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Event-specific Method for the Quantification of Soybean DAS-81419-2 by Real-time PCR

Validation Report

European Union Reference Laboratory for Genetically Modified Food and Feed

2015

Joint Research Centre

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European Union Reference Laboratory for GM Food & Feed

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Institute for Health and Consumer Protection
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13 March 2015

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate[a], the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting and quantifying the soybean event DAS-81419-2 (unique identifier DAS-81419-2). The validation study was conducted according to the EURL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and internationally accepted guidelines [1, 2].

In accordance with current EU legislation[b], Dow AgroSciences LLC provided the detection method and the samples (genomic DNA extracted from soybean grains harbouring the DAS-81419-2 event as positive control DNA, genomic DNA extracted from conventional soybean grains as negative control DNA). The EURL GMFF prepared the validation samples (calibration samples and blind samples at test GM percentage [DNA/DNA]), organised an international collaborative study and analysed the results.

The study confirms that the method meets the method performance requirements as established by the EURL GMFF and the ENGL, in line with the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004[b] and it fulfils the analytical requirements of Regulation (EU) No 619/2011[c].

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

[a] Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed".

[b] Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003". For GM plants, since July 2013 Annex III of Regulation (EU) No 503/2013 applies. This annex defines the same method performance criteria as Annex I of Regulation (EC) No 641/2004.

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

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172 (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

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

The EURL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EURL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection (IHCP) provided by SGS.

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1. Timeline

In line with Regulation (EC) No 1829/2003, Dow AgroSciences LLC provided the EURL GMFF with a copy of the official application for authorisation of an event-specific method for the detection and quantification of soybean (*Glycine max*) event DAS-81419-2 (unique identifier DAS-81419-2) together with negative and positive control samples (May 2013).

In response to an earlier submission of the method, the EURL GMFF started its step-wise validation procedure (step 1: dossier reception) before the formal approval by EFSA of the official dossier (February 2014).

The scientific dossier assessment (step 2) focused on the reported method performance characteristics assessed against the ENGL method acceptance criteria[d] (see http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf for a summary of method acceptance criteria and method performance requirements) and it was positively concluded in March 2014.

In step 3 of its validation procedure (experimental testing), 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 Reg. (EC) No 1829/2003, were found of good quality. The method characteristics were verified by quantifying five blind GM levels within the range 0.1%-5.0% on a genome copy number basis. The EURL GMFF performed the experiments under repeatability conditions and found that the PCR efficiency, linearity, trueness and precision of the optimized method were within the limits established by the ENGL.

In addition, and in line with the requirements of Reg. (EU) No 619/2011, the EURL GMFF also verified *i)* the zygosity ratio of the submitted positive control sample in order to determine the conversion factor between copy numbers and mass fractions, and *ii)* the method's precision (relative repeatability standard deviation, RSD_r) at the 0.1% level related to mass fraction of GM material. Step 3 was completed in June 2014 and concluded that the method could be finally tested in a collaborative study.

The collaborative study (step 4) took place in June-July 2014. It demonstrated that the method is well suited for analysing DNA, appropriately extracted from food or feed, and for identifying and quantifying the presence of GM event DAS-81419-2 down to a level of 0.1% m/m. The method is therefore applicable for this purpose.

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

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

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

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

The specificity of the event-specific assay was verified by the applicant and confirmed at MBG Unit by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant. The detection method reported spans the junction between the 5' genomic region and the transgenic insert. The DAS81419-f2 primer binding site was found in the genomic border adjacent to the insertion. The DAS81419-r1 primer and the DAS81419-p3 probe bind in the insert, in a region that corresponds to recombined fragments of the Cry1Ac gene. It should be noted that the reverse primer has two additional almost perfect binding sites (1 and 2 bases different, according to the submitted sequence). However, these binding sites are found far (about 5kb and 8kb) from the amplicon, and in the wrong orientation, so no secondary amplicon should be produced.

The amplicon size is expected to be 105 bp, consistent with data reported by the applicant. The sequence of the amplicon was analysed by BLAST[3] against local copies of the Nucleotide collection (nr/*nt*) and patents (patnt) databases available at ftp://ftp.ncbi.nih.gov/blast/db/ on 20 June 2013, and no significant similarity was found with any other published sequence. In addition, the primers were tested against the sequences of the other GM events present in the Central Core Sequence Information System of the JRC, as well as the whole genomes of *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays* using an e-PCR prediction tool[4, 5], and no potential amplicon was identified.

The applicant determined the slope and R^2 coefficient parameters of the event-specific method for the detection and quantification of soybean (*Glycine max*) event DAS-81419-2 on eight calibration curves and the bias and precision of quantification over five levels, respectively 5.0%, 2.0%, 0.9%, 0.5% and 0.085% expressed in copy numbers. All parameters were within ENGL acceptance criteria.

The parameters of the calibration curve (slope, R^2 coefficient) were appropriately determined by the applicant for DAS-81419-2 event-specific and *Le1* soybean-specific assays by quantifying in eight runs five test samples at different GM levels expressed in haploid genome copy number (see Table 1). The assays were performed on Agilent Mx3005P®.

Table 1. Values of slope and R^2 obtained by the applicant for DAS-81419-2 event-specific and *Le1* soybean-specific assay

	DAS-81419-2		*Le1*	
	Slope	**R^2**	**Slope**	**R^2**
Run 1	-3.39	1.00	-3.44	1.00
Run 2	-3.46	1.00	-3.48	1.00
Run 3	-3.44	1.00	-3.44	1.00
Run 4	-3.44	1.00	-3.42	1.00
Run 5	-3.43	1.00	-3.41	1.00
Run 6	-3.43	1.00	-3.45	1.00
Run 7	-3.45	1.00	-3.46	1.00
Run 8	-3.43	1.00	-3.51	1.00
Mean	-3.43	1.00	-3.45	1.00

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall be within the range of -3.1 to -3.6, and the R^2 coefficient shall be ≥ 0.98. Table 1 indicates that the mean slope of the standard curves is -3.43 and -3.45 for the DAS-81419-2 and for the *Le1* methods, respectively, and that the mean R^2 coefficient for the DAS-81419-2 and for the soybean-specific reference system (*Le1*) is 1.00. Therefore all values are within ENGL acceptance criteria.

Table 2 reports precision (measured as relative repeatability standard deviation RSD_r) and trueness (bias) for the five GM levels tested by the applicant. Sixteen values for each GM level were provided, which is fully in line with EURL GMFF guidance. The mean values of trueness and precision were within the ENGL acceptance criteria (trueness ± 25%, RSD_r ≤ 25% across the entire dynamic range).

Table 2. Mean %, precision and trueness (measured at five GM levels by the applicant)

	Test results				
Expected GMO %	**0.085**	**0.5**	**0.9**	**2.0**	**5.0**
Measured mean %	0.078	0.51	0.93	2.2	5.3
Precision (RSDr %)	11	5.7	9.1	6.4	9.1
Trueness (bias %)	-8.2	2.0	3.3	10.0	6.0

Acceptable performance was also observed when the method was tested by the applicant on ABI 7900HT and ABI 7500 (data not shown).

3. Step 3 (EURL GMFF experimental testing of the samples and method) and step 4 (international collaborative study)

3.1 DNA extraction

A "CTAB-Anion-Exchange" DNA extraction method from soybean grains was submitted by the method developer for extracting genomic DNA from event DAS-81419-2 and non-GM soybean grains. This DNA extraction method was previously assessed for DAS-68416-4 by the EURL GMFF. The assessment report is published at http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-11-10-XP-Report-DNA-Ext.pdf. In agreement with the ENGL position, which endorses the modularity principle (see also Annex I to Reg. (EC) No 641/2004), and given the similarity in the matrix, the EURL GMFF considers the above mentioned DNA extraction protocol applicable for the validation of the method for soybean event DAS-81419-2.

The positive and negative DNA control samples submitted to the EURL GMFF by the applicant were isolated using a large-scale DNA extraction method that combines cetyltrimethyl ammonium bromide (CTAB)-based lysis, phenol/chloroform/isoamylalcohol and chloroform/isoamylalcohol extraction of the lysate with isopropanol precipitation of the DNA, different from the DNA extraction method submitted for this application. After re-suspension, the precipitated DNA was further purified by anion exchange chromatography.

3.2 Method protocol for the PCR analysis

The PCR method provided by the applicant (see the corresponding Validated Method at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and Annex 1 of this report) and subsequently validated in a collaborative trial by the EURL GMFF is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event DAS-81419-2 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean *lectin* (*Le1*) specific assay and the GM target assay (DAS-81419-2) are performed in separate wells.

For the specific detection of soybean event DAS-81419-2, a 105 bp fragment of the region spanning the 5′ plant-to-insert junction in soybean event DAS-81419-2 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein), as a reporter at its 5′ end, and TAMRA (carboxytetramethylrhodamine) as a quencher at its 3′ end.

As a *Glycine max* specific system a fragment of the *lectin* (*Le1*) endogenous gene (Accession Number GeneBank: K00821 and M30884), is amplified using gene-specific primers and a probe, labelled with FAM as reporter dye at its 5′ end, and TAMRA as quencher at its 3′ end. The amplified *Le1* fragment is 74 bp long. This soybean-specific PCR method had been previously validated (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

For relative quantification of event DAS-81419-2 in a test sample, standard curves are generated for both the DAS-81419-2 and the *Le1* systems by plotting the Ct 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 copy numbers in the test sample DNA are estimated by interpolation from the standard curves. The DAS-81419-2 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 to obtain the percentage value (GM% = DAS-81419-2/*Le1* x 100).

The GM % content of the calibration samples is calculated considering the 1C value for soybean genomes as equivalent to 1.13 pg (Plant DNA C-values Database [(6)]).

3.3 EURL GMFF experimental testing (step 3)

3.3.1 Determination of the zygosity ratio in the positive control sample

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

The copy number of the DAS-81419-2 and *Le1* targets was determined by digital PCR (dPCR) on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix no UNG (Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probe at the reaction concentrations indicated in the corresponding Validated Method document (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx) and 1 µL of DNA at a concentration of 1.13 ng/µL, to avoid panel saturation after analysis (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). A volume of 9 µL of reaction mix was loaded into each well of which only 4.6 µL were distributed into the 765 partitions (or chambers) constituting one panel. Five replicates of the same dilution were loaded in five panels for both the GM- and the taxon-specific assay. The experiments were repeated three times for a total number of fifteen data sets for both targets. No template controls were included. Amplification conditions were as reported in the Validated Method document (see Annex 1). Data analysis and copy number calculations were performed using the BioMark digital PCR Analysis software using a range of Ct retention from 20 to 40.

Calculations of mean and variances were carried out according to the procedure outlined for random variables in the Annex 4 of the ENGL guidance document 'Verification of analytical

methods for GMO testing when implementing inter-laboratory validated methods' (http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf).

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

The method performance characteristics were verified (EURL GMFF step 3) by quantifying on a copy number basis five blinded test samples with known GM levels, within the range 0.1%-5.0% copy/copy (equivalent to mass/mass). The experiments were performed on an ABI 7900, ABI 7500 and Agilent Mx3005P® real-time platforms under repeatability conditions. Test samples with GM levels 5.0%, 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% copy/copy (equivalent to 0.1% in mass fractions of GM material) was tested in 15 replicates in an additional real-time PCR run. Average values of the slope and of the R^2 coefficient of the standard curves and method trueness and precision of quantification over the dynamic range were evaluated for compliance against the ENGL method acceptance criteria.

In order to assess the method compliance with Reg. (EU) No 619/2011, the EURL GMFF estimated also the method precision (RSD_r) at 0.1% GM level in mass fraction (m/m) on 15 replicates.

3.4 International collaborative study (step 4)

The international collaborative study (EURL GMFF step 4) involved 12 laboratories, all being "National Reference Laboratories (NRL), assisting the CRL for testing and validation of methods for detection", as listed in annex to Commission Implementing Regulation (EU) No 120/2014. The study was carried out in accordance with the following internationally accepted guidelines:

- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) (1)
- ISO 5725 (1994) (2)

The objective of the international collaborative study was to verify in experienced laboratories the trueness and reproducibility of the PCR analytical method that was provided by the applicant and which is described under 3.2 above and in the "Validated Method" (Annex 1).

3.4.1 List of participating laboratories

The 12 participants in the DAS-81419-2 validation study (see Table 3) were randomly selected from the 30 NRLs that offered to participate.

Clear guidance was given to the selected laboratories for strictly following the standard operational procedures provided for the execution of the protocol (the protocol is detailed in

the Validated Method, available in Annex 1 and at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Table 3. Laboratories participating in the international validation study of the detection method for soybean event DAS-81419-2

Laboratory	Country
AGES - Austrian Agency for Health and Food Safety	AT
Agricultural Institute of Slovenia	SI
BioGEVES - Groupement d'Intérêt Public - Groupe d'Etude et de contrôle des Variétés et des Semences	FR
CRA-SCS Sede di Tavazzano - Laboratorio, Via Emilia km 307 - 26838 Tavazzano (LO) Italy	IT
Federal Office of Consumer Protection and Food Safety - Berlin	DE
State Veterinary and Food Institute Dolny Kubin	SK
Institute for National Investigation for the Health and Veterinarian Nature Saxonia-Dresden	DE
LGC Limited	UK
National Food Agency, Science Department	SE
National Institute of Biology	SI
Plant Health Laboratory	FR
Walloon Agricultural Research Centre - Department Valorization des productions (D4) - Unit 16 - Authentication and traceability	BE

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: ABI 7500 (three laboratories), ABI 7500 fast (two laboratories), ABI 7900 (three laboratories), ABI 7900 fast (one laboratory), Roche LC480 (one laboratory), Bio-Rad iQ5 (one laboratory) and Agilent Mx3005P (one laboratory).

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

3.4.3 Materials used in the international collaborative study

For the validation of the quantitative event-specific method control samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11[e]. The genomic DNA was extracted by the applicant from:

i) soybean grains harbouring the event DAS-81419-2 in homozygous status, and
ii) conventional soybean grains genetically similar to those harbouring the DAS-81419-2 event.

These positive and negative control samples were also used by the EURL GMFF for preparing standards (of known GM-content) and test samples (of unknown GM-content), containing mixtures of DAS-81419-2 soybean DNA and non-GM soybean DNA. The GM-DNA copy numbers were calculated in terms of haploid genomes in relation to target taxon-specific DNA copy numbers.

The calibration sample S1 was prepared as 10% GM solution. Calibration samples S2 – S5 were prepared by serial four-fold dilution from the S1 sample.

The total amount of DNA/reaction of standards S1 to S5 is reported in Table 4.

Table 4. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	125	31.2	7.8	1.95	0.49
Target taxon *Le1* copies	110619	27655	6914	1728	432
DAS-81419-2 soybean GM copies	11062	2765	691	173	43

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 4),
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution each at 20 ng/µL) labelled from U1 to U20, representing five GM levels (Table 5).

[e] 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).

Table 5. DAS-81419-2 GM contents in blinded DNA samples

DAS-81419-2 GM % GM copy number/soybean genome copy number x 100
5.00
2.00
0.90
0.50
0.10

- ✓ Reaction reagents:
  - TaqMan® Universal Master Mix, No UNG (2x), one vial: 8 mL
  - distilled sterile water, one vial: 3.5 mL

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

  Le1 taxon-specific assay
  - Lec for2 (10 µM): 520 µL
  - GMO3-126 Rev (10 µM): 520 µL
  - Lec probe (10 µM): 144 µL

  DAS-81419-2 assay
  - DAS81419-f2 (10 µM): 320 µL
  - DAS81419-r1 (10 µM): 320 µL
  - DAS81419-p3 (10 µM): 96 µL

3.4.4 Design of the collaborative study

Participating laboratories received a detailed validation protocol that included, inter alia, the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the DAS-81419-2 event-specific system and for the *Le1* taxon-specific system.

Each participant received twenty blind samples containing mixtures of soybean DAS-81419-2 genomic DNA and non-GM soybean genomic DNA at five GM contents, ranging from 0.1% to 5% (copy/copy). Each laboratory received each GM level in four blind replicates. Each test sample was analysed by PCR in three repetitions. Two replicates of each GM level were analysed on the same PCR plate. In total, two plates were run per participating laboratory.

The laboratories prepared the master-mixes for the soybean event DAS-81419-2 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 and amplified following the cycling program specified in the protocol. The raw data were reported to the EURL GMFF on an excel sheet that was designed, validated and distributed by the EURL GMFF. Participants determined the GM % in the test samples according to the instructions and using the excel sheet provided. All data were stored by the EURL GMFF on a dedicated and protected server.

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

3.4.5 Deviations reported from the protocol

One laboratory performed the two PCR runs with 45 cycles instead of 40, and reported amplification at 38.9 Cq in one no template control replicate.

Data sets from three laboratories were analysed using automatic baseline and /or threshold, instead of manual baseline and threshold. In both cases the laboratories were able to quantify the unknown samples with acceptable trueness and precision, and the parameters of the standard curves did not change substantially when the data were re-analysed by the EURL GMFF with manual baseline and threshold.

4. Results

4.1 EURL GMFF experimental testing

4.1.1 Zygosity ratio in the positive control sample

A summary of the dPCR analysis conducted on the positive control sample for both the event DAS-81419-2 and the *Le1* targets is shown in Table 6. The results were determined on a total of fifteen data sets.

Table 6. Summary of dPCR analysis conducted on the DAS-81419-2 and *Le1* targets in the positive control sample.

Mean ratio (DAS-81419-2/*Le1)* *	0.986
Standard deviation	0.071
RSD_r %	7.2
Standard error of the mean	0.018
Upper 95 % CI of the mean	1.024
Lower 95 % CI of the mean	0.949

* Mean of fifteen datasets

As indicated in the table the 95% confidence interval (CI) spans around 1 (from 0.949 to 1.024) and therefore the mean ratio is not significantly different from an expected ratio of 1, assuming a homozygous GM target and a single copy reference target, for an alpha = 0.05.

Hence: GM % in DNA copy number ratio = GM % in mass fraction

The GM concentration of 0.1%, expressed in terms of GM DNA copy numbers in relation to target taxon specific copy numbers, corresponds to the same GM concentration (0.1%) related to mass fraction of GM material.

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

Test samples with GM levels 5.0%, 2.0%, 0.9% and 0.5% (copy/copy), were tested in two real-time PCR runs (run A and B on ABI 7900, run D and E on ABI 7500 and run G and H on Agilent Mx3005P) 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% mass/mass was tested in 15 replicates in one run (run C on ABI 7900, run F on ABI 7500 and run I on Agilent Mx3005P).

The corresponding standard curve parameters and the results of efficiency, linearity (R^2), trueness and precision are shown in Tables 7a, 7b, 7c and in Tables 8a, 8b and 8c.

Table 7a. Standard curve parameters of the real-time PCR testing carried out on ABI 7900

	DAS-81419-2 method			***Le1* reference method**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.39	97	1.00	-3.32	100	1.00
Run B	-3.38	98	1.00	-3.39	97	1.00
Run C	-3.44	95	1.00	-3.41	96	1.00

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

Table 7b. Standard curve parameters of the real-time PCR testing carried out on ABI 7500

	DAS-81419-2 method			***Le1* reference method**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run D	-3.39	97	1.00	-3.38	98	1.00
Run E	-3.33	100	1.00	-3.36	98	1.00
Run F	-3.39	97	1.00	-3.35	99	1.00

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

Table 7c. Standard curve parameters of the real-time PCR testing carried out on Agilent Mx3005P

	DAS-81419-2 method			***Le1* reference method**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run G	-3.46	95	0.99	-3.48	94	1.00
Run H	-3.36	98	1.00	-3.44	95	1.00
Run I	-3.29	101	1.00	-3.45	95	1.00

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

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall fall into the range -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98.
Tables 7a, 7b and 7c document that the slope of the standard curve and the R^2 coefficient were within the limits established by the ENGL.

Table 8a. Outcome of the *in-house* tests, with regards to the quantification of the five test samples. Testing carried out on ABI 7900.

Run	Target GM-levels % (copy/copy)	Measured GM level %	Bias %	Precision (RSD_r %)
Run A and Run B	5.0	5.0	0.18	4.4
	2.0	1.78	-11	5.1
	0.9	0.83	-7.4	6.8
	0.5	0.45	-9.6	5.0
Run C	0.1	0.09	6.9	8.5

Table 8b. Outcome of the *in-house* tests, with regards to the quantification of the five test samples. Testing carried out on ABI 7500.

Run	Target GM-levels % (copy/copy)	Measured GM level %	Bias %	Precision (RSD_r %)
Run D and Run E	5.0	5.1	2.4	1.45
	2.0	1.89	-5.6	2.6
	0.9	0.82	-8.8	6.9
	0.5	0.45	-9.5	5.6
Run F	0.1	0.09	14	12

Table 8c. Outcome of the *in-house* tests, with regards to the quantification of the five test samples. Testing carried out on Agilent Mx3005P.

Run	Target GM-levels % (copy/copy)	Measured GM level %	Bias %	Precision (RSD_r %)
Run G and Run H	5.0	5.4	7.7	2.6
	2.0	2.0	0.71	6.3
	0.9	0.92	2.3	1.55
	0.5	0.49	-1.95	2.8
Run I	0.1	0.08	21	13

According to the ENGL method acceptance criteria, the method trueness (measured as bias %) should be within ±25% of the target value over the entire dynamic range. The method's precision estimated through relative standard deviation of repeatability (RSD_r) should be ≤25% over the entire dynamic range. Tables 8a, 8b and 8c document that trueness and precision of quantification were within the limits established by the ENGL. The EURL GMFF *in-house* results confirmed the data provided by the applicant.

4.2 International collaborative study

4.2.1 PCR efficiency and linearity

The PCR efficiency (%) and R^2 values (expressing the linearity of the regression) for the standard curves, reported by participating laboratories are displayed in Table 9. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

$$\text{Efficiency} = (10 * (-1/\text{slope})) - 1) \times 100.$$

Table 9 indicates that the efficiency of amplification for the DAS-81419-2 system ranges from 78% to 98% and the linearity from 0.98 to 1.00; the amplification efficiency for the soybean specific system ranges from 88% to 98% and the linearity from 0.99 to 1.00. The mean PCR efficiency is 92% for the DAS-81419-2 assay and 93% for the *Le1* assay. Both values were within the ENGL acceptance criteria. The average R^2 of the methods is 1.00 for both the DAS-81419-2 and *Le1* assays.

Table 9. Values of slope, PCR efficiency and R^2 obtained during the validation study

		DAS-81419-2			***Le1***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.38	98	0.98	-3.51	93	1.00
	B	-3.47	94	0.99	-3.49	93	1.00
2	A	-3.55	91	1.00	-3.48	94	1.00
	B	-3.40	97	1.00	-3.45	95	1.00
3	A	-3.59	90	1.00	-3.62	89	1.00
	B	-3.44	95	0.99	-3.44	95	0.99
4	A	-3.56	91	1.00	-3.45	95	1.00
	B	-3.55	91	1.00	-3.38	98	1.00
5	A	-3.51	93	1.00	-3.56	91	1.00
	B	-3.47	94	1.00	-3.53	92	1.00
6	A	-3.99	78	0.99	-3.66	88	1.00
	B	-3.67	87	1.00	-3.66	88	1.00
7	A	-3.56	91	1.00	-3.48	94	1.00
	B	-3.56	91	1.00	-3.47	94	1.00
8	A	-3.67	87	1.00	-3.61	89	1.00
	B	-3.50	93	1.00	-3.62	89	1.00
9	A	-3.48	94	1.00	-3.37	98	1.00
	B	-3.52	92	1.00	-3.40	97	1.00
10	A	-3.49	93	1.00	-3.57	91	1.00
	B	-3.47	94	1.00	-3.53	92	1.00
11	A	-3.71	86	1.00	-3.58	90	1.00
	B	-3.60	89	1.00	-3.56	91	1.00
12	A	-3.53	92	1.00	-3.45	95	1.00
	B	-3.45	95	1.00	-3.46	94	1.00
	Mean	**-3.55**	**92**	**1.00**	**-3.51**	**93**	**1.00**

These results confirm the appropriate performance of the method tested in terms of efficiency and linearity. The different machines used (see chapter 3.4.2) had no significant influence on the results.

4.2.2 GMO quantification

Table 10 reports the values of quantification for the four replicates of each GM level as generated by each of the 12 participating laboratories, before application of the Cochran's and Grubbs' tests, which according to ISO 5725 are to be performed for identifying outlying values.

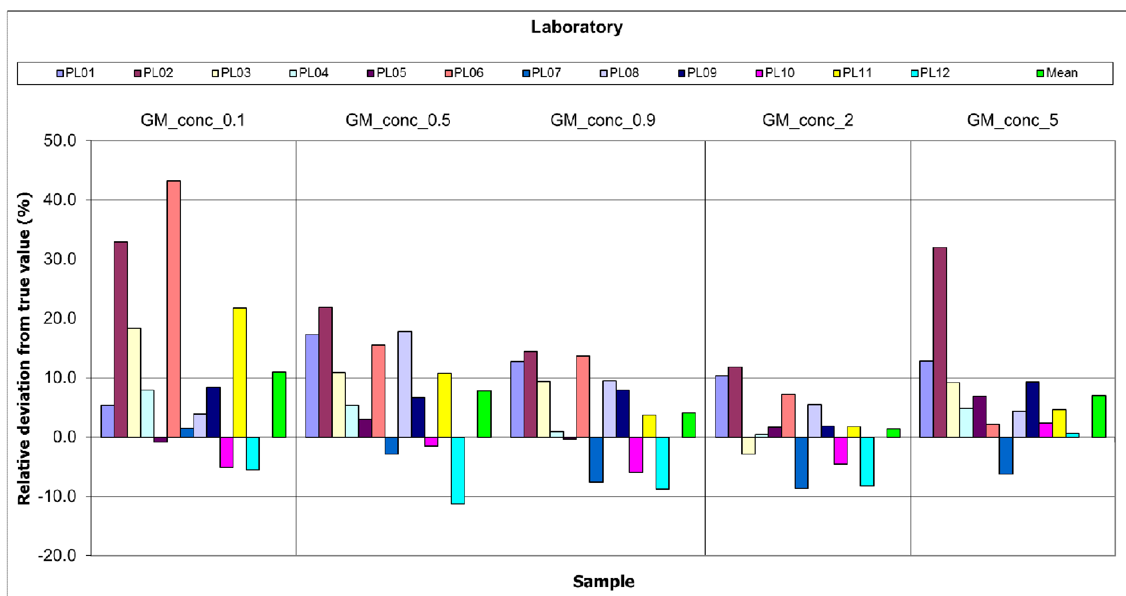
Table 10. GM% values determined by laboratories for test samples, including outliers

	GMO content (%) *																			
Lab	**0.1**				**0.5**				**0.9**				**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	0.09	0.12	0.11	0.10	0.55	0.61	0.57	0.61	1.09	1.07	0.96	0.94	2.12	2.11	2.39	2.21	5.81	5.03	6.17	5.56
2	0.17	0.10	0.12	0.14	0.53	0.64	0.69	0.58	1.02	1.04	0.99	1.07	2.15	2.71	2.10	1.99	6.84	7.48	5.37	6.72
3	0.13	0.11	0.09	0.15	0.50	0.47	0.61	0.65	0.87	0.98	1.06	1.02	1.87	1.64	2.37	1.89	5.29	4.94	5.68	5.94
4	0.12	0.10	0.09	0.12	0.52	0.52	0.53	0.54	0.96	0.86	0.89	0.92	2.02	2.05	2.11	1.87	5.42	5.36	5.03	5.17
5	0.11	0.09	0.09	0.11	0.50	0.51	0.54	0.52	0.89	0.91	0.90	0.89	2.04	2.01	2.00	2.10	5.46	5.36	5.13	5.43
6	0.17	0.11	0.12	0.17	0.51	0.51	0.71	0.58	1.04	0.85	1.11	1.09	2.07	2.17	2.26	2.08	4.94	5.24	5.13	5.13
7	0.10	0.10	0.11	0.10	0.48	0.51	0.45	0.50	0.84	0.85	0.79	0.84	1.89	1.81	1.91	1.69	4.67	4.45	4.93	4.71
8	0.11	0.09	0.10	0.11	0.54	0.59	0.59	0.64	0.93	0.96	0.99	1.06	2.13	2.11	2.05	2.15	5.11	5.22	5.41	5.15
9	0.11	0.09	0.11	0.12	0.48	0.48	0.59	0.59	0.89	0.99	0.98	1.02	1.93	1.96	1.99	2.27	5.74	5.17	4.86	6.10
10	0.10	0.10	0.09	0.09	0.48	0.52	0.50	0.47	0.79	0.96	0.80	0.84	1.78	1.91	1.91	2.04	5.10	5.36	5.03	4.97
11	0.13	0.10	0.12	0.13	0.53	0.49	0.59	0.60	0.88	0.95	0.98	0.92	2.11	1.94	1.97	2.13	5.20	5.52	4.90	5.32
12	0.10	0.09	0.09	0.10	0.43	0.41	0.51	0.43	0.85	0.79	0.85	0.79	2.10	1.81	1.80	1.63	5.07	5.05	5.16	4.86

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

A graphical representation of the data reported in Table 10 is provided in Figure 1 where the relative deviation from the target value for each GM level tested is shown for each laboratory. The coloured bars represent the deviation of the GM level measured by the respective laboratory in % (copy/copy) of the true GM level; the light green bar on the right represents the mean relative deviation for each true GM level over all 12 participating laboratories.

Figure 1. Relative deviation (%) from the target value of event DAS-81419-2 concentration (% copy/copy,) for all laboratories



Two laboratories overestimated the GM-content of the 0.1 % sample of more than 25%; one laboratory overestimated the 5% sample of more than 25%. Overall the relative deviations from the target values were within a maximum of ±25%, with a trend towards overestimation at lower levels for all laboratories.

All data were retained for the statistical analysis and for tests of outliers (Cochran and Grubbs) whose results are reported in Table 11.

4.2.3 Method performance requirements

According to the method performance requirements established by the ENGL and adopted by the EURL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), reproducibility is assessed through an international collaborative trial. Table 11 illustrates the estimation of reproducibility at the various GM levels tested during the collaborative trial.

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

As it can be observed in Table 11, the method satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R is 19% at the 0.1% GM level, thus within the acceptance criterion.

Table 11. Summary of validation results for the DAS-81419-2 method

	Test Sample expected GMO % (*)				
	0.1	**0.5**	**0.9**	**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
Reason for exclusion	-	-	-	-	C
Mean value of measured GM content (%)	0.11	0.54	0.94	2.0	5.2
Relative repeatability standard deviation, RSD_r (%)	15	9.9	6.5	8.2	5.7
Repeatability standard deviation	0.017	0.053	0.061	0.167	0.297
Relative reproducibility standard deviation, RSD_R (%)	19	12	9.9	9.7	6.9
Reproducibility standard deviation	0.021	0.067	0.093	0.196	0.362
Bias (absolute value)	0.011	0.039	0.037	0.027	0.232
Bias (%)	11	7.8	4.1	1.4	4.6

* GMO % expressed as copy/copy.
C= Cochran's test; G= Grubbs' test; DG= Double Grubbs test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2 and bias is estimated according to ISO 5725 data analysis protocol (2)

Table 11 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for a collaborative study, the EURL GMFF requires the RSD_r value to be below 25%, as indicated by the ENGL (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 method showed a relative repeatability standard deviation below 25% at all GM levels, with the highest value of RSD_r being 15% at the 0.1% GM level.

The trueness of the method is estimated in the collaborative trial 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 (%) being 11% at the 0.1% GM level.

5. Compliance of the method for detection of event DAS-81419-2 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 assessments were carried out:

- at step 2 of the validation process (scientific assessment of the dossier), the applicant's data (Table 2) indicated that the RSD_r at the level of 0.085%, expressed as ratio of GM-DNA copy numbers to target taxon-specific DNA copy numbers, was 11%, hence below 25%. The results had been determined on 16 replicates. The EURL GMFF accepted the applicant's data;

- at step 3 of the validation process (*in-house* testing of the method), the EURL GMFF determined the RSD_r at the level of 0.1% (m/m) (expressed as mass fraction of GM-material and corresponding to the same level expressed in terms of copy number ratio). The measurements were carried out under repeatability conditions on 15 replicates. The RSD_r resulted to be 8.5% on real-time PCR platform ABI 7900, 12% on real-time PCR platform ABI 7500 and 13% on real-time PCR platform Agilent Mx3005P, respectively (Tables 8a, 8b and 8c), hence below 25%;

- the collaborative study (step 4 of the validation process) established that over the twelve participating laboratories the mean RSD_r of the method at the level of 0.1% (m/m, corresponding to the same level expressed in terms of copy number ratio) was 15%, therefore below the limit of 25%.

The outcome of these tests is summarised in Table 12.

Table 12. Precision of the method for quantitative detection of event DAS-81419-2 at or around 0.1% level related to mass fractions of GM material

Source	**Real-time PCR**	**GM %**	**RSD_r %**
Applicant's method optimisation	Agilent Mx3005P	0.085%	11%
EURL GMFF in-house verification	ABI 7900	0.1%	8.5%
	ABI 7500		12%
	Agilent Mx3005P		13%
Collaborative study	§ 3.4.2	0.1%	15%

Based on the results of the *in-house* verification and of the 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 for quantitative detection of soybean event DAS-81419-2 meets the requirement laid down in Regulation (EU) No 619/2011.

6. Conclusion

A method for detection, identification and quantification of GM event DAS-81419-2 was provided by the applicant. It is described in detail under 3.2 (and available as "Validated Method" at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1). This method has been fully validated in accordance to the EURL GMFF validation scheme (step 1, 2, 3 and 4), 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 3.4.3), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004 and (EU) No 619/2011. The method is therefore valid to be used for control purposes, including the quantification of low level presence of 0.1% (m/m). It can be assumed that it is applicable to any appropriately extracted soybean DNA.

7. References

1. Horwitz W. Protocol for the design, conduct and interpretation of method- performance studies, *Pure and Appl. Chem.* 1995; 67: 331-343.
2. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
3. Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res, 1997; 25: 3389-3402.
4. Schuler G.D. Sequence mapping by electronic PCR. Genome Res, 1997; 7(5):541-50
5. Rotmistrovsky K., Jang W., Schuler G.D. A web server for performing electronic PCR. Nucleic Acids Res, 2004; 32(Web Server issue):W108-112.
6. Plant DNA C-values Database, http://data.kew.org/cvalues/.

Annex 1: Event-specific Method for the Quantification of Soybean DAS-81419-2 by Real-time PCR

Validated Method

Method development:

Dow AgroSciences LLC

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 (*Glycine max*) event DAS-81419-2 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 a PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of soybean event DAS-81419-2, a 105 bp fragment of the region spanning the 5′ plant-to-insert junction in soybean event DAS-81419-2 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein), as a reporter at its 5′ end, and TAMRA (carboxytetramethylrhodamine) as a quencher at its 3′ end.

For the relative quantification of soybean event DAS-81419-2 DNA, a *Glycine max* specific system amplifies a fragment of the *lectin (Le1)* endogenous gene, using *(Le1)* gene-specific primers and a *(Le1)* gene-specific probe, labelled with FAM as reporter dye at its 5′ end, and TAMRA as quencher at its 3′ end. The amplified *Le1* fragment is 74 bp long.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of DAS-81419-2 DNA in a test sample, Ct values for the DAS-81419-2 and *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DAS-81419-2 DNA to total soybean DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional soybean grains. The reproducibility 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 12 participating laboratories in June-July 2014.

Each participant received twenty blind samples containing mixtures of soybean DAS-81419-2 genomic DNA and non-GM soybean genomic DNA at five GM contents, ranging from 0.1% to 5% (copy/copy). Each laboratory received each GM level in four blind replicates. Each test sample was analysed by PCR in three repetitions. Two replicates of each GM level were analysed on the same PCR plate.

The 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 0.04% (GM DNA copy numbers to target taxon-specific DNA copy numbers) in 100 ng of total 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 0.085% (GM-DNA copy numbers to target taxon-specific DNA copy numbers) in 100 ng of total soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1% (mass/mass) equivalent to level 0.1% expressed in terms of copy number ratio).

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' plant-to-insert junction in soybean event DAS-81419-2; the sequence is specific to event DAS-81419-2 and thus imparts event-specificity to the method.

The specificity of the event-specific assay was assessed by the method developer in real-time PCR using genomic DNA (100 ng) containing 1% of the GMO extracted from DAS-81419-2 soybean (positive control sample) and from maize events GA21, Bt176, Bt11, NK603, MON863, TC1507, MIR604, MON810, DAS59122, 3272, MON88017, MON89034, T25, MIR162, 98140; soybean DAS-28129-1, DAS-81615-9, DAS-28151-5, DAS-68416-4, A2704-12, A5547-127, DP305423, DP356043, GTS 40-3-2, MON89788, FG72, MON87701, CV127, DAS-44406-6; oilseed rape Ms8, RF3, T45, GT73/RT73, Topas 19/2, Ms1, Rf1, Rf2; cotton GHB614, MON1445, MON531, MON15985, 281-24-236 x 3006-210-23, LLCotton25, GHB119, T304-40, MON88913; sugar beet H7-1; potato EH92-527-1 and AM04-1020; rice LLRICE62 and conventional soybean, oilseed rape, rice, maize, potato, sugar beet, wheat, cotton.

According to the method developer, the forward and reverse oligonucleotide primers and the TaqMan® probe of the DAS-81419-2 method showed no amplification signals in the quantitative PCR analysis of the samples, apart from the soybean event DAS-81419-2. Specificity was further verified *in silico* by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

The detection method reported spans the junction between the 5' genomic region and the transgenic insert.

The DAS81419-f2 primer binding site was found in the genomic border adjacent to the insertion. The DAS81419-r1 primer and the DAS81419-p3 probe bind in the insert, in a region that corresponds to recombined fragments of the Cry1Ac gene.

It should be noted that the reverse primer has two additional almost perfect binding sites (1 and 2 bases different, according to the submitted sequence). However, these binding sites are found far (about 5kb and 8kb) from the amplicon, and in the wrong orientation, so no secondary amplicon should be produced.

The amplicon size is expected to be 105 bp, consistent with data reported by the applicant. The sequence of the amplicon was analysed by BLAST[1] against local copies of the Nucleotide collection (nr/*nt*) and patents (patnt) databases available at ftp://ftp.ncbi.nih.gov/blast/db/ on 20 June 2013, and no significant similarity was found with any other published sequence.
In addition, the primers were tested against the sequences of the other GM events present in the Central Core Sequence Information System of the JRC, as well as the whole genomes of *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays* using the e-PCR prediction tool[2, 3], and no potential amplicon was identified.

3. Procedure

3.1 General instructions and precautions

- The procedure requires 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 employed previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All equipment should be sterilised prior to use and any residue of DNA has to be 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 frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps, unless specified otherwise, should be carried out at 0 – 4°C.
- In order to avoid repeated freeze/thaw cycles, aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of soybean event DAS-81419-2

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*Le1)* and for the GMO (event DAS-81419-2) target sequence is carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated and is therefore not recommended.

The method is developed and validated for a total volume of 25 µL per reaction mixture with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

To establish the calibration curve five samples should be prepared and analysed. The range of GM contents in the calibration curve should be equal or included in the range validated during the international collaborative trail.

For the collaborative trial, the calibration curve was established on the basis of five samples. The first point of the calibration curve (S1) contained 10% soybean event DAS-81419-2 DNA (GM-DNA copy numbers to target taxon-specific DNA copy numbers) in a total of 125 ng of soybean DNA (GM% calculated considering the 1C value for soybean genome as 1.13 pg)[(4)]. Standards S2 to S5 were prepared by serial four-fold dilution of the S1 sample.

The copy number values of the calibration samples and total amount of DNA/reaction are reported in Table 1.

Table 1. Total amount of DNA in PCR reaction and copy number values of the standard curve samples.

Sample	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	125	31.2	7.8	1.95	0.49
Target taxon *Le1* copies	110619	27655	6914	1728	432
DAS-81419-2 soybean GM copies	11062	2765	691	173	43

A calibration curve is produced by plotting the Ct 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 of the real-time PCR equipment.

3.3 Real-time PCR set-up

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.

2. To prepare the amplification reaction mixtures add the following components (Table 2 and 3) in two reaction tubes (one for the DAS-81419-2 assay and one for the *Le1* assay) on ice in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the DAS-81419-2 assay.

Component	Final concentration	µL/reaction
2x TaqMan® Universal Master Mix, No UNG	1x	12.5
DAS81419-f2 forward primer (10 µM)	400 nM	1.00
DAS81419-r1 reverse primer (10 µM)	400 nM	1.00
DAS81419-p3 probe (10 µM)	120 nM	0.30
Nuclease free water	/	5.20
Template DNA	/	5.0
Total reaction volume:		25 µL

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

Component	Final concentration	µL/reaction
2x TaqMan® Universal Master Mix, No UNG	1x	12.5
Lec for2 forward primer (10 µM)	650 nM	1.625
GMO3-126 Rev reverse primer (10 µM)	650 nM	1.625
Lec probe (10 µM)	180 nM	0.45
Nuclease free water	/	3.80
Template DNA	/	5.0
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.
4. Prepare two reaction tubes (one for the soybean DAS-81419-2 and one for the *Le1* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).

5. Add to each reaction tube the correct amount of reaction mix for 3.5 PCR repetitions (e.g. (70 µL for the *Le1* reference system and 70 µL for the DAS-81419-2 soybean system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (e.g. 17.5 µL DNA). The additional 0.5 repetition included will ensure adequate volume when loading the samples.

Vortex each tube for approx. 10 sec. This step is mandatory for minimising the variability among the repetitions of each sample.

6. Spin down the tubes in a micro-centrifuge. Aliquot 25 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4°C) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for DAS-81419-2/*Le1* assays.

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

3.4 Data analysis

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

a) Set the threshold: display the amplification curves of one assay (e.g. DAS-81419-2) 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 Ct 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: 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 Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a), b) and c) on the amplification plots of the other system (e.g. *Le1*).

e) Save the settings and export all the data to a text file for further calculations.

3.5 Calculation of results

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

The standard curves are generated both for the *Le1* and the DAS-81419-2 specific assays by plotting the Ct 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 DAS-81419-2 DNA in the unknown sample, the DAS-81419-2 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 (GM% = DAS-81419-2 /*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)
- Plastic reaction vessels suitable for real-time PCR instruments (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Centrifuge for PCR-plates
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix, No AmpErase® UNG. Applied Biosystems Part No 4324020.

4.3 Primers and Probes

Oligonucleotide	Name	DNA Sequence (5' to 3')	Length (nt)
Event DAS-81419-2			
Forward primer	DAS81419-f2	5'-TCT AgC TAT ATT Tag CAC TTg ATA TTC AT-3'	29
Reverse primer	DAS81419-r1	5'-gCT TCA AgA TCC CAA CTT gCg-3'	21
Probe	DAS81419-p3	5'-FAM-ATC AAC Agg CAC CgA TgC gCA CCg-TAMRA-3'	24
Le1			
Forward primer	Lec F	5'-CCA gCT TCg CCg CTT CCT TC-3'	20
Reverse primer	Lec R	5'-gAA ggC AAg CCC ATC TgC AAg CC-3'	23
Probe	Lec P	5'-FAM-CTT CAC CTT CTA TgC CCC TgA CAC-TAMRA-3'	24

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine

5. References

1. Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res, 1997; 25: 3389-3402
2. Schuler G.D. Sequence mapping by electronic PCR. Genome Res, 1997; 7(5):541-50
3. Rotmistrovsky K., Jang W., Schuler G.D. A web server for performing electronic PCR. Nucleic Acids Res, 2004; 32(Web Server issue):W108-112
4. Plant DNA C-values Database, http://data.kew.org/cvalues/

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European Commission
Joint Research Centre – Institute for Health and Consumer Protection

Title: **Event-specific Method for the Quantification of Soybean DAS-81419-2 by Real-time PCR**

Author(s): European Union Reference Laboratory for GM Food and Feed

2015 – 34 pp. – 21.0 x 29.7 cm

Abstract

In line with its mandate , the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting and quantifying the soybean event DAS-81419-2 (unique identifier DAS-81419-2). The validation study was conducted according to the EURL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and internationally accepted guidelines (1, 2).

In accordance with current EU legislation , Dow AgroSciences LLC provided the detection method and the samples (genomic DNA extracted from soybean grains harbouring the DAS-81419-2 event as positive control DNA, genomic DNA extracted from conventional soybean grains as negative control DNA). The EURL GMFF prepared the validation samples (calibration samples and blind samples at test GM percentage [DNA/DNA]), organised an international collaborative study and analysed the results.

The study confirms that the method meets the method performance requirements as established by the EURL GMFF and the ENGL, in line with the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004b and it fulfils the analytical requirements of Regulation (EU) No 619/2011 .

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