European Commission

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Event-specific Method for the Quantification of Soybean DBN-09004-6 Using Real-time PCR

Validation Report

Zaoui X., Sacco M. G., Jacchia S., Mazzara M., Vincent U.

European Union Reference Laboratory for Genetically Modified Food and Feed

2023

Joint Research Centre

This publication is a Validated Methods, Reference Methods and Measurements report by the Joint Research Centre (JRC), the European Commission's science and knowledge service. It aims to provide evidence-based scientific support to the European policymaking process. The contents of this publication do not necessarily reflect the position or opinion of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication. For information on the methodology and quality underlying the data used in this publication for which the source is neither Eurostat nor other Commission services, users should contact the referenced source. The designations employed and the presentation of material on the maps do not imply the expression of any opinion whatsoever on the part of the European Union concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

Contact information
European Commission
Directorate General Joint Research Centre
Directorate F – Health and Food
European Union Reference Laboratory for GM Food and Feed
Food & Feed Compliance (F.5)
Via E. Fermi, 2749.
I-21027 Ispra (VA), Italy

Functional mailbox: JRC-EURL-GMFF@ec.europa.eu

EU Science Hub
https://joint-research-centre.ec.europa.eu

JRC134862

Ispra: European Commission, 2023

How to cite this report: Zaoui X., Sacco M. G., Mazzara M., Vincent U. "Event-specific Method for the Quantification of Soybean DBN-09004-6 Using Real-time PCR." European Union Reference Laboratory for GM Food and Feed, Joint Research Centre, Ispra, 2023, JRC134862. http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F – Health and Food
Food and Feed Compliance (F.5)

Event-specific Method for the Quantification of Soybean DBN-09004-6 Using Real-time PCR

Validation Report

25 August 2023

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate [1] the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), validated an event-specific real-time polymerase chain reaction (qPCR) method for detecting and quantifying soybean event DBN-09004-6 (unique identifier DBN-Ø9ØØ4-6). 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], Beijing DaBeiNong Biotechnology Co., Ltd. represented for the GM Food & feed authorization in the EU by Perseus provided the detection method and the positive and negative control samples (genomic DNA from seeds of DBN-09004-6 soybean as positive control DNA, and genomic DNA from seeds 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]), 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 17025:2017 accredited [certificate number: Belac 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) in food and feed by DNA extraction, DNA identification and Real-time PCR and for determination of Genetically Modified content in % (cp/cp) in food and feed by DNA extraction and digital PCR)].

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

Address of contact laboratory:
European Commission
Directorate General Joint Research Centre
Directorate F – Health and Food
European Union Reference Laboratory for GM Food and Feed
Food & Feed Compliance (F.5)
Via E. Fermi, 2749. TP201
I-21027 Ispra (VA), Italy

Functional mailbox: JRC-EURL-GMFF@ec.europa.eu

1. Introduction

In line with Regulation (EC) No 1829/2003 [1], Beijing DaBeiNong Biotechnology Co., Ltd. represented for the GM food & feed authorisation in the EU by Perseus, provided the EURL GMFF with an event-specific method for detection and quantification of soybean event DBN-09004-6 (unique identifier DBN-Ø9ØØ4-6) 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 DBN-09004-6, 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)

Beijing DaBeiNong Biotechnology Co., Ltd. represented for the GM food & feed authorisation in the EU by Perseus, submitted the identification and quantification method, data demonstrating its adequate performance when applied to genomic DNA extracted from soybean DBN-09004-6 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, on the basis of the sequence data provided by the -applicant.

3.1. Specificity assessment conducted by the applicant

The specificity of the event-specific assay was assessed by the applicant in triplicate real-time PCR reactions, according to the method described in Annex 1 (Tables 1, 2, 3, 4 and 5), using approximately 250 haploid genome copies/reaction of the target DNA (DBN-09004-6) and of 2500 haploid genome copies/reaction of the following non-target DNA samples: oilseed rape Ms1, Ms8, Rf1, Rf2, Rf3, Topas19/2, T45, RT73, MON 88302, 73496; soybean A2704-12, A5547-127, FG72, GTS40-3-2, MON 89788, MON 87701, MON 87708, 356043, 305423, CV127, MON 87769, MON 87705, DAS 68416-4, DAS-81419-2, DAS-44406-6, MON 87751; maize T25, Bt176, Bt11, MON 810, GA21, NK603, MON 863, 1507, 3272, MIR604, MIR162, 59122, 98140, MON 88017, MON 89034, MON 87460, MON 87427, MON 87403, MON 87411, VCO-1981-5, DAS-40278-9, 5307, 4114, MZHG0JG; cotton LLCotton25, T304-40, GHB614, GHB119, MON 1445, MON 531, MON 15985, MON 88913, MON 88701, DAS-81910-7, COT 102, 281-24-236 x 3006-210-23; sugar beet H7-1; potato EH92-527-1, AM04-1020, AV43-6-G7, PH05-026-0048; rice LLRICE62 and conventional oilseed rape, conventional soybean, conventional maize, conventional cotton, conventional sugar beet, conventional potato, conventional rice. According to the method developer the DBN-09004-6 method did not react with any sample, except the positive control.

In addition, the applicant performed an in-silico specificity analysis by using the amplicon sequence as a query for BLASTN 2.9.0+ algorithm search against public sequence of National Center for Biotechnology Information (NCBI) and Patent Genbank databases. No sequence showed alignment of both forward and reverse primers or with the full length of the probe (29th March 2019).

A previously validated soybean-specific PCR method (https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-04-12-VP.pdf), which amplifies a 74 base pair (bp) fragment of the *lectin* (*Le1*) of *Glycine max*, was used as a reference method.
The specificity of the taxon-specific assay was assessed by the applicant in triplicate real-time PCR reactions, according to the method described in Annex 1 (Tables 1, 2, 3, 4 and 5), using minimum 50 ng of genomic DNA per reaction extracted from: conventional oilseed rape, conventional soybean, conventional maize, conventional cotton, conventional sugar beet, conventional potato and conventional rice. According to the method developer the *Le1* assay did not react with any sample except the positive control.

In addition, the applicant performed an in-silico specificity analysis by using the amplicon sequence as a query for BLASTN 2.9.0+ algorithm search against public sequence of National Center for Biotechnology Information (NCBI). No sequence showed alignment of both forward and reverse primers or with the full length of the probe, except 2x Soybean *Lectin* (*Le1*) mRNA, Soybean *Lectin* (*Le1*) coding sequence and a synthetic construct with a Soybean *Lectin* (*Le1*) fusion protein. (22nd March 2019).

3.2. Specificity assessment conducted by the EURL GMFF

The detection method spans the 3' insert-to-plant junction in soybean DBN-09004-6. The forward primer "S17A01_F" binds to the insert. The reverse primer "S17A01_R" binding site was found in the soybean (*Glycine max*) genomic border adjacent to the insertion. The probe "S17A01_P" binds to the junction between the insert and the 3' genomic region of *Glycine max*.

The amplicon size is expected to be 89 bp, consistent to what reported by the applicant. The sequence of the amplicon was analysed by BLAST (NCBI) against local copies of the "nt" and "patents" databases, and no significant similarity was found with any other published sequence. In addition, the primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System (CCSIS) of the JRC, as well as the whole genomes of more than 80 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays*) using the e-PCR prediction tool (NCBI), and no potential amplicon was identified. A perfect match of the amplicon and of the primers was identified with the sequence deposited for DBN-09004-6.

3.3. Verification of the ENGL acceptance parameters

The applicant prepared the calibration curve from a DNA solution (S1) of 10 % soybean event DBN-09004-6 genomic DNA (expressed as copy number ratio) which was serially diluted (1:5) to obtain solutions S2 and S3, while S3 was diluted 10-times to obtain S4. The parameters (slope, R2 coefficient) of eight runs of the calibration curve are reported as provided by the applicant (Table 1).

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

	DBN-09004-6		***Le1***	
	Slope	**R^2**	**Slope**	**R^2**
Run 1	-3.32	1.00	-3.38	1.00
Run 2	-3.41	1.00	-3.37	1.00
Run 3	-3.28	1.00	-3.42	1.00
Run 4	-3.34	1.00	-3.46	1.00
Run 5	-3.37	1.00	-3.32	1.00
Run 6	-3.25	1.00	-3.36	1.00
Run 7	-3.32	1.00	-3.42	1.00
Run 8	-3.34	1.00	-3.36	1.00
Mean	-3.33	1.00	-3.39	1.00

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

Table 1 indicates that the slope and R^2 coefficient of the standard curves for the GM-system (DBN-09004-6) and the soybean-specific *lectin* (*Le1*) system, as established by the applicant, were within the ENGL acceptance criteria.

Precision and trueness of the method were established by the applicant on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System and 16 values for each of five GM levels (expressed as copies GM/total haploid genome copies) were provided. Table 2A reports precision and trueness mean values for the five GM-levels as provided by the applicant. Both parameters were within the ENGL acceptance criteria (trueness ± 25 %, RSD_r ≤ 25 % across the entire dynamic range).

Table 2A. Mean %, precision and trueness values provided by the applicant estimated for single measurements

	Test results				
Expected GM %	**5.00**	**2.00**	**0.900**	**0.100**	**0.058**
Measured mean GM %	5.67	2.00	0.854	0.087	0.050
Precision (RSD_r %)	5.1	5.1	5.7	11.3	13.6
Trueness (bias %)	13.4	0.0	-5.1	-13.0	-13.8

* 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. 0.058 % (expressed as copy number ratio)]. 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 DBN-09004-6 event specific and the soybean reference real-time PCR methods was assessed by the applicant in 60 PCR replicates. The LOD_{abs} was found to be below 22 haploid genome copies for the DBN-09004-6 event-specific method and below 20 haploid genome copies for the *Le1* reference method. The relative LOD (LOD_{rel}) of the combined method was found to be at least 0.025 % (related to copies GM/total haploid genome copies) in 100 ng of total soybean DNA per reaction. The LOD_{abs} and LOD_{rel} are in line with the ENGL acceptance criteria (below 0.045 % or 25 copies with a level of confidence of 95 %).

The robustness of the method was assessed in sixteen combinations of the following variations to the method: ABI 7500/ABI 7900HT PCR cycler, exact/+/- 10 % master mix concentration, exact/+/- 30 % primer concentration, exact/+/- 30 % probe concentration, exact/+/- 1 µl master mix volume and +/-1 °C annealing temperature. The RSD_r and the trueness calculated for each combination of variations on a sample at the LOQ level (0.058 %) did not exceed 30 %, thus meeting the ENGL acceptance criteria.

Precision and trueness of the method were tested in a transferability study involving three Eurofins laboratories using two Bio-Rad CFX96 Touch™ and an Applied Biosystems 7500 instruments: the

values provided for each of the five GM levels (expressed as copies GM/total haploid genome copies) are the mean of two sets of triplicates. Table 2B reports precision and trueness values for the five GM-levels as provided by three laboratories different from the method developer. Both parameters were within the ENGL acceptance criteria (trueness ≤ 25 %, RSDr ≤ 25 % across the entire dynamic range).

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

		Test results				
	Expected GM %	**5.00**	**2.00**	**0.900**	**0.100**	**0.058**
Laboratory 1	Measured mean GM %	4.86	1.82	0.890	0.094	0.050
	Precision (RSD_r %)	0.4	6.6	7.3	0.7	12.8
	Trueness (bias %)	-2.8	-9.0	-1.1	-6.0	-13.8
Laboratory 2	Measured mean GM %	4.88	1.85	0.801	0.090	0.050
	Precision (RSD_r %)	2.6	2.4	6.9	1.6	0.0
	Trueness (bias %)	-2.4	-7.5	-11.0	-10.0	-13.8
Laboratory 3	Measured mean GM %	4.38	1.80	0.782	0.076	0.052
	Precision (RSD_r %)	24.4	20.8	18.5	2.8	1.3
	Trueness (bias %)	-12.4	-10.0	-13.1	-24.0	-10.3

3.4. DNA extraction

Genomic DNA was isolated from ground soybean seeds, using a CTAB-based method followed by silica resin purification using commercially available columns (Wizard® DNA Clean-Up System, Promega) and size-exclusion spin columns with Sephacryl matrix (Mobi Spin S-300, MoBiTec).

According to the experimental data submitted by the applicant, the protocol for DNA extraction generated DNA of suitable quantity and quality for PCR based applications when applied to ground seeds from the soybean event DBN-09004-6. This method was validated by the EURL GMFF for the extraction of DNA from ground soybean seeds. The protocol for DNA extraction and a report on testing are published at http://gmo-crl.jrcec.europa.eu/summaries/EURL-VL-08-19-XP.pdf.

Annex III to Regulation (EU) No 503/2013 [7] requires the applicant to discuss the validity and limitations of the detection methods in the various types of foods and feeds (matrices) that are expected to be placed on the market. To this regard the applicant stated that the method is applicable to any matrix from which genomic DNA with sufficient quantity and quality can be purified, although the DNA extraction method was developed to extract DNA from soybean seeds, which is of high molecular weight.

If the soybean seeds are processed to produce food ingredients, the quality of the DNA would likely change. Therefore, it is recommended that, in order to ensure high quality results, all DNA samples used for event-specific real-time PCR should be monitored and thoroughly controlled for intactness, concentration and required quality prior to running any analysis.

Many factors affect the applicability and reliability of DNA-based qualitative and quantitative GMO-detection. While the efficiency of the qPCR method depends on DNA quality and purity, food processes involving mechanical stress, high temperature, pH variations, enzymatic activities, and fermentations, affect the primary structure of DNA and cause, for example, hydrolysis, oxidation, and deamination of the DNA [10-11]. Although food processing can lead to increased homogeneity, it can result in significant degradation and reduction of the fragment size of DNA or removal of DNA from the sample, thereby reducing the sensitivity of the analysis an affecting the limits of detection and quantification [11-14]. This may alter the results of a qualitative [15] and quantitative [16] GMO analysis.

Whenever DNA is extracted from more complex and difficult matrices, it is recommended to control the DNA to ensure 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 seeds harbouring the DBN-09004-6 event, and
- genomic DNA extracted by the applicant from conventional soybean seeds genetically similar to those harbouring the DBN-09004-6 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 DBN-09004-6 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean specific assay targeting the endogenous gene *lectin* (*Le1*), and the GM target assay for DBN-09004-6 are performed in separate wells. The validated method protocol is published by the EURL GMFF at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and can be found in Annex 1 to this report.

For the detection of GM event DBN-09004-6, an 89 bp fragment of the region spanning the 3' insert-to-plant junction in soybean DBN-09004-6 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 MGBNFQ (Minor Groove Binder Non-Fluorescent-Quencher) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event DBN-09004-6, a soybean taxon-specific system amplifies a 74 bp fragment of an soybean *lectin* (*Le1*) endogenous gene (Accession number, GeneBank: K00821), using *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with FAM 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 DBN-09004-6 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 DBN-09004-6 DNA in a test sample, the DBN-09004-6 copy number is divided by the copy number of the soybean haploid genome and multiplied by 100 to obtain the percentage value (GM % = DBN-09004-6 / soybean haploid genome 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) [17]. 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
Total amount of soybean DNA in the reaction (ng)	125	25	5.0	0.50
Target taxon haploid genome copies	110619	22124	4425	442
Target DBN-09004-6 copies	11062	2212	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 Regulation (EU) No 619/2011 [8].

The copy number of the DBN-09004-6 and of the *Le1* targets in the positive control sample were determined by digital PCR (dPCR) performed on the Bio Rad QX200 Droplet Reader.

Reaction mixes were prepared in order to test the zygosity in five replicates to a final volume of 22 µL and contained 1X ddPCR Super Mix no dUTP (Bio Rad, Cat. number 64299440), primers and probes at the concentrations indicated in the corresponding validated method (S17A01_F and S17A01_R primers at 600 nM each, S17A01_P probe at 200 nM; Lec F and Lec R primers at 600 nM each, Lectin_P probe at 200 nM), and 1 µL of DNA at a concentration of 25 ng/µL; the DNA concentration was chosen in order to avoid panel saturation (optimal between 200<positive partitions<700).

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

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

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

The sealed 96-well plate was then placed in the QX200 Droplet Reader to determine through cytofluorimetry the fraction of fluorescent PCR-positive droplets with respect of the total number of droplets in the original sample by selecting the proper fluorescent dye used. Data analysis and copy number calculations were performed using the Bio-Rad QX200 Droplet Reader Analysis software (QuantaSoft version 1.7.4).

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'[18].

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

The method performance characteristics were verified by quantifying on a copy number basis five blind test samples distributed over a range of GM levels (5 % - 0.10 %, 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 DBN-09004-6 soybean DNA and non-GM soybean DNA.

Table 4. DBN-09004-6 blinded samples GM % contents

DBN-09004-6 GM % GM copy number/soybean haploid genome copy number x 100
5.0
2.0
0.90
0.45
0.10

The calibration sample S1 was prepared from the genomic DNA provided by the applicant by mixing the appropriate amount of DBN-09004-6 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-S3 were prepared by 5-fold serial dilutions from the S1 sample and sample S4 by a 10-fold serial dilution from the S3 sample (see Table 3).

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

Test samples with GM levels 5.0 %, 2.0 %, 0.90 % and 0.45 % 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 0.10 % was tested in 15 replicates in an additional run for each platform. Average values of the slope and of the R^2 coefficient of the standard curves and method trueness and precision over the dynamic range were evaluated against the ENGL method acceptance criteria. On Roche LC480 platform the method was run at 45 cycles as described in the validated method published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1, and analysed with the second derivative maximum method.

4.4. International collaborative study (step 4)

The international collaborative trial involved twelve randomly selected laboratories, all being "national reference laboratories, assisting the EURL GMFF for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 120/2014 [19] 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 DBN-09004-6 international collaborative study were randomly selected from 27 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 DBN-09004-6

Laboratory	**Country**
National Food and Veterinary Risk Assessment Institute	LT
Federal Office of Consumer Protection and Food Safety	DE
Crop Research Institute - Reference Laboratory for GMO Detection and DNA fingerprinting	CZ
Center for Agricultural Technology Augustenberg	DE
Hellenic Agricultural Organisation Demeter	GR
Laboratory for the Detection of GMO in Food - Bad Langensalza	DE
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE
Instituto Nacional de Investigação Agrária e Veterinária, I.P.	PT
BioGEVES - Groupement d'Intérêt Public – Groupe d'Étude et de contrôle des Variétés et des Semences	FR
Plant Breeding and Acclimatization Institute – National Research Institute	PL
Walloon Agricultural Research Centre	BE
LUFA Speyer	DE

4.4.2. Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: four laboratories used ABI 7500, one used ABI 7500 Fast, two Quantstudio 5, one Quantstudio 6, one QuantStudio 7Flex, one Roche LC 480 II, one PCRmax ECO48 and one qTower3G.

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

4.4.3. Materials used in the international collaborative study

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

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

- ✓ Four calibration samples with known concentrations of GM-event (175 µL of DNA solution each) labelled from S1VL0819 to S4VL0819 (Table 3).

- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution, each at 20 ng/µL) labelled from U1VL0819 to U20VL0819, representing five GM levels, each in four replicates (Table 4)

- ✓ Reaction reagents:
  - TaqMan Universal Master Mix (no UNG), one vial: 8 mL
  - Distilled sterile water, one vial: 3 mL

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

  Le1 taxon-specific assay
  - Lec F primer (10 µM): 480 µL
  - Lec R primer (10 µM): 480 µL
  - Lec P probe (10 µM): 160 µL

  DBN-09004-6 assay
  - S17A01_F primer (10 µM): 480 µL
  - S17A01_R primer (10 µM): 480 µL
  - S17A01_P 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 DBN-09004-6 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 DBN-09004-6 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 also reported the raw data to the EURL GMFF on an Excel sheet that was designed, validated and distributed by the EURL GMFF. All data are stored by the EURL GMFF on a dedicated and protected server.

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

4.4.5. Deviations reported from the protocol

Only one laboratory reported deviations from the validation protocol, adding DNA directly in PCR tubes (instead of mixing it with the master mix prior to loading the PCR tubes).

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 DBN-09004-6 and *Le1* targets to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Zygosity ratio of the DBN-09004-6 and *Le1* targets in the positive control sample.

Mean ratio (DBN-09004-6/*Le1*)	0.97
Standard deviation	0.05
RSD_r (%)	5.2
Standard error of the mean	0.01
Upper 95 % CI of the mean	0.99
Lower 95 % CI of the mean	0.94

The mean ratio (DBN-09004-6/*Le1*) was 0.97. The 95 % confidence interval (CI) spans around 0.97, the expected ratio for a soybean control sample, homozygous for the GM-locus, and assuming single copy endogenous gene target. Therefore, the measured mean ratio is not significantly different from the expected ratio, for an alpha = 0.05.

Hence:

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

Note: the zygosity ratio herein reported is valid for the positive control sample DNA in the context of the present validation study. It is used to assess the method performance at 0.1% GM level - expressed as mass fraction of GM material- in relation to the provisions of Regulation (EU) No 619/2011. When analytical results of official laboratories are primarily expressed as ratio of GM-DNA copy numbers, they shall be translated into mass fraction results by means of the specific conversion factor published in the document "Conversion factors (CF) for certified references materials (CRM)" (https://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

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

Test samples with GM levels from 5.0 % to 0.45 % (GM copies/copies haploid genomes) 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 0.10 % GM-level was tested for its precision in quantification in 15 replicates in three separate runs.

Tests were conducted on ABI 7500, Q7 (QuantStudio 7 Flex System) and Roche LC480 for robustness.

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

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

Table 7A. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, Q7 and Roche LC480 to quantify GM-levels in the range 5.0 % to 0.45 % in four replicates each.

	DBN-09004-6 system			***Le1* system**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.6	90	0.99	-3.4	96	1.0
Run B	-3.5	94	1.0	-3.5	95	1.0
Run C	-3.4	97	1.0	-3.4	97	1.0
Run D	-3.5	94	1.0	-3.4	95	1.0
Run E	-3.5	91	1.0	-3.5	95	1.0
Run F	-3.5	94	1.0	-3.5	94	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 Q7; runs E and F were carried out on Roche LC480.

Table 7B. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, Q7 and Roche LC480 to quantify the GM-level 0.10 % in 15 replicates.

	DBN-09004-6 system			***Le1* system**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run G	-3.5	92	1.0	-3.5	94	1.0
Run H	-3.5	93	1.0	-3.5	94	1.0
Run I	-3.6	90	0.99	-3.5	94	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 Q7; run I was carried out on Roche LC480.

According to the ENGL method acceptance criteria the method trueness (measured as bias in % of the target GM level) should be within ± 25 % of the accepted reference value over the entire dynamic range and the precision, expressed as RSD_r % (relative standard deviation of repeatability), should be ≤ 25 %, also over the entire dynamic range.

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

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

Target GM-levels %	**Measured GM-level %**	**Bias % of the target GM-level**	**Precision (RSD_r %)**
5.0	5.0	-1.1	3.5
2.0	2.0	2.2	2.2
0.90	0.92	2.6	2.9
0.45	0.46	2.6	4.0
0.10	0.12	21	14

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

Target GM-levels %	**Measured GM-level %**	**Bias % of the target GM-level**	**Precision (RSD_r %)**
5.0	5.0	-0.75	1.6
2.0	2.1	2.7	4.5
0.90	0.91	1.6	2.6
0.45	0.44	-3.1	7.0
0.10	0.12	22	10

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

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	4.9	-2.2	3.0
2.0	2.0	1.3	0.97
0.90	0.93	3.0	2.3
0.45	0.46	3.0	2.9
0.10	0.12	22	13

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 DBN-09004-6 system ranges from 85 % to 101 % and the linearity is equal to 1.0; the amplification efficiency for the soybean-specific system ranges from 90 % to 98 % and the linearity is equal to 1.0. The mean PCR efficiency was 94 % for both the DBN-09004-6 and the *Le1* assays. The average R^2 of the methods was 1.0 for the DBN-09004-6 and the *Le1* assays. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

Table 11. Values of slope, PCR efficiency and R^2 obtained during the international collaborative trial.

		DBN-09004-6			***Le1***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.5	91	1.0	-3.5	94	1.0
	B	-3.5	92	1.0	-3.5	93	1.0
2	A	-3.5	94	1.0	-3.4	96	1.0
	B	-3.5	94	1.0	-3.5	95	1.0
3	A	-3.4	98	1.0	-3.4	97	1.0
	B	-3.4	96	1.0	-3.4	98	1.0
4	A	-3.6	91	1.0	-3.6	90	1.0
	B	-3.5	95	1.0	-3.6	90	1.0
5	A	-3.3	100	1.0	-3.6	90	1.0
	B	-3.4	97	1.0	-3.5	92	1.0
6	A	-3.4	97	1.0	-3.4	96	1.0
	B	-3.4	97	1.0	-3.4	97	1.0
7	A	-3.3	99	1.0	-3.4	95	1.0
	B	-3.3	101	1.0	-3.4	97	1.0
8	A	-3.6	91	1.0	-3.5	93	1.0
	B	-3.8	85	1.0	-3.6	91	1.0
9	A	-3.5	92	1.0	-3.4	95	1.0
	B	-3.5	93	1.0	-3.5	93	1.0
10	A	-3.6	91	1.0	-3.5	94	1.0
	B	-3.5	95	1.0	-3.4	95	1.0

11	A	-3.4	97	1.0	-3.5	95	1.0
	B	-3.6	91	1.0	-3.4	97	1.0
12	A	-3.5	94	1.0	-3.4	97	1.0
	B	-3.5	93	1.0	-3.4	95	1.0
	Mean	-3.5	94	1.0	-3.5	94	1.0

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.

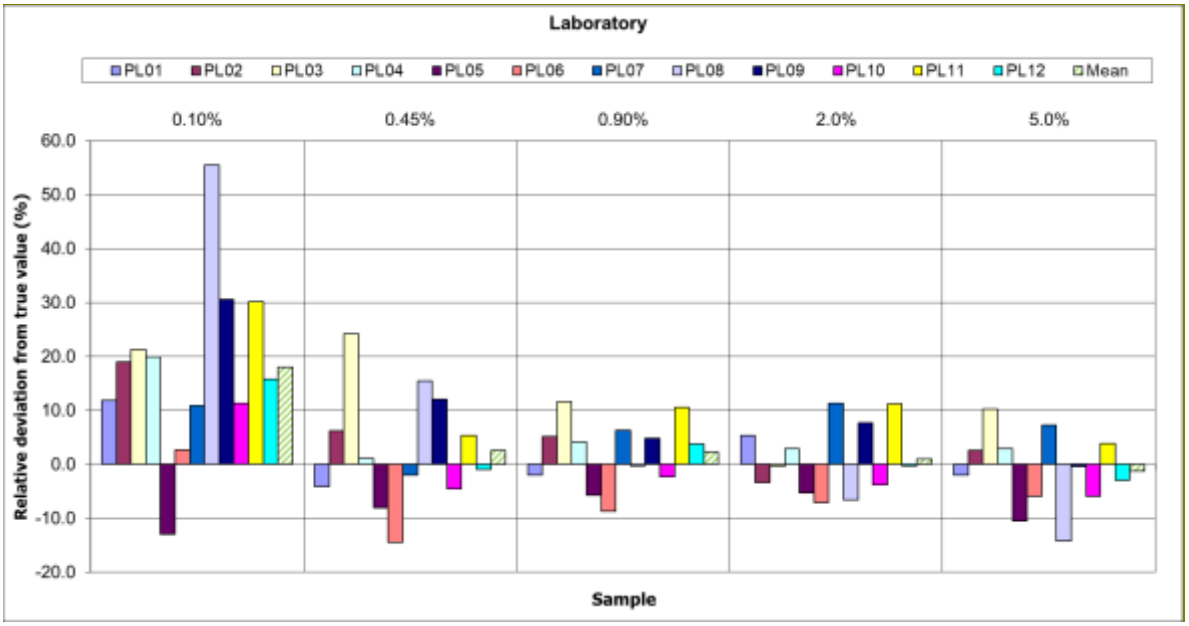
Table 12. GM % values determined by laboratories for test samples

	GMO content (%) *																			
LAB	**0.10**				**0.45**				**0.90**				**2.0**				**5.0**			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.13	0.11	0.11	0.10	0.41	0.47	0.42	0.43	0.83	0.87	1.0	0.82	2.1	2.2	2.1	2.0	5.1	5.1	4.6	4.9
2	0.11	0.12	0.12	0.12	0.48	0.46	0.49	0.48	0.91	0.96	0.91	1.0	1.3	2.1	2.1	2.2	5.0	5.0	5.2	5.3
3	0.13	0.12	0.13	0.11	0.51	0.55	0.52	0.66	0.97	0.95	1.1	1.0	1.8	2.3	1.8	2.1	5.2	5.8	5.6	5.5
4	0.14	0.10	0.12	0.12	0.45	0.46	0.46	0.45	0.86	1.0	0.92	0.94	2.0	2.1	2.1	2.1	5.3	5.3	5.1	5.0
5	0.09	0.09	0.09	0.08	0.50	0.47	0.35	0.34	0.91	0.81	0.81	0.86	1.9	2.0	1.9	1.8	5.0	4.8	4.0	4.1
6	0.10	0.10	0.09	0.12	0.40	0.38	0.39	0.38	0.75	0.92	0.81	0.80	1.9	1.8	1.9	1.9	5.0	4.3	4.8	4.8
7	0.12	0.11	0.10	0.12	0.35	0.48	0.45	0.47	0.91	0.97	1.0	0.94	2.2	2.3	2.2	2.2	5.5	5.4	5.2	5.3
8	0.12	0.16	0.23	0.11	0.54	0.70	0.45	0.39	0.97	0.88	1.0	0.71	1.9	2.2	1.8	1.6	4.3	5.0	3.4	4.5
9	0.13	0.13	0.14	0.12	0.48	0.49	0.55	0.50	0.85	0.97	0.99	0.96	2.2	2.2	2.1	2.1	5.2	4.9	4.8	5.0
10	0.10	0.11	0.11	0.12	0.48	0.43	0.40	0.41	0.86	0.93	0.85	0.88	2.0	2.0	1.8	1.9	5.1	4.7	4.3	4.7
11	0.12	0.13	0.16	0.11	0.49	0.52	0.45	0.43	1.0	0.95	1.1	0.96	2.3	2.3	2.1	2.2	5.2	5.4	5.1	5.1
12	0.12	0.11	0.12	0.11	0.47	0.44	0.46	0.42	0.93	0.95	0.94	0.91	1.9	2.1	2.0	2.1	5.1	4.7	4.8	4.9

* 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 by the participating laboratory. The coloured bars represent the deviation of the GM level measured in % of the true GM level; the green stripes bar on the right represents the mean relative deviation over all data before eliminating outliers for each GM level.

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



PL = participating laboratory.

Overall, most laboratories' mean relative deviations from the true values were within a maximum of ± 25 %. At GM-level 0.45 %, 0.90 %, 2.0 % and 5.0 %, twelve laboratories were within the limit; at GM-level 0.10 %, nine laboratories were within the limit. Three laboratories overestimated GM-level 0.10 % by more than 25 %. However, overall, 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 14 % at the 0.10 % GM level, thus within the acceptance criterion.

Table 13. Summary of validation results for the DBN-09004-6 method, expressed as GM copy numbers in relation to target taxon haploid genome copy numbers.

	Test Sample Expected GMO %				
	0.10	**0.45**	**0.90**	**2.0**	**5.0**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	1	1	0	1	0
Reason for exclusion	C	C	-	C	-
Mean value	0.11	0.46	0.92	2.0	4.9
Relative repeatability standard deviation, RSD_r (%)	9.7	9.3	7.3	6.1	6.1
Repeatability standard deviation	0.01	0.04	0.07	0.12	0.30
Relative reproducibility standard deviation, RSD_R (%)	14	13	8.7	8.6	9.0
Reproducibility standard deviation	0.02	0.06	0.08	0.17	0.44
Bias** (absolute value)	0.02	0.006	0.02	0.03	-0.06
Bias (%)	15	1.4	2.3	1.4	-1.3

* 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 13 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for collaborative study, the EURL GMFF and ENGL require that the RSD_r value indicated by the applicant and confirmed by the EURL GMFF through in-house experiments, is below 25 % (see ENGL document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). As it can be observed from the values reported, the repeatability standard deviation is below 25 % at all GM levels, with the highest value of 9.7 % at the 0.10 % GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25 % across the entire dynamic range. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 15 % at the 0.10 % GM level.

6. Compliance of the method for detection and quantification of event DBN-09004-6 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 0.10 % level shown by the applicant's dossier (expressed in mass fraction of GM-material and corresponding to 0.10 % in terms of copy number ratio to haploid genome copy numbers) was 11 %, based on 16 replicates (Table 2A), and between 0.7 % and 2.8 % based on 6 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 0.10 % in mass fraction of GM-material (corresponding to 0.10 % 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 10 % and 14 % (Table 8, 9 and 10) depending on the qPCR platform applied, hence also below 25 %;

- the collaborative study (step 4 of the validation process) established that over the twelve participating laboratories at the level of 0.1 % related to mass fraction of GM-material the RSD_r of the method was 9.7 %, 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 DBN-09004-6 at or around 0.1 % level related to mass fractions of GM material.

Source	**RSD_r %**	**GM %**
Applicant's method optimisation	11 %	0.1 %
Applicant's transferability study	0.7 - 2.8 %	0.1 %
EURL GMFF tests	10 - 14 %	0.1 %
Collaborative study	9.7 %	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.

8. References

1. Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed.
2. Horwitz W. Protocol for the design, conduct and interpretation of method-performance studies, *Pure and Appl. Chem.* 1995; 67: 331-343.
3. 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.
4. ISO 5725-1:1994/Cor 1:1998.
5. 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.
6. ISO 5725-2:1994/Cor 1:2002.
7. Commission Implementing Regulation (EU) 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.

8. Commission 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.
9. European Network of GMO Laboratories (ENGL). Definition of minimum performance requirements for analytical methods of GMO testing. 2015. http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm.
10. Meyer, R. (1999). Development and application of DNA analytical methods for the detection of GMOs in food. Food control, 10(6), 391-399.
11. Kharazmi, M., Bauer, T., Hammes, W. P., & Hertel, C. (2003). Effect of food processing on the fate of DNA with regard to degradation and transformation capability in Bacillus subtilis. Systematic and Applied Microbiology, 26(4), 495-501.
12. Weighardt, F. (2007). GMO quantification in processed food and feed. Nature biotechnology, 25(11), 1213-1214.
13. Lipp, M., Shillito, R., Giroux, R., Spiegelhalter, F., Charlton, S., Pinero, D., & Song, P. (2005). Polymerase chain reaction technology as analytical tool in agricultural biotechnology. Journal of AOAC International, 88(1), 136-155.
14. Spiegelhalter, F., Lauter, F. R., & Russell, J. M. (2001). Detection of genetically modified food products in a commercial laboratory. Journal of Food Science, 66(5), 634-640.
15. Gryson, N., Dewettinck, K., & Messens, K. (2007). Detection of genetically modified soy in doughs and cookies. Cereal chemistry, 84(2), 109-115.
16. Cankar, K., Štebih, D., Dreo, T., Žel, J., & Gruden, K. (2006). Critical points of DNA quantification by real-time PCR–effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. Bmc Biotechnology, 6(1), 1-15.
17. CEN/TS 17329-1:2021 (Foodstuffs - General guidelines for the validation of qualitative real-time PCR methods - Part 1: Single-laboratory validation)
18. European Network of GMO Laboratories (ENGL). Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. 2017. http://gmo-crl.jrc.ec.europa.eu/ENGL/docs/WG-MV-Report-version-2.pdf.
19. Commission Implementing Regulation (EU) No 120/2014 of 7 February 2014 amending Regulation (EC) No 1981/2006 on detailed rules for the implementation of Article 32 of Regulation (EC) No 1829/2003 of the European Parliament and the Council as regards the Community reference laboratory for genetically modified organisms.

Annex 1: Event-specific Method for the Quantification of soybean DBN-09004-6 by Real-time PCR

Validated Method

Method development:

Beijing DaBeiNong Biotechnology Co., Ltd.

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 DBN-09004-6 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 DBN-09004-6, an 89 bp fragment of the region spanning the 3' insert-to-plant junction in soybean DBN-09004-6 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 MGBNFQ (Minor Groove Binder Non-Fluorescent-Quencher) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event DBN-09004-6, a soybean taxon-specific system amplifies a 74 bp fragment of a soybean *lectin* (*Le1*) endogenous gene (Accession number, GeneBank: K00821), using *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with FAM as reporter dye at its 5' end and BHQ-1 (Black Hole Quencher® 1) as non-fluorescent quencher dye at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Cq" value. For quantification of the amount of DBN-09004-6 DNA in a test sample, Cq values for the DBN-09004-6 and the *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DBN-09004-6 DNA to total soybean DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from genetically modified and conventional soybean 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 June 2022.

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 0.025 % (related to copies GM/total haploid genome copies) in 100 ng of total suitable soybean 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 0.058 % (related to copies GM/total haploid genome copies) in 100 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 3' insert-to-plant junction in soybean DBN-09004-6 and is therefore event-specific for the event DBN-09004-6.

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 DBN-09004-6

3.2.1 General

The real-time PCR set-up for the taxon (*Le1*) and the GMO (event DBN-09004-6) 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 DBN-09004-6) 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 four samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % soybean DBN-09004-6 DNA in a total of 125 ng of soybean DNA (corresponding to 110619 soybean haploid genome copies with one haploid genome assumed to correspond to 1.13 pg of soybean genomic DNA) [(1)]. Standards S2 to S4 are to be prepared by serial dilutions (dilution factor 5 for samples S2-S3 and dilution factor 10 for standard S4) according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4
Total amount of soybean DNA in reaction (ng)	125	25	5.0	0.50
Target taxon haploid genome copies	110619	22124	4425	442
Target DBN-09004-6 copies	11062	2212	442	44

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 DBN-09004-6 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 DBN-09004-6 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix 2x (no UNG)	1x	12.5
S17A01_F (10 µM)	600 nM	1.5
S17A01_R (10 µM)	600 nM	1.5
S17A01_P* (10 µM)	200 nM	0.5
Nuclease free water	-	4.0
DNA	-	5.0
Total reaction volume:		25 µL

*TaqMan® probe labelled with 6-FAM at its 5'-end and MGBNFQ 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 (no UNG)	1x	12.5
Lec F (10 µM)	600 nM	1.5
Lec R (10 µM)	600 nM	1.5
Lec P* (10 µM)	200 nM	0.5
Nuclease free water	-	4.0
DNA	-	5.0
Total reaction volume:		25 µL

*TaqMan® probe is labelled with FAM 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 DBN-09004-6 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 DBN-09004-6 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 the DBN-09004-6 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 DBN-09004-6 and for the *Le1* reference system. Define MGBNFQ or non-fluorescent as quencher dye for DBN-09004-6 specific system and BHQ1 or non-fluorescent for *Le1* reference system. Select ROX as the passive reference dye. Enter the correct reaction volume (25 µL).
10. Run the PCR with the cycling program described in Table 4. Users of Roche LC480 instrument who plan to use the second derivative maximum analysis method are advised to program 45 cycles instead of 40, in order to be able to quantify down to Cq 40.

Table 4. Cycling program for DBN-09004-6/*Le1* assays.

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

* see comment above for Roche LC480 instruments

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 the DBN-09004-6 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 DBN-09004-6 DNA in the unknown sample, the DBN-09004-6 copy number is divided by the copy number of the soybean endogenous gene *Le1* and multiplied by 100 (GM% = DBN-09004-6/*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 mL, 1.5 mL and 5 mL or 15 mL DNAse free reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (no UNG). Applied Biosystems Cat. No. 4326614

4.3 Primers and Probes

Table 5. Primers and probes for the DBN-09004-6 and *Le1* methods

	Name	DNA Sequence (5' to 3')	Length (nt)
DBN-09004-6			
Forward primer	S17A01_F	GCC GTA TCC GCA ATG TGT TA	20
Reverse primer	S17A01_R	GCT CCA TAA ACG TGT GCT TTC A	22
Probe	S17A01_P	6-FAM-TTG TTT ACA ACT CTG TGA CCC-MGBNFQ	21
Le1			
Forward primer	Lec F	CCA GCT TCG CCG CTT CCT TC	20
Reverse primer	Lec R	GAA GGC AAG CCC ATC TGC AAG CC	23
Probe	Lec P	6-FAM-CTT CAC CTT CTA TGC CCC TGA CAC-BHQ1	24

6-FAM: 6-carboxyfluorescein; MGBNFQ: minor groove non-fluorescent quencher; BHQ1: Black Hole Quencher 1.

5. References

1. CEN/TS 17329-1:2021 (Foodstuffs - General guidelines for the validation of qualitative real-time PCR methods - Part 1: Single-laboratory validation)

GETTING IN TOUCH WITH THE EU

In person

All over the European Union there are hundreds of Europe Direct centres. You can find the address of the centre nearest you online (european-union.europa.eu/contact-eu/meet-us_en).

On the phone or in writing

Europe Direct is a service that answers your questions about the European Union. You can contact this service:

— by freephone: 00 800 6 7 8 9 10 11 (certain operators may charge for these calls),

— at the following standard number: +32 22999696,

— via the following form: european-union.europa.eu/contact-eu/write-us_en.

FINDING INFORMATION ABOUT THE EU

Online

Information about the European Union in all the official languages of the EU is available on the Europa website (european-union.europa.eu).

EU publications

You can view or order EU publications at op.europa.eu/en/publications. Multiple copies of free publications can be obtained by contacting Europe Direct or your local documentation centre (european-union.europa.eu/contact-eu/meet-us_en).

EU law and related documents

For access to legal information from the EU, including all EU law since 1951 in all the official language versions, go to EUR-Lex (eur-lex.europa.eu).

Open data from the EU

The portal data.europa.eu provides access to open datasets from the EU institutions, bodies and agencies. These can be downloaded and reused for free, for both commercial and non-commercial purposes. The portal also provides access to a wealth of datasets from European countries.

Science for policy

The Joint Research Centre (JRC) provides independent, evidence-based knowledge and science, supporting EU policies to positively impact society

EU Science Hub
joint-research-centre.ec.europa.eu

@EU_ScienceHub

EU Science Hub - Joint Research Centre

EU Science, Research and Innovation

EU Science Hub

@eu_science