3005p.

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.

3.4.3 Materials used in the international collaborative study

For the validation of the quantitative event-specific method, test samples were provided by the EURL GMFF to the participating laboratories. They were derived from:

i) genomic DNA extracted by the applicant from homogenized hemizygous maize seeds harbouring the DP4114 event, the DP-004114-3 maize event was inherited from the female parent, and

ii) genomic DNA extracted by the applicant from homogenized ground seeds of hybrid near-isogenic maize.

The control samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11[f].

The control samples were used by the EURL GMFF to prepare standards (of known GMO content) and test samples (of undisclosed GM content = blind samples) by mixing DP4114 maize DNA and non-GM maize DNA.

The calibration sample S1 was prepared by mixing the appropriate amount of DP4114 genomic DNA with control non-GM maize genomic DNA to obtain a 10 % (in copy number ratio) GM sample. Calibration samples S2-S5 were prepared by 5-fold serial dilutions from the S1 sample (Table 3).

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

- ✓ Five calibration samples with known concentrations of GM event (180 µL of DNA solution each) labelled from S1 to S5 (Table 3).
- ✓ Twenty blinded test DNA samples (90 µL of DNA solution, each at 60 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 5)

Table 5. DP4114 blinded samples GM percentage

Nominal DP4114 GM % GM copy number / maize genome copy number x 100
5.0
2.61
0.90
0.52
0.058

- ✓ Reaction reagents:
  - 2x SsoAdvanced Universal Probes Supermix, one vial: 6.4 mL
  - HPLC Molecular Biology Grade Water, one vial: 3.0 mL
  - Bovine Serum Albumin 208 µL

[f] 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).

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

  hmg taxon-specific

  - ZM1-F (QT-TAX-ZM-002) (10 µM): 384 µL
  - ZM1-R (QT-TAX-ZM-002) (10 µM): 384 µL
  - PHN149436 (10 µM): 80 µL

  DP4114

  - PHN164689 (10 µM): 384 µL
  - PHN1641690 (10 µM): 384 µL
  - PHN164691 (10 µM): 90 µL

3.4.4 Design of the collaborative study

Participating laboratories received a detailed protocol including the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the DP4114 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 DP4114 and the *hmg* in according to 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 template, 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.

3.4.5 Deviations reported from the protocol

No laboratory reported deviations from the validation protocol.

4. Results

4.1 EURL GMFF experimental testing

4.1.1 Zygosity ratio in the positive control sample

The results of the digital PCR analysis conducted by the EURL GMFF on the DP4114 and *hmg* targets to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Zygosity ratio of the DP4114 and *hmg* targets in the positive control sample.

Mean ratio (DP4114/*hmg*)	0.58
Standard deviation	0.05
RSD_r (%)	8.2
Standard error of the mean	0.01
Upper 95 % CI of the mean	0.61
Lower 95 % CI of the mean	0.56

The mean ratio (DP4114/*hmg*) is 0.58. The 95 % confidence interval (CI) spans around 0.58, the expected theoretical ratio for a maize hemizygous control sample with a GM parental contribution of female origin and a single-copy endogenous gene target; therefore, the measured mean ratio is not significantly different from the expected ratio, for an alpha = 0.05.

Hence:

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

The zygosity value obtained by the EURL GMFF is in agreement with the information received from the applicant.

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

Test samples with GM levels from 0.52 % to 5.0 % were tested twice on three different instruments with two replicates for each GM level on each plate (total of four replicates per GM level). Tests were conducted on ABI 7500 and on ABI 7900HT.

The sample at 0.058 % GM level (corresponding to 0.1 % in mass fractions of GM material) was tested in 15 replicates in ABI 7500 and on ABI 7900HT.

Additionally the method was run on Roche LC480. The five GM levels were analysed in two replicates on each plate (total of four replicates per GM level).

The corresponding standard curve parameters and the results of efficiency, linearity, trueness and precision are shown in Tables 7A, 7B, 8A, 8B and 8C.

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

Table 7A. Standard curve parameters of the real-time PCR tests carried out on ABI 7500, ABI 7900HT, and Roche LC480 for quantifying GM levels 0.52 % to 5.0 %. Slope and R^2 coefficient values were rounded to two digits.

	DP4114			***hmg***		
	Slope	PCR efficiency %*	R^2	Slope	PCR efficiency %*	R^2
Run A	-3.49	93	1.0	-3.47	94	1.0
Run B	-3.51	93	1.0	-3.52	92	1.0
Run C	-3.45	95	1.0	-3.41	96	1.0
Run D	-3.43	96	1.0	-3.40	97	1.0
Run E	-3.44	95	1.0	-3.28	102	1.0
Run F	-3.51	93	1.0	-3.38	98	1.0

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

Table 7B. Standard curve parameters of the real-time PCR tests carried out on ABI 7500, and ABI 7900HT for quantifying the GM level 0.058 % in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	DP4114			***hmg***		
	Slope	PCR efficiency %*	R^2	Slope	PCR efficiency %*	R^2
Run G	-3.46	95	1.0	-3.50	93	1.0
Run H	-3.57	91	1.0	-3.39	97	1.0

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$
Run G was carried out on ABI 7500; Run H was carried out on ABI 7900HT.

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

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

Table 8A. Values of trueness and precision as established by the EURL GMFF in its in-house verification using ABI 7500. GM percentage in copy number ratio.

Nominal GM levels %	Measured GM level %	Bias % of the target GM level	Precision (RSD_r %)
5.0	5.2	4.7	2.8
2.61	2.8	8.6	7.2
0.90	0.92	2.6	2.9
0.52	0.53	1.2	5.2
0.058	0.054	-6.7	12

Table 8B. Values of trueness and precision as established by the EURL GMFF in its in-house verification using ABI 7900HT. GM percentage in copy number ratio.

Nominal GM levels %	Measured GM level %	Bias % of the target GM level	Precision (RSD_r %)
5.0	5.1	2.6	3.3
2.61	2.5	-6.0	8.2
0.90	0.78	-13	4.9
0.52	0.46	-12	6.3
0.058	0.060	2.9	16

Table 8C. Values of trueness and precision as established by the EURL GMFF in its in-house verification using Roche LC480. GM percentage in copy number ratio.

Nominal GM levels %	Measured GM level %	Bias % of the target GM level	Precision (RSD_r %)
5.0	5.7	13	6.4
2.61	2.9	12	5.4
0.90	0.97	7.7	3.6
0.52	0.57	8.9	8.7
0.058	0.072	24	7.2

4.2 Results of the international collaborative study

4.2.1 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 shown in Table 9. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

Efficiency (%) = (10 * (-1/slope)) – 1) x 100

Table 9. 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.

		DP4114			***hmg***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.57	91	0.99	-3.66	87	1.00
	B	-3.49	93	0.99	-3.59	90	1.00
2	A	-3.55	91	1.00	-3.54	92	1.00
	B	-3.67	87	1.00	-3.12	109	0.89
3	A	-3.90	81	0.99	-3.74	85	1.00
	B	-3.77	84	0.99	-3.70	86	1.00
4	A	-3.49	94	0.99	-3.56	91	1.00
	B	-3.69	87	0.99	-3.59	90	0.98
5	A	-3.78	84	1.00	-3.50	93	1.00
	B	-3.68	87	1.00	-3.64	88	1.00
6	A	-3.78	84	1.00	-3.65	88	1.00
	B	-3.69	87	0.99	-3.66	87	1.00
7	A	-3.59	90	1.00	-3.56	91	1.00
	B	-3.61	89	1.00	-3.50	93	1.00
8	A	-3.68	87	1.00	-3.69	87	1.00
	B	-3.77	84	1.00	-3.66	88	1.00
9	A	-3.83	83	1.00	-3.68	87	1.00
	B	-3.73	85	1.00	-3.69	87	1.00
10	A	-3.92	80	0.99	-3.82	83	1.00
	B	-3.86	82	1.00	-3.81	83	1.00
11	A	-3.88	81	0.99	-3.73	85	1.00
	B	-3.74	85	1.00	-3.67	87	1.00
12	A	-3.64	88	1.00	-3.64	88	1.00
	B	-3.75	85	1.00	-3.64	88	1.00
	Mean	-3.71	86	1.00	-3.63	89	0.99

Table 9 indicates that the efficiency of amplification for DP4114 ranges from 80 % to 94 % and the R^2 from 0.99 to 1.00; the amplification efficiency for *hmg* ranges from 83 % to 109 % and the R^2 from 0.99 to 1.00. The mean PCR efficiency was 86 % for DP4114 and 89 % for *hmg*. The average R^2 was 1.00 and 0.99 for DP4114 and *hmg*, respectively. PCR efficiency was slightly lower than the ENGL acceptance criteria both for DP4114 and *hmg*, while the R^2 values were within the ENGL acceptance criteria; however, the correctness of the quantification is not affected by this sub-optimal PCR efficiency, apart from the lower end of the quantification range (see Section 4.2.3).

4.2.2 GMO quantification

Table 10 reports the values of quantification for the four replicates of each GM level as reported by each of the twelve participating laboratories.

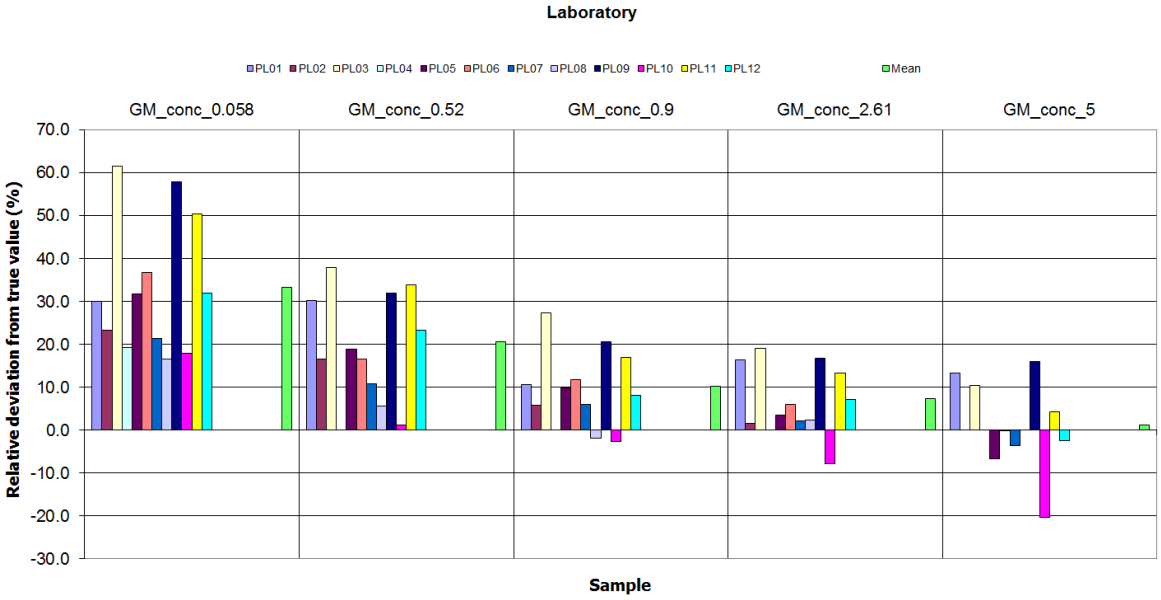
Table 10. GM % values determined by laboratories for test samples

	GMO content (%)																			
	0.058				0.52				0.90				2.61				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	0.07	0.07	0.08	0.08	0.75	0.63	0.67	0.66	0.97	0.90	1.01	1.10	3.02	2.72	3.09	3.30	5.78	5.30	5.71	5.83
2	0.06	0.07	0.08	0.08	0.65	0.52	0.64	0.61	0.83	0.91	1.02	1.05	2.43	2.36	3.00	2.80	3.86	4.24	5.29	5.71
3	0.09	0.09	0.09	0.10	0.74	0.67	0.68	0.78	1.10	1.14	1.16	1.18	3.19	3.11	3.15	2.97	5.27	5.79	5.57	5.42
4	0.06	0.08	0.07	0.07	0.79	0.55	0.47	0.64	0.67	0.55	1.14	1.12	1.82	2.41	3.27	2.59	4.35	4.56	6.33	6.95
5	0.07	0.07	0.09	0.07	0.60	0.64	0.60	0.64	0.87	1.01	1.05	1.02	2.75	2.65	2.89	2.51	4.49	4.53	4.83	4.81
6	0.08	0.08	0.09	0.07	0.62	0.60	0.64	0.57	1.02	0.98	1.06	0.96	2.98	2.78	2.65	2.66	5.07	5.08	4.85	4.98
7	0.08	0.07	0.07	0.06	0.56	0.60	0.61	0.53	1.01	0.90	0.99	0.92	2.66	2.63	2.66	2.70	4.55	4.83	5.11	4.76
8	0.07	0.07	0.06	0.06	0.44	0.62	0.58	0.56	0.89	0.91	0.91	0.83	2.73	2.77	2.68	2.50	4.00	3.93	5.00	3.78
9	0.09	0.09	0.10	0.09	0.70	0.64	0.69	0.71	1.06	1.14	1.04	1.10	3.12	3.36	2.77	2.92	5.81	6.01	5.67	5.68
10	0.07	0.06	0.07	0.07	0.50	0.60	0.51	0.50	0.83	0.79	0.96	0.92	2.48	2.24	2.44	2.45	3.95	3.95	4.24	3.76
11	0.08	0.10	0.10	0.07	0.72	0.65	0.71	0.70	1.00	0.99	1.14	1.08	3.00	2.87	3.10	2.84	5.15	5.35	4.94	5.41
12	0.08	0.09	0.07	0.07	0.59	0.67	0.63	0.68	1.02	0.96	0.93	0.98	2.87	2.68	2.97	2.67	5.12	4.67	4.94	4.77

* GMO % = (GMO copy number/maize genome copy number) x 100
n.a. not available

A graphical representation of the data reported in Table 10 is provided in Figure 1 showing the relative deviation from the true value for each GM level tested for the participating laboratories. 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 after eliminating outliers (PL4 for GM levels 0.52 %, 0.9 %, 2.61 % and 5.0 %; for the latter level, PL2 and PL8 were also eliminated as outliers). Outliers were identified by performing Cochran and Grubbs tests.

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



* For PL4, levels 0.52 %, 0.9 %, 2.6 % and 5.0 %, the corresponding histograms are not shown because the laboratory was eliminated as outlier. Similarly, level 5 % for PL2 and PL8 was eliminated as outlier. PL = participating laboratory.

Overall, the mean relative deviations from the true values were within a maximum of ± 25 % for most laboratories for all levels except at 0.058 %. At GM level 0.058 % five laboratories out of 12 showed a bias within the limit of 25 %; seven and ten laboratories were within the ENGL limit, respectively at GM level 0.52 % and 0.9 %; eleven and nine laboratories were within the limit, respectively at GM level 2.6 % and 5.0 %. Seven laboratories overestimated GM level 0.058 % by more than 25 %; similarly, four laboratories overestimated GM level 0.52 % and one overestimated GM level 0.9 % by more than 25 %. Overall a trend can be observed for overestimation of the GM content for almost all laboratories at all GM levels. The overestimation increased progressively with the decreasing GM concentration in the sample.

4.2.3 Method performance requirements

Among the performance requirements established by ENGL and adopted by the EURL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), repeatability and reproducibility are assessed through an international collaborative trial. Table 11 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study (see Table 4 for a list of the participant laboratories).

According to the ENGL method performance requirements, the relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 35 % at the target concentration and over the majority of the dynamic range, while it should be below 50 % at the lower end of the dynamic range.

As it can be observed in Table 11, the method satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R (%) is 15 % at the 0.058 % GM level, thus within the acceptance criterion.

Table 11. Summary of validation results for the DP4114 method expressed as GM copy numbers in relation to target taxon copy numbers. Standard deviation values and absolute bias values are rounded to three digits.

	Test Sample nominal GMO %				
	0.058	**0.52**	**0.90**	**2.6**	**5.0**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	1	1	1	3
Reason for exclusion	-	C*	C*	C*	3C*
Mean value	0.077	0.63	0.99	2.8	5.1
Relative repeatability standard deviation, RSD_r (%)	10	7.3	6.5	6.2	3.9
Repeatability standard deviation	0.008	0.046	0.064	0.17	0.20
Relative reproducibility standard deviation, RSD_R (%)	15	12	9.9	9.4	12
Reproducibility standard deviation	0.011	0.074	0.098	0.26	0.59
Bias** (absolute value)	0.019	0.11	0.091	0.19	0.056
Bias (%)	33	21	10	7.2	1.1

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

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

Table 11 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 in its in-house testing 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 10 % at the 0.058 % 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. In this case, the method satisfies this requirement across the tested dynamic range, including the 0.9 % GM level (legal threshold for labelling of adventitious presence of GM material according to Regulation (EC) No 1829/2003); however, the method overestimates the GM content at the 0.058 % level with a bias of 33 %.

5. Compliance of the method for detection and quantification of event DP4114 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 12:

- at step 2 of the validation process (scientific assessment of the dossier), the EURL GMFF acknowledged that the RSD_r % value at the 0.09 % level shown by the applicant's dossier (expressed as mass fraction of GM-material) was 16 %, hence below the maximum value of 25 % required by the ENGL. The value was calculated on three real-time PCR runs each with 14-15 replicates (Table 2). 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 0.058 % in terms of copy number ratio corresponding to 0.1 % in mass fraction of GM-material. The experiments were carried out under repeatability conditions on fifteen replicates. The RSD_r resulted to range between 12 % and 16 % (Table 8A and 8B) 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 0.058 % in terms of copy number ratio, corresponding to 0.1 % related to mass fraction of GM-material, the RSD_r of the method was 10 %, lower than 25 % and therefore respecting the ENGL method performance requirements.

The outcome of the different steps is summarised in Table 12.

Table 12. Precision of the event-specific method for quantitative detection of DP4114 at or around 0.1 % level related to mass fractions of GM material.

Source	**RSD_r %**	**GM %**
Applicant's method optimisation	16 %	0.09 %
EURL GMFF tests	12-16 %	0.1 %
Collaborative study	10 %	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 requirements laid down in Regulation (EU) No 619/2011.

6. Conclusion

A method for detection, identification and quantification of GM event DP4114 was provided by the applicant. The method has been fully validated in accordance to the EURL GMFF validation scheme, respecting the requirements of the relevant EU legislation and international standards for method validation.

This validation study confirms that the method is applicable to the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex III-3.C.2 to Commission Regulation (EU) No 503/2013 and (EU) No 619/2011 and meets most of the method performance requirements established by the ENGL and the EURL GMFF down to the 0.52 % GM level in copy number ratio.

At the lowest level of the dynamic range, 0.058 % in copy ratio (corresponding to 0.1 % in mass fractions of GM DNA) the method shows a positive bias of 33 %, but at that low GM level this is deemed acceptable for the following reasons: a) the positive bias would determine an overestimation of the GM content and not an underestimation which would instead cause a problem b) the bias is not significant as the true value falls within the 95 % CI around the mean (0.077 % ± 0.022).

The method is therefore valid to be used for regulatory purposes, including the quantification of low level presence [corresponding to 0.1 % (mass/mass)] of the GM event. It can be assumed that it is applicable to any appropriately extracted maize genomic DNA.

Regarding the method performance requirements that were not met by the results of the collaborative study, i.e. trueness at the 0.058 %, the EURL GMFF, in collaboration with the European Network of GMO Laboratories (ENGL), will conduct a monitoring by asking laboratories using the method for the purpose of Regulation (EU) No 619/2011 to provide their experimental data to the EURL GMFF in order to clarify if the found minor deviations are confirmed in practice and are of any significance.

The validated method is described in detail as "Validated Method" at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1.

7. References

1. Horwitz W. Protocol for the design, conduct and interpretation of method- performance studies, *Pure and Appl. Chem.* 1995; 67: 331-343.
2. International Standard (ISO) 5725-1, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 1: General principles and definitions. International Organization for Standardization, Genève, Switzerland.
3. ISO 5725-1:1994/Cor 1:1998.
4. International Standard (ISO) 5725-2, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method. International Organization for Standardization, Genève, Switzerland.
5. ISO 5725-2:1994/Cor 1:2002.
6. Plant DNA C-values Database, http://data.kew.org/cvalues/

Annex 1: Event-specific Method for the Quantification of maize DP-ØØ4114-3 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 DP4114 (unique identifier DP-ØØ4114-3) genomic DNA to total maize genomic 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 DP4114, an 80 bp fragment spanning the junction between the 5' genomic region and the transgenic insert DP4114 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled FAM (6-carboxyfluorescein) as reporter dye at its 5' end and BHQ (Black Hole quencher) at its 3' end.

For the relative quantification of maize GM event DP4114 DNA, a maize taxon-specific reference system amplifies a 79 bp fragment of a maize *high mobility group* (*hmg*) endogenous gene (GeneBank accession number AJ131373.1), using *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM as reporter dye at its 5' end and Minor Groove Binding (MGB) 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 DP4114 genomic DNA in a test sample, Cq values for the DP4114 and the *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DP4114 genomic DNA to total maize genomic DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from genetically modified and conventional maize seeds. 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 October 2016.

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 0.01 % (related to mass fraction of GM material) 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 0.09 % (related to mass fraction of GM material) in 300 ng of total suitable maize DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.058 % calculated in terms of GM copy number ratio, corresponding to 0.1 % mass fraction of GM-material.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the junction between the 5' genomic region and the transgenic insert DP4114 and is therefore event-specific for the event DP4114. This was confirmed by bioinformatics analyses.

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).

- 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 DP4114

3.2.1 General

The real-time PCR set-up for the taxon (*hmg*) and the GMO (event DP4114) 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 DP4114) and the taxon (*hmg*) assay with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curves are established on at least five samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % (relative to copy number fractions) maize DP4114 genomic DNA in a total of 325 ng of maize genomic DNA (corresponding to 119048 maize genome copies with one haploid genome assumed to correspond to 2.73 pg of maize genomic DNA) [(1)]. Standards S2 to S5 are to be prepared by serial dilutions (dilution factor 5.0) according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	**S1**	**S2**	**S3**	**S4**	**S5**
Total amount of maize DNA in reaction (ng) *	325	65	13	2.6	0.52
hmg copies	119048	23810	4762	952	190
DP4114 copies	11905	2381	476	95	19

* 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 DP4114 maize specific system (Table 2) and the *hmg* reference gene system (Table 3). Please note that additional volume is included in the total to cover pipetting variability due to the viscosity of the solution.

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the DP4114 event-specific assay.

Component	**Final concentration**	**µL/reaction**
SsoAdvanced Universal Probe Supermix (2x)	1x	10
PHN164689 (10 µM)	600 nM	1.20
PHN1641690 (10 µM)	600 nM	1.20
PHN164691 (10 µM)	140 nM	0.28
BSA (Bovine Serum Albumin)	0.081 %	0.324
HPLC Molecular Biology Grade Water	/	1.996
(DNA)	/	(5.0)
Total volume		20 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* taxon-specific assay.

Component	**Final concentration**	**µL/reaction**
SsoAdvanced Universal Probe Supermix (2x)	1x	10
ZM1-F (10 µM)	600 nM	1.20
ZM1-R (10 µM)	600 nM	1.20
PHN149436 (10 µM)	120 nM	0.24
BSA	0.081 %	0.324
HPLC Molecular Biology Grade Water	/	2.036
(DNA)	/	(5.0)
Total volume		20 µL

3. Mix well and centrifuge briefly.
4. Label two 0.5 mL reaction tubes (one for the DP4114 and one for the *hmg* system) for each DNA sample to be tested (standard curve samples, blind samples and the C0 amplification reagent control).
5. Add to each reaction tube the amount of reaction mix for 3.5 PCR repetitions (52.5 µL for the *hmg* system and 52.5 µL for the DP4114 maize system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (17.5 µL DNA). The additional 0.5 repetition included will ensure adequate volume when loading the samples.
6. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
7. Spin down the tubes. Aliquot 20 µL for the DP4114 system and for the *hmg* reference system in each well.
8. Place an optical cover on the reaction plate and briefly centrifuge the plate.
9. 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.
10. Select FAM as reporter dye for the DP4114 and FAM for the *hmg* reference system. Define BHQ as quencher dye for DP4114 specific system and MGB for *hmg* reference system. Select ROX as the passive reference dye. Enter the correct reaction volume (20 µL).
11. Run the PCR with the cycling program described in Table 4. Users of Roche LC480 real-time PCR instrument should set the instrument to 45 cycles, instead of 40, and use the settings of the ramp rate as indicated at the important note below.

Table 4. Cycling program for DP4114/*hmg* assays.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	Initial Enzyme Activation		95°C	180	No	1
3	Amplification	Denaturation	95°C	15	No	40x*
		Annealing & Extension	60°C	30	Yes	

** see point 11 above for Roche LC480 instruments*

Note:
Applied Biosystems 7900HT users: uncheck the 9600 emulation mode.
Roche LC480 users: ramp rates for the Roche LC480 are: 2.6°C/second Initial Enzyme Activation and Denaturation, and 1.6°C/second for Annealing and Extension.
According to the applicant users of ViiA7™ should select the default ramp rate; the EURL GMFF did not test the method on this instrument

3.3 Data analysis

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

a) Analyse data: automatic baseline and threshold settings have given the best results at the EURL GMFF. For users of Roche LC480 instruments, it is recommended that 45 cycles are run and the analysis is carried out with the Absolute Quantification/Second Derivative Maximum method with High Confidence algorithm.

b) 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, the instrument's software calculates the Cq values for each reaction.

The standard curves are generated both for the *hmg* and the DP4114 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 DP4114 DNA in the unknown sample, the DP4114 copy number is divided by the copy number of the maize endogenous gene *hmg* and multiplied by 100 % (GM % = DP4114 / *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
- Micropipettes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL, 1.5mL and 2.0 mL reaction tubes, centrifuge for 96-Well reaction plates

- Vortex
- Racks for reaction tubes, also cooled
- 0.5, 1.5 mL or 2 mL DNAse free reaction tubes

4.2 Reagents

- SsoAdvanced™ Universal Probes Supermix, Biorad Catalogue No 1725281.
- Bovine Serum Albumin (BSA), Ambion Catalogue No AM2618
- Nuclease free water

4.3 Primers and Probes

Table 5. Primers and probes for the DP4114 and *hmg* methods

	Name	DNA Sequence (5' to 3')	Length (nt)
DP4114			
Forward primer	PHN164689	gCT TTg gAg CCT CTC gTT TgT A	22
Reverse primer	PHN1641690	gCg TTT AAA CTA TCA gAT CTg TgT TgA A	28
Probe	PHN164691	6'FAM-CAC TTg CAC gTA gTT ACC Cgg ACC gAA-BHQ*	27
hmg			
Forward primer	ZM1-F	TTg gAC TAg AAA TCT CgT gCT gA	23
Reverse primer	ZM1-R	gCT ACA TAg ggA gCC TTg TCC T	22
Probe	PHN149436	6'FAM-CAA TCC ACA CAA ACg C-MGB**	16

* Black Hole Quencher
** Minor Groove Binding (MGB) quencher

5. References

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

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