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Event-specific Method for the Quantification of Soybean GMB151 Using Real-time PCR

Validation Report

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Genetically Modified Food and Feed

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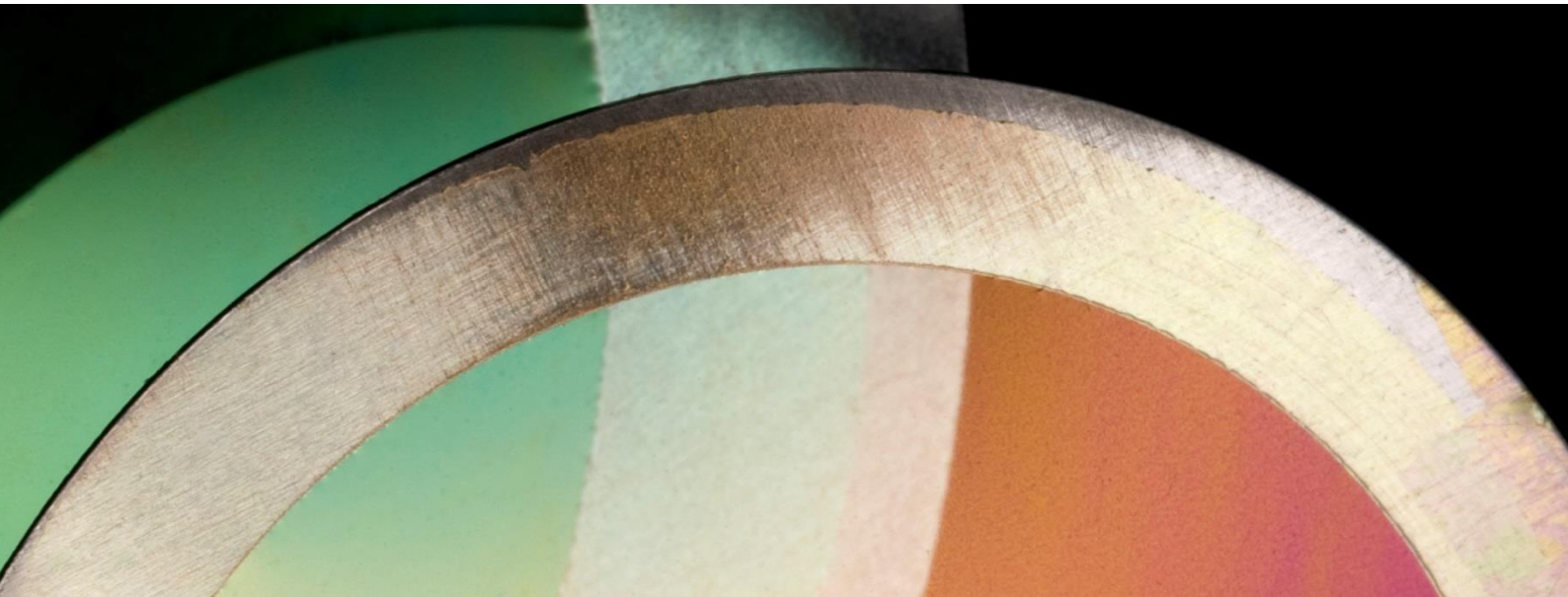

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EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers & Reference Materials
Food and Feed Compliance

Event-specific Method for the Quantification of Soybean GMB151 Using Real-time PCR

Validation Report

5 June 2020

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate [1] the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), validated an event-specific real-time polymerase chain reaction (qPCR) method for detecting and quantifying soybean event GMB151 (unique identifier BCS-GM151-6). The validation study was conducted according to the EURL GMFF validation procedure (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and the relevant internationally accepted guidelines [2-6].

In accordance with current EU legislation [7], Bayer CropScience provided the detection method and the positive and negative control samples (genomic DNA from leaves of GMB151 soybean as positive control DNA, and genomic DNA from leaves of conventional soybean 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 soybean haploid genome copies, equivalent to mass fractions of GM DNA), organised an international collaborative study and analysed the results.

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

Content

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: Belac 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) by DNA extraction, DNA identification and real Time PCR in food and feed) 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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1. Introduction

In line with Regulation (EC) No 1829/2003 [1], Bayer CropScience provided the EURL GMFF with an event-specific method for detection and quantification of soybean event GMB151 (unique identifier BCS-GM151-6) 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 soybean GMB151, appropriately extracted from food or feed, down to a GM content level of 0.1 % m/m.

The preparation of the report (step 5) was aligned with the timeline communicated by EFSA for its risk assessment.

2. Dossier reception and acceptance (step 1)

Bayer CropScience submitted the identification and quantification method, data demonstrating its adequate performance when applied to genomic DNA extracted from soybean GMB151 GM event and from non-GM soybean, and the corresponding positive and negative control DNA samples.

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

3. Scientific assessment and bioinformatics analysis (step 2)

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

The specificity of the event-specific assay was verified by the applicant and confirmed by the EURL GMFF by means of bioinformatics analysis, based on the sequence data provided by the applicant.

3.1. Specificity assessment conducted by the applicant

The specificity of the event-specific assay was assessed by the applicant in duplicate real-time PCR reactions, according to the method described in Annex 1 (Tables 2, 3 and 4), using 100 copies of genomic DNA of the target DNA (GMB151) and 2500 copies of the following non-target DNA

samples: rice LLRICE62; oilseed rape MS1, MS8, MS11, RF1, RF2, RF3, Topas19/2, T45, OXY-235, RT73, MON 88302, event 73496; soybean A2704-12, A5547-127, GMB471, GTS 40-3-2, MON 89788, MON87701, MON 87708, DP-356043-5, DP-305423-1, CV127, MON 87769, MON 87705, DAS-68416-4, DAS-81419-2, DAS-44406-6, FG72; maize T25, BT176, BT11, MON 810, GA21, NK603, MON 863, TC1507, event 3272, MIR604, MIR162, 59122, event 98140, MON 88017, MON 89034, MON 87460, MON 87427, DAS-40278-9; cotton LLCOTTON25, T304-40, GHB614, GHB119, GHB811, COT102, MON 1445, MON 531, MON 15985, MON 88913, 281-24-236x3006-210-23; conventional oilseed rape, cotton, rice and maize. According to the method developer the GMB151 assay did not react with any sample except the positive control. All non-target DNA samples were amplifiable when tested with respective taxon-specific reference methods.

In addition, the applicant performed an *in-silico* specificity analysis by using the amplicon sequence of GMB151 as a query for Basic Local Alignment Search Tool (BLASTN 2.2.28+) algorithm against public sequences of National Center for Biotechnology Information (NCBI) GenBank and patent databases. The applicant concluded that the results of the *in silico* BLASTN sequence similarity search with Patent and Genbank databases showed that the amplicon of the GMB151 soybean system reaction is unique.
A previously validated soybean-specific PCR method (https://gmo-crl.jrc.ec.europa.eu/summaries/A5547_validated%20Method.pdf), which amplifies a 102 base pair (bp) fragment of the *Lectin1* (*Le1*) gene of *Glycine max*, was used as a reference method. The specificity of the taxon-specific assay was assessed by the applicant in duplicate real-time PCR reactions, according to the method described (Tables 2, 3 and 4), using 200 ng genomic DNA extracted from conventional oilseed rape, rice, soybean, cotton and maize. According to the method developer the *Le1* assay did not react with any sample except the positive control.

3.2. Specificity assessment conducted by the EURL GMFF

The detection method spans the 5' insert-to-plant junction in GMB151 soybean. The forward primer "PRIM1040" binds to the 5' soybean (*Glycine max*) genomic border adjacent to the insertion site. The reverse primer "PRIM1041" binding site was found in the insert. The probe "TM1789" binds mostly to the insert close to the junction at the 5' genomic region of GMB151, with the two bases on the 5' end of the probe binding to the sequence in the genomic region. The amplicon size is expected to be 84 bp, consistent with what is reported by the applicant.

The sequence of the amplicon was analysed by BLAST (NCBI) against local copies of the "nt" and "patents" databases, and no significant similarity was found with any other published sequence. In addition, the primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System of the JRC, as well as the whole genomes of more than 80 plants (including *Brassica rapa, Glycine max, Oryza sativa, Solanum lycopersicum and Zea mays*) using the e-PCR prediction tool (NCBI), and no potential amplicon was identified.

3.3. Verification of the ENGL acceptance parameters

The applicant prepared the calibration curve from a DNA solution (S1) of 10 % soybean event GMB151 genomic DNA (expressed as mass fraction of GM material) which was diluted 1:3 in water to prepare sample S2; S2 was diluted 1:5 to prepare sample S3; S3 was diluted 1:4 to prepare sample S4 which was diluted approximately 1:11 to prepare sample S5. The parameters (slope, R^2 coefficient) of six 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

GMB151		***Le1***	
Slope	**R^2**	**Slope**	**R^2**
-3.5	0.9995	-3.5	0.9995
-3.4	0.9999	-3.5	0.9997
-3.5	0.9998	-3.5	0.9993
-3.6	0.9987	-3.4	0.9994
-3.5	0.9998	-3.5	0.9992
-3.5	0.9999	-3.5	0.9999

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98.

Table 1 indicates that the slope and R^2 coefficient of the standard curves for the GM-system (GMB151) and the soybean-specific *Lectin* 1 (*Le1*) system, as established by the applicant, were within the ENGL acceptance criteria.

Precision and trueness of the method were established by the applicant and 18 values for each of four GM levels (expressed as mass fraction of GM-DNA) 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

	Test results			
Expected GM %	**0.08**	**0.90**	**2.00**	**4.50**
Measured mean GM %	0.084	0.92	2.05	4.38
Precision (RSD_r %)	7.7	6.2	7.9	7.1
Trueness (bias %)	4.7	1.9	2.7	-2.6

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

The method met the ENGL acceptance criteria for trueness and precision at the lowest GM level i.e. 0.08 %. 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 GMB151 event specific and the soybean reference real-time PCR methods was assessed by the applicant in 60 PCR replicates at 10, 5 and 1 haploid genome copies per reaction of GMB151 event and of soybean reference DNA. The LOD_{abs} was found to be below 10 haploid genome copies for GMB151 event-specific method and below 10 haploid genome copies for *Le1* reference method. The relative LOD (LOD_{rel}) of the combined method was assessed by the applicant in 18 PCR replicates and it was found to be at least 0.03 % (related to mass fraction of GM material) in 200 ng of total soybean DNA per reaction. The LOD_{abs} and LOD_{rel} were 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 with eight combinations of the following variations compared to the original method: enzyme mix concentration: +5 %/-5 %; primers concentration: +10 %/-10 %; probe concentration: +10 %/-10 %; reaction volume: +1 µL/-1 µL; annealing temperature: +/-1 °C. The RSD_r and the trueness calculated for each combination of variations on a sample at the LOQ level (0.08 %) did not exceed 30 %, thus meeting the ENGL acceptance criteria.

Precision and trueness of the method were tested in a transferability study: 18 values for each of five GM levels, namely 0.08 %, 0.4 %, 0.9 %, 2.0 %, 4.5 %, (expressed as mass fraction of GM material) were provided. Table 2B reports precision and trueness values for the five GM-levels as provided by a laboratory different from the method developer. Both parameters were within the ENGL acceptance criteria (trueness ≤ 25 %, RSDr ≤ 25 % across the entire dynamic range).

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

	Test results				
Expected GM %	**0.08**	**0.40**	**0.90**	**2.00**	**4.50**
Measured mean GM %	0.08	0.39	0.87	2.02	4.90
Precision (RSD_r %)	18.2	8.9	11.4	10.4	10.3
Trueness (bias %)	-2.9	-3.1	-2.9	1.2	8.9

3.4. DNA extraction

Genomic DNA was isolated from soybean leaves, using a "Dellaporta-derived" protocol previously submitted for detection of soybean event A2704-12, already validated in-house by the EURL GMFF on soybean seeds. The protocol for DNA extraction and a validation report are published at https://gmo-crl.jrc.ec.europa.eu/summaries/A2704-12_soybean_DNAExtr_report.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 leaves from the soybean event GMB151.

In agreement with the ENGL position, endorsing the modularity principle (see also Annex III to Reg. (EU) No 503/2013), the EURL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for soybean event GMB151.

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 submitted method for DNA extraction is suitable for the isolation of genomic DNA from a wide variety of matrices (e.g. soybean seed, grain and other soybean tissues). The suitability of isolated DNA as an analyte for PCR based detection of GMOs will depend on the quality, purity, and quantity of the DNA. Although the DNA extraction method can be applied to different food and feed matrices, the application of the method to certain complex and difficult processed matrices may require adaptation. In fact, food processes can influence the quality and intactness of the DNA contained in the final processed products [10-13]. Other challenges of working with processed food and feed matrices is the presence of PCR inhibitors, which can reduce the efficiency and/or reproducibility of PCR and thus may contribute to inaccurate PCR results [5,6]. Therefore, DNA extraction from certain of these processed matrices may require additional rounds of purification in order to achieve the quality standards needed for quantitative real-time PCR [14,15]."*

Whenever DNA is extracted from more complex and difficult matrices, a thorough control of the quality of the DNA is recommended in order to ensure that it has the required quality for subsequent PCR analysis.

4. Materials and method

4.1. Samples

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

- genomic DNA extracted by the applicant from homozygous soybean leaves harbouring the GMB151 event, and
- genomic DNA extracted by the applicant from conventional soybean leaves genetically similar to those harbouring the GMB151 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 GMB151 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean specific assay targeting the endogenous gene *Lectin 1* (*Le1*) and the GM target assay for GMB151 are run 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 GMB151, a 84 bp fragment of the region spanning the 5' insert-to-plant junction in soybean GMB151 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-1 (Black Hole Quencher® 1) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event GMB151, a soybean taxon-specific system amplifies a 102 bp fragment of a soybean *Lectin 1* (*Le1*) endogenous gene, using *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with JOE 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 GMB151 and the *Le1* 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 GMB151 DNA in a test sample, the GMB151 copy number is divided by the copy number of the soybean haploid genome and multiplied by 100 to obtain the percentage value (GM % = GMB151 copies/ soybean haploid genome copies x 100)

[a] Control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample).

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 soybean genome (1.13 pg) [16]. The copy number values used in the quantification, the GMO contents of the calibration samples, and the total DNA quantity used in the PCR reactions are listed in Table 3.

Note: Numerical values presented in the following tables were rounded keeping two digits for values ≤ 1, one digit for values between 1 and 10 and no digit for values ≥ 10, unless otherwise 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 of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of soybean DNA in the reaction (ng)	300	100	20	5	0.5
Target taxon [haploid genome copies]	265487	88496	17699	4425	442
Target GMB151 copies	26549	8850	1770	442	44

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 Reg. (EU) No 619/2011 [8].

The copy number of the GMB151 and of the *Le1* 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 (Applied Biosystems, Cat. number 4318157), 1X GE sample loading reagent (Fluidigm PN 85000746), primers and probes at concentrations indicated in the corresponding validated method (PRIM1040 and PRIM1041 primers at 400 nM each, TM1789 probe at 200 nM; KVM164 and KVM165 primers at 200 nM each, TM1242 probe at 200 nM]), and 1 µL of DNA at a concentration of 1.5 ng/µL; the DNA concentration was chosen in order to avoid panel saturation (optimal between 200<positive partitions<700).

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 each for the GM target and 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 15 to 35.

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' [17].

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 (4.5 % - 0.1 %, 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 GMB151 soybean DNA and non-GM soybean DNA.

Table 4. GMB151 blinded samples GM % contents

GMB151 GM % GM copy number/soybean haploid genome copy number x 100
4.5
2.0
0.9
0.5
0.1

The calibration sample S1 was prepared from the genomic DNA provided by the applicant by mixing the appropriate amount of GMB151 DNA with control non-GM soybean DNA to obtain a 10 % (in copy number ratio related to haploid genome copies) GM sample. Calibration samples S2-S5 were prepared by serial dilutions from the S1 sample (see Table 3).

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

Test samples with GM levels 4.5 %, 2.0 %, 0.9 % and 0.5 % were tested in two real-time PCR runs with two replicates for each GM-level on each plate (total of four replicates per GM-level). The test sample with GM level 0.1 % was tested in 15 replicates in an additional run for each platform. Average values of the slope and of the R^2 coefficient of the standard curves and method trueness

and precision over the dynamic range were evaluated against the ENGL method acceptance criteria.

4.4. International collaborative study (step 4)

The international collaborative trial involved twelve randomly selected laboratories, all being "national reference laboratories, assisting the EURL GMFF for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 120/2014 [18] who had expressed their interest in participation. The study was carried out in accordance with the following internationally accepted guidelines:

> The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) [2]
> ISO 5725 "Accuracy (trueness and precision) of measurement methods and results", Part 1 and Part 2 (ISO, 1994); ISO 5725-1:1994/Cor 1 (ISO 1998) and ISO 5725-2:1994/Cor 1 (ISO, 2002) [3-6]

The objective of the international collaborative study was to verify in experienced laboratories the trueness and precision of the PCR analytical method provided by the applicant and verified in-house by the EURL GMFF.

4.4.1. List of participating laboratories

The twelve laboratories participating in the GMB151 international collaborative study were randomly selected from 28 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 soybean event GMB151

Laboratory	**Country**
AGES -Austrian Agency for Health and Food Safety	AT
Departamento de OGM/Técnicas Biomoleculares Laboratorio Arbitral Agroalimentario	ES
Environment Agency Austria	AT
Federal Office of Consumer Protection and Food Safety- Berlin	DE
Italian National Institute of Health (ISS) Food Safety, Nutrition and Veterinary Public Health	IT
National Centre for Food, Spanish Agency for Consumer Affairs, Food Safety and Nutrition	ES
National Food Chain Safety Office, Food and Feed Safety Directorate, Food Microbiology	HU
National Health Laboratory	LU
National Institute of Biology	SI
National Veterinary Research Institute, Department of Hygiene of Animal Feedingstuff	PL
Plant Breeding and Acclimatization Institute – National Research Institute, GMO Control	PL
Walloon Agricultural Research Centre - Department Valorization des productions	BE

4.4.2. Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: six laboratories used ABI 7500, two used BioRad CFX96, one used ABI 7900HT, one used Roche LC480 II, one used Roche LC 480 and one a QuantStudio 5.

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

4.4.3. Materials used in the international collaborative study

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

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

- ✓ Five calibration samples with known concentrations of GM-event (175 µL of DNA solution each) labelled from S1 to S5 (Table 3).

- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution, each at 40 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 4)

- ✓ Reaction reagents:
  - TaqMan® Universal PCR Master Mix (2x), one vial: 8.0 mL
  - distilled sterile water, one vial: 4.0 mL

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

  Le1 taxon-specific assay
  - KVM164 primer (10 µM): 160 µL
  - KVM165 primer (10 µM): 160 µL
  - TM1242 probe (10 µM): 160 µL

  GMB151 assay
  - PRIM1040 primer (10 µM): 320 µL
  - PRIM1041 primer (10 µM): 320 µL
  - TM1789 probe (10 µM): 160 µL

4.4.4. Design of the collaborative study

Participating laboratories received a detailed validation protocol that included the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the GMB151 event-specific system and for the *Le1* taxon-specific system. In total, two plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for the soybean event GMB151 and the *Le1* assay in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per pre-determined plate layout.

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

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

4.4.5. Deviations reported from the protocol

Eleven laboratories reported no deviations from the validation protocol. One laboratory did 45 cycles of amplification instead of 40 cycles, in order to apply the 2nd Derivative Maximum Analysis method with the Roche LC480II system.

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 GMB151 and *Le1* targets to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Zygosity ratio of the GMB151 and *Le1* targets in the positive control sample.

Mean ratio (GMB151/*Le1*)	0.95
Standard deviation	0.16
RSD_r (%)	17
Standard error of the mean	0.036
Upper 95 % CI of the mean	1.03
Lower 95 % CI of the mean	0.88

The mean ratio (GMB151/*Le1*) equals 0.95. The 95 % confidence interval (CI) spans around 1.0, the expected ratio for a soybean control sample, homozygous for the GM-locus and assuming 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:

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

Thus, given the zigosity ratio of the positive control sample, the dynamic range over which the GMB151 method performance has been investigated (from 4.5 % to 0.1 %) is equally expressed in copy number ratio related to haploid genome copies or in mass fractions of GM DNA.

Note: the zygosity ratio herein reported is valid for the positive control sample 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 Reg. (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 4.5 % to 0.5 % 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 0.1 % GM-level was tested for its precision in quantification in 15 replicates in separate runs.

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

The standard curve parameters and the results of efficiency, linearity, trueness and precision obtained in the three real-time PCR equipment 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 $\geq$ 0.98. Table 7A and 7B document that the slopes of the standard curves and the R^2 coefficients were within the limits established by the ENGL. The EURL GMFF in-house results confirm the data provided by the applicant.

Table 7A. Standard curve parameters of the real-time PCR tests, carried out on ABI 7900HT, ABI 7500 and Roche LC480 to quantify GM-levels in the range 4.5 % to 0.5 % in four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	GMB151			***Le1***		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.39	97	1.00	-3.34	99	1.00
Run B	-3.38	98	1.00	-3.33	100	1.00
Run C	-3.44	95	1.00	-3.30	101	1.00
Run D	-3.45	95	1.00	-3.35	99	1.00
Run E	-3.46	94	1.00	-3.40	97	1.00
Run F	-3.44	95	1.00	-3.42	96	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$
Runs A-B were carried out on ABI 7900; runs C-D were carried out on ABI 7500; 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 7900HT, ABI 7500 and Roche LC480] to quantify the GM-level 0.1 % in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	GMB151			***Le1***		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run G	-3.42	96	1.00	-3.36	98	1.00
Run H	-3.42	96	1.00	-3.34	99	1.00
Run I	-3.49	93	1.00	-3.44	95	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$
Run G was carried out on ABI 7900HT; run H was carried out on ABI 7500; 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 7900 HT. GM % in copy/copy haploid genomes, equivalent to mass fractions of GM DNA.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
4.5	4.3	-4.6	1.0
2.0	1.9	-4.5	5.1
0.90	0.88	-2.7	3.8
0.50	0.47	-6.9	1.4
0.10	0.10	0.21	12

Table 9. 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, equivalent to mass fractions of GM DNA.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
4.5	4.6	1.3	0.88
2.0	1.9	-5.4	1.9
0.90	0.88	-2.7	3.4
0.50	0.47	-5.9	3.4
0.10	0.10	2.0	9.6

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, equivalent to mass fractions of GM DNA.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
4.5	4.6	2.6	1.1
2.0	2.0	1.7	3.6
0.90	0.93	3.3	2.6
0.50	0.52	4.0	3.9
0.10	0.11	14	8.6

5.2. Results of the international collaborative study

5.2.1. PCR efficiency and linearity

The PCR efficiency (%) and R^2 values (expressing the linearity of the regression) for the standard curve, reported by participating laboratories are displayed in Table 11. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

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

Table 11 indicates that the efficiency of amplification for the GMB151 system ranges from 92 % to 100 % and the mean R^2 is 1.00.

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.

		GMB151			***Le1***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.51	93	1.00	-3.35	99	1.00
	B	-3.53	92	1.00	-3.33	100	1.00
2	A	-3.44	95	1.00	-3.38	97	1.00
	B	-3.47	94	1.00	-3.37	98	1.00
3	A	-3.41	96	1.00	-3.36	98	1.00
	B	-3.50	93	1.00	-3.36	98	1.00
4	A	-3.45	95	1.00	-3.37	98	1.00
	B	-3.45	95	1.00	-3.38	97	1.00
5	A	-3.33	100	1.00	-3.26	103	1.00
	B	-3.40	97	1.00	-3.26	103	1.00
6	A	-3.40	97	1.00	-3.27	102	1.00
	B	-3.51	93	1.00	-3.35	99	1.00
7	A	-3.46	94	1.00	-3.38	98	1.00
	B	-3.46	94	1.00	-3.36	98	1.00
8	A	-3.41	97	1.00	-3.39	97	1.00
	B	-3.39	97	1.00	-3.39	97	1.00
9	A	-3.48	94	1.00	-3.41	96	1.00
	B	-3.44	95	1.00	-3.40	97	1.00
10	A	-3.48	94	0.99	-3.38	98	1.00
	B	-3.44	95	1.00	-3.29	101	1.00
11	A	-3.49	93	1.00	-3.34	99	1.00
	B	-3.46	94	1.00	-3.36	98	1.00
12	A	-3.45	95	1.00	-3.44	95	1.00
	B	-3.38	98	1.00	-3.40	97	1.00
	Mean	**-3.45**	**95**	**1.00**	**-3.36**	**99**	**1.00**

The amplification efficiency for the soybean-specific system ranges from 95 % to 103 % and the mean R^2 is 1.00. The mean PCR efficiency was 95 % forGMB151 and 99 % for *Le1*. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

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.

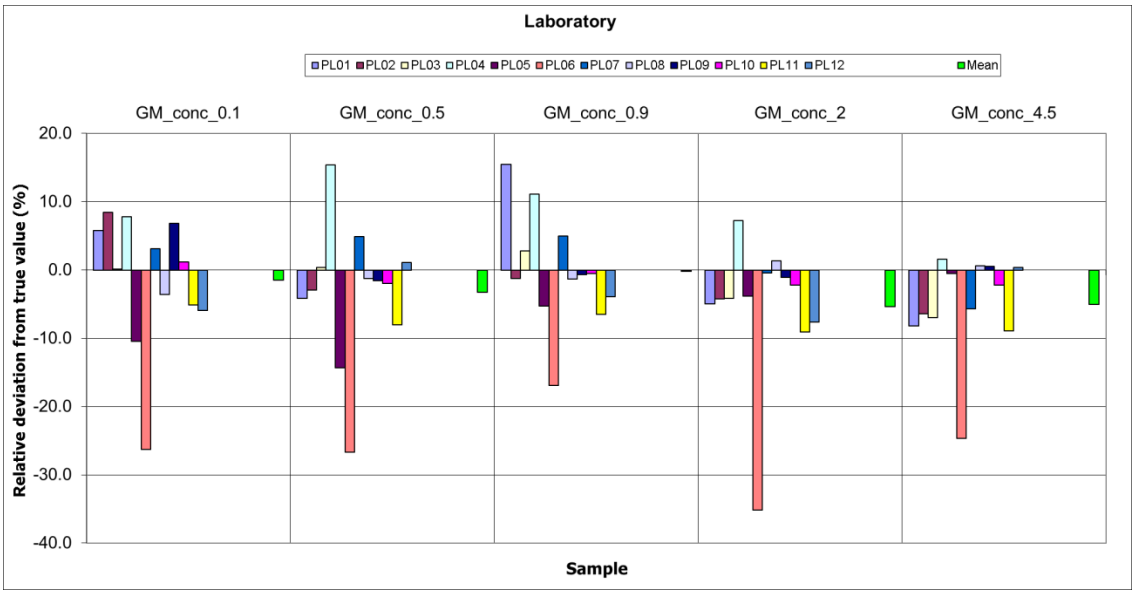
Table 12. GM % values determined by laboratories for test samples

	GMO content (%) *																			
LAB	0.1				0.5				0.9				2.0				4.5			
	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	0.11	0.10	0.11	0.10	0.49	0.47	0.52	0.44	1.4	0.96	0.93	0.88	1.9	1.8	2.1	1.8	4.1	3.9	4.1	4.4
2	0.12	0.10	0.11	0.10	0.49	0.50	0.47	0.48	0.85	0.95	0.89	0.87	1.9	1.9	1.9	1.9	4.1	4.1	4.2	4.4
3	0.10	0.10	0.11	0.10	0.52	0.51	0.49	0.49	0.87	0.97	0.92	0.94	1.9	2.0	1.9	1.8	4.2	4.0	4.2	4.4
4	0.12	0.10	0.10	0.11	0.60	0.49	0.62	0.60	1.0	0.93	0.91	1.1	1.9	2.2	2.3	2.2	4.6	4.1	4.5	5.1
5	0.10	0.09	0.09	0.08	0.41	0.44	0.46	0.40	0.85	0.87	0.86	0.84	2.1	1.7	2.0	2.0	4.5	4.7	4.3	4.4
6	0.08	0.07	0.08	0.07	0.42	0.34	0.40	0.31	0.62	0.77	0.85	0.74	1.4	1.4	1.4	1.0	4.0	2.6	3.2	3.8
7	0.10	0.10	0.11	0.10	0.53	0.51	0.50	0.55	0.94	1.0	0.90	0.95	2.0	2.1	2.0	1.8	4.2	4.1	4.3	4.4
8	0.09	0.10	0.10	0.10	0.50	0.49	0.49	0.49	0.91	0.89	0.88	0.87	2.0	2.1	2.0	2.0	4.6	4.6	4.5	4.4
9	0.10	0.11	0.11	0.11	0.48	0.49	0.50	0.50	0.92	0.90	0.87	0.89	1.9	2.0	2.1	1.9	4.5	4.5	4.6	4.5
10	0.10	0.10	0.10	0.11	0.46	0.46	0.53	0.50	0.91	0.85	0.88	0.94	2.0	1.8	2.0	2.0	4.1	4.2	4.6	4.7
11	0.09	0.10	0.09	0.09	0.47	0.45	0.49	0.44	0.81	0.86	0.83	0.88	1.8	1.7	1.8	1.9	4.1	4.1	4.1	4.1
12	0.09	0.09	0.10	0.11	0.51	0.49	0.52	0.51	0.82	0.82	0.87	0.95	2.0	2.0	1.5	2.0	4.6	4.5	4.3	4.6

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

A graphical representation of the data reported in Table 12 is provided in Figure 1 that shows the relative deviation from the true value for each GM level tested and for each participating laboratory. The coloured bars represent the deviation of the GM level measured in % of the true GM level; the green bar on the right represents the mean relative deviation for each GM level before eliminating outliers.

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



* For PL03 at level 0.1 % a very small relative deviation from the true value was observed and therefore the corresponding histogram does not show up in Figure 1. PL = participating laboratory.

Overall, most laboratories' mean relative deviations from the true values were within a maximum of ± 25 %. The mean relative deviations from true value was within the limit for twelve laboratories at GM-levels 0.9 % and 4.5 % for eleven laboratories at GM-levels 0.1 %, 0.5 % and 2.0 %. One laboratory underestimated GM-level 0.1 %, 0.5 %, 2.0 % and 4-5 % by more than 25 %. No clear trend for over- or underestimation was observed.

5.2.3. Method performance requirements

Among the performance requirements established by ENGL and adopted by the EURL GMFF (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 12 % at the 0.1 % and 0.5 % GM levels, thus within the acceptance criterion.

Table 13. Summary of validation results for the GMB151 method, expressed as GM copy numbers in relation to target taxon haploid genome copy numbers, equivalent to mass fractions of GM DNA.

	Test Sample Expected GMO %				
	0.1	**0.5**	**0.9**	**2.0**	**4.5**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	1	1	1
Reason for exclusion	-	-	C	G	C
Mean value	0.10	0.48	0.89	1.9	4.4
Relative repeatability standard deviation, RSD_r (%)	7.4	6.3	6.1	6.5	4.6
Repeatability standard deviation	0.007	0.031	0.054	0.13	0.20
Relative reproducibility standard deviation, RSD_R (%)	12	12	9.0	7.3	5.8
Reproducibility standard deviation	0.012	0.058	0.079	0.14	0.25
Bias** (absolute value)	-0.002	-0.016	-0.014	-0.053	-0.15
Bias (%)	-1.5	-3.3	-1.6	-2.6	-3.3

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

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

Table 13 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for collaborative study, the EURL GMFF and ENGL require that the RSD_r value indicated by the applicant and confirmed by the EURL GMFF through in-house experiments, is below 25 % (see ENGL document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). As it can be observed from the values reported, the repeatability standard deviation is below 25 % at all GM levels, with the highest value of 7.4 % at the 0.1 % 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 largest value of bias (%) of -3.3 % % at the 0.5 % and 4.5 % GM levels.

6. Compliance of the method for detection and quantification of event GMB151 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 0.08 % level shown by the applicant's dossier (expressed as mass fraction of GM-material) was 7.7 %, based on 18 replicates (Table 2A), and 18.2 % based on 18 replicates in the transferability study (Table 2B), hence below the maximum value of 25 % required by the ENGL. The EURL GMFF therefore concluded that it could accept the applicant's data on method performance:

- at step 3 of the validation process (experimental testing of samples and methods), the EURL GMFF determined the RSD_r % value at the level of 0.1 % in mass fraction of GM-material (corresponding to 0.1 % 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 8.6 % and 12 % (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 7.4 %, 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 GMB151 at or around 0.1 % level related to mass fractions of GM material.

Source	**RSD_r %**	**GM %**
Applicant's method optimisation	7.7 %	0.08 %
Applicant's transferability study	18.2 %	0.08 %
EURL GMFF tests	8.6 - 12 %	0.1 %
Collaborative study	7.4 %	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 soybean genomic DNA.

In any case the user of the method is advised to verify the quality of the extracted genomic DNA in order to ensure that it is suitable for the subsequent PCR analysis. This is particularly relevant for more complex matrices of samples from food and feed products.

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

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Annex 1: Event-specific Method for the Quantification of soybean GMB151 by Real-time PCR

Validated Method

Method development:

Bayer CropScience

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 soybean event GMB151 DNA to total soybean 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 GMB151, an 84 bp fragment of the region spanning the 5' insert-to-plant junction in soybean GMB151 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-1 (Black Hole Quencher® 1) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event GMB151, a soybean taxon-specific system amplifies a 102 bp fragment of a soybean lectin 1 (Le1) endogenous gene (Accession number, GeneBank: K00821.1 M30884), using *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with JOE 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 GMB151 DNA in a test sample, Cq values for the GMB151 and the *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of GMB151 DNA to total soybean DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA that can be extracted from genetically modified and conventional soybean seeds, grains and leaves. 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 April 2019.

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 0.03 % (related to mass fraction of GM material) in 200 ng of total suitable soybean DNA. The sample was analysed in six replicates in three real-time PCR runs (total eighteen values, with GM target detected in all reactions). According to the method developer, the LOD_{abs} of the GMB151 and of the Le1 systems is below 10 haploid genome copies). The 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 0.08 % (related to mass fraction of GM material) in 200 ng of total suitable soybean 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 5' insert-to-plant junction in soybean GMB151 and is therefore event-specific for the event GMB151. This was confirmed by the applicant's specificity studies and by in silico analysis performed by the applicants and the EURL GMFF.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. "forward flow direction" during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.
- 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 soybean event GMB151

3.2.1 General

The real-time PCR set-up for the taxon (*Le1*) and the GMO (event GMB151) 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 GMB151) and the taxon (*Le1*) assay with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curves have to be established on at least five samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % soybean GMB151 DNA in a total of 300 ng of soybean DNA (corresponding to 265487 soybean haploid genome copies with one haploid genome assumed to correspond to 1.13 pg of soybean genomic DNA) [(1)]. Standards S2 to S5 are to be prepared by serial dilutions (dilution factor 3 for samples S2, dilution factor 5 for sample S3, dilution factor 4 for sample S4 and dilution factor 10 for standard S5) according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of soybean DNA in reaction (ng)*	300	100	20	5	0.5
Soybean haploid genome copies	265487	88496	17699	4425	442
GMB151 copies	26549	8850	1770	442	44

* 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 GMB151 soybean specific system (Table 2) and the *Le1* 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 GMB151 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
PRIM1040 (10 µM)	400 nM	1.0
PRIM1041 (10 µM)	400 nM	1.0
TM1789* (10 µM)	200 nM	0.5
Nuclease free water	-	5
DNA	-	5
Total reaction volume:		25 µL

*TaqMan® probe labelled with 6-FAM at its 5'-end and BHQ-1 at its 3'-end

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the soybean *Le1* assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM164 (10 µM)	200 nM	0.5
KVM165 (10 µM)	200 nM	0.5
TM1242* (10 µM)	200 nM	0.5
Nuclease free water	-	6
DNA	-	5
Total reaction volume:		25 µL

*TaqMan® probe is labelled with JOE at its 5'-end and BHQ-1 at its 3'-end]

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for the soybean GMB151 and one for the *Le1* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).

5. Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (70 µL for the GMB151 soybean system and 70 µL for the *Le1* system). 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 GMB151 system and for the *Le1* reference system in each well.
7. Place an optical cover on the reaction plate and briefly centrifuge the plate.
8. Place the reaction plate in the real-time PCR apparatus (possibly apply a compression pad, depending on the model), according to the manufacturer's instructions and your Standard Operating Procedures and start the run.
9. Select FAM as reporter dye for the GMB151 and JOE for the *Le1* reference system. Define BHQ-1 or non-fluorescent as quencher dye for GMB151 specific system and BHQ or non-fluorescent for *Le1* reference system. Select ROX as the passive reference dye, according to the instructions of your instrument. Enter the correct reaction volume (25 µL).
10. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for GMB151/*Le1* assays.

Step	**Stage**		**T (°C)**	**Time (s)**	**Acquisition**	**Cycles**
1	[UNG*]		50	120	No	1
2	Initial denaturation		95	600	No	1
3	Amplification	Denaturation	95	15	No	40
		Annealing & Extension	60	60	Yes	

*UNG: Uracil-N-glycosylase

3.3 Data analysis

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

a) Set the threshold following the automatic or the manual mode. In the manual mode display the amplification curves of the event specific assay in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the "update" button to ensure changes affect Cq values (only needed for some analysis software). Switch to the linear view mode by clicking on the Y axis of the amplification plot and check that the threshold previously set falls within the exponential phase of the curves.

b) Set the baseline following the automatic or the manual mode. In the manual mode: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Cq = 25, set the baseline crossing at Cq = 25 – 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a), b) and c) on the amplification plots of the taxon specific system.

e) Save the settings and export all the data for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Cq values for each reaction.

The standard **curves** are generated both for the *Le1* and for the GMB151 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 GMB151 DNA in the unknown sample, the GMB151 copy number is divided by the copy number of the soybean endogenous gene *Le1* and multiplied by 100 (GM% = GMB151/*Le1* 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

- TaqMan® Universal PCR Master Mix. Applied Biosystems Part No 4304437.

4.3 Primers and Probes

Table 5. Primers and probes for the GMB151 and *Le1* methods

		DNA Sequence (5' to 3')	**Length (nt)**
GMB151			
Forward primer	PRIM1040	TCA AAT CAA CAT ggg TgA CTA gAA A	25
Reverse primer	PRIM1041	CAT TgT gCT gAA TAg gTT TAT AgC TAT gAT	30
Probe	TM1789	FAM-5'- CAg TAC Tgg gCC CTT gTg gCg CT-3'-BHQ1	23
Le1			
Forward primer	KVM164	CTT TCT CgC ACC AAT TgA CA	20
Reverse primer	KVM165	TCA AAC TCA ACA gCg ACg AC	20
Probe	TM1242	JOE-5'- CCA CAA ACA CAT gCA ggT TAT CTT gg-3'-BHQ1	26

FAM: 6-carboxyfluorescein; JOE: 4,5-dichloro-dimethoxy-fluorescein; BHQ1: black hole quencher.

5. References

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

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