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Event-specific Method for the Quantification of Soybean DAS-44406-6 by Real-time PCR

Validation Report

European Union Reference Laboratory for Genetically Modified Food and Feed

2015

Joint Research Centre

EURL European Union Reference Laboratory for GM Food & Feed

European Commission
Joint Research Centre
Institute for Health and Consumer Protection

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17 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 soybean event DAS-44406-6 (unique identifier DAS-444Ø6-6). 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 [(1-5)].

In accordance with current EU legislation[b], Dow AgroSciences LLC provided the detection method and the positive and negative control samples (genomic DNA extracted from soybean seeds harbouring the DAS-44406-6 event as positive control DNA, genomic DNA extracted from conventional soybean seeds as negative control DNA). The EURL GMFF verified the performance data provided by the applicant, where necessary experimentally, prepared the validation samples (calibration samples and blind samples at different GM percentage [DNA/DNA]), organised an international collaborative study, and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL and according to Annex I-2.C.2 to Regulation (EC) No 641/2004 and it fulfils the analytical requirements of Regulation (EU) No 619/2011[c].

[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 apply 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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Content

1. Timeline

In line with Regulation (EC) No 1829/2003, Dow AgroSciences 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 event DAS-44406-6 (unique identifier DAS-444Ø6-6) together with genomic DNA as negative and positive control samples (January 2012).

In response to an early submission of the method, the EURL GMFF started its step-wise validation procedure (step 1: dossier reception) already in advance to the official dossier (August 2012), before EFSA declared the dossier as complete and valid (April 2013).

The scientific dossier assessment (step 2) focused on the reported method performance characteristics assessed against the ENGL method acceptance criteria (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 positively concluded in August 2012.

In step 3 of the procedure (experimental testing), the EURL GMFF verified the purity of the control samples and conducted an in-house testing of the method provided.

The positive and negative control DNA samples, submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Regulation (EC) No 1829/2003, were found of good quality.

The method characteristics were verified in-house by quantifying five blind GM levels within the range of 0.1%-5% on a genome copy number basis. The experiments, performed under repeatability conditions, demonstrated that the PCR efficiency, linearity, trueness and precision were within the limits established by the ENGL.

In addition, and in line with the requirements of Regulation (EU) No 619/2011, the EURL GMFF also verified *i)* the zygosity ratio of the positive control sample submitted by the applicant in order to determine the conversion factor between copy numbers and mass fractions; and *ii)* the method precision (relative repeatability standard deviation, RSDr %) at 0.1% related to mass fraction of GM material. Step 3 was completed in October 2012 with the conclusion that the method could be submitted to collaborative study (step 4).

The collaborative study (step 4) took place in October/November 2012. It demonstrated that the method is well suited for analysing and identifying DNA of GM soybean DAS-44406-6 appropriately extracted from food or feed down to the level of 0.1% (m/m).

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

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

Documentation and the data provided by the applicant were evaluated by the EURL GMFF for completeness in step 1 and compliance with the ENGL acceptance criteria in step 2.

The specificity of the event-specific assay was assessed by the applicant in real-time PCR using genomic DNA (100 ng) extracted from DAS-44406-6 (1%) as positive control sample and from soybean DAS-44210-8, DAS-14536-7, DAS-14524-4, DAS-68416-4, A2704-12, A5547-127 DP305423, DP356043, 40-3-2, MON89788-1, maize DAS 40278-9, GA21, Bt176, Bt11, NK603, MON863, TC1507, MIR604, MON810, 59122, 3272, MON88017, MON89034, T25, MIR162, 98140, rapeseed MS8, RF3, T45, RT73, cotton GBH614, MON1445, MON531, MON15985, 281-24-236 x 3006-210-23, LL25, GBH119, T304-40, sugar beet H7-1, potato EH92-527-1, LL62 rice, soybean rapeseed, rice, maize, potato, wheat, cotton, sugar beet.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the TaqMan® probe of the DAS-44406-6 event showed no amplification signals following quantitative PCR analysis.

The specificity of the soybean taxon-specific assay was not assessed by the method developer since a previously validated *lectin (le1)* system was used (http://gmo-crl.jrc.ec.europa.eu/summaries/356043-5_val_report.pdf).

The specificity of the event-specific assay was also 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. The detection method spans the junction between the transgenic insert and the 5' genomic region. The forward primer "DAS-44406-5F" binding site was found in the 5' genomic border adjacent to the insertion. The reverse primer "DAS-44406-5R" binds in the insert. The probe "DAS-44406-6-5p1" binds in the junction region including a 3 bp insertion. The amplicon size is expected to be 99 bp, consistent to that reported by the applicant. The sequence of the amplicon was analysed by BLAST against local copies of the "nt" and "patents" databases, and no significant similarity was found with any other published sequence, except with the corresponding sequences of the related Patents from the applicant of GMO Event DAS-44406-6, soybean.

In addition, the primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System of the JRC, as well as the whole genomes of more than 80 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Triticum aestivum* and *Zea mays*) using the e-PCR prediction tool (NCBI). Only one potential amplicon in the *G. max* genome was identified that probably, under less stringent PCR conditions, might have influence on the detection method. The EURL GMFF confirmed experimentally that, using the PCR conditions of the event-specific method, no unspecific amplification occurs.

The parameters of the calibration curves (slope, R^2 coefficient) were determined by the applicant by quantifying in eight runs five test samples at different GM-levels expressed in haploid genome copy number (see Table 1).

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

	DAS-44406-6		***Le1***	
	Slope	**R^2**	**Slope**	**R^2**
Run 1	-3.50	1.00	-3.39	1.00
Run 2	-3.38	1.00	-3.41	1.00
Run 3	-3.49	1.00	-3.43	1.00
Run 4	-3.48	1.00	-3.45	1.00
Run 5	-3.41	0.99	-3.40	1.00
Run 6	-3.37	1.00	-3.35	1.00
Run 7	-3.52	1.00	-3.42	1.00
Run 8	-3.54	1.00	-3.39	1.00
Mean	**-3.46**	**1.00**	**-3.41**	**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 shall be ≥ 0.98.
Table 1 indicates that the mean slope of the standard curves is -3.46 and -3.41 for the GM-system (DAS-44406-6) and for the soybean–specific reference system (*Le1*) methods respectively, and that the mean R^2 coefficient for the DAS-44406-6 and for the soybean–specific reference system (*Le1*) is 1.00 and therefore all values are within ENGL acceptance criteria.

Table 2 reports precision and trueness for the five GM-levels tested by the applicant. Sixteen values for each GM-level were provided. Both parameters were within the ENGL acceptance criteria (trueness ± 25%, RSDr ≤ 25% across the entire dynamic range).

Table 2. Mean %, precision and trueness (measured by the applicant)

	Test results				
Expected GMO %	**0.085**	**0.5**	**0.9**	**2.0**	**5.0**
Measured mean GMO%	0.084	0.493	0.945	2.25	4.97
Precision (RSDr %)	14.9	10.1	9.1	9.1	11.1
Trueness (bias %)	-1.2	-1.4	5.0	12.5	-0.6

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

3.1 DNA extraction

The applicant referred to a EURL GMFF in-house verified method to extract genomic DNA from ground soybean grains for the quantitative analysis of DAS-44406-6 soybean event[d].

In agreement with the ENGL position, which endorses the modularity principle (see also Annex III to Reg. (EU) No 503/2013), and given the similarity in the matrix, the EURL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for soybean event DAS-44406-6.

3.2 Method protocol for the PCR analysis

The PCR method provided by the applicant and subsequently validated by the EURL GMFF (see http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx) is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event DAS-44406-6 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean *Le1* specific assay, and the target assay (DAS-44406-6) are performed in separate wells.

For the specific detection of soybean event DAS-44406-6, a 99-bp fragment of the region spanning the 5' plant-to-insert junction in soybean DAS-44406-6 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 TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of GM event DAS-44406-6, a soybean specific reference system amplifies a 74-bp fragment of lectin (*Le1*), a soybean 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.

Standard curves are generated for both the DAS-44406-6 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 standard curves are used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves.

For relative quantification of event DAS-44406-6 DNA in a test sample, the DAS-44406-6 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-44406-6/*Le1* x 100).

[d]'CTAB-Anion-Exchange method' previously submitted for detection of event DAS-68416-4. This method had already been evaluated and tested by the EURL GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on testing were published in 2014 at http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-11-10-XP-Report-DNA-Ext.pdf

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) [6]. The copy number values used in the quantification, the GMO contents of the calibration samples and total DNA quantity used in PCR are listed in Table 3.

Table 3. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	100	50	16.67	4.17	0.83
Target taxon *Le1* copies	88496	44248	14749	3687	737
DAS-44406-6 Soybean GM copies	8850	4425	1475	369	74

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 assessed the zygosity (GM-target to reference target ratio) in the positive control sample submitted by the applicant.

To this end, the copy number of the DAS-44406-6 and of the *Le1* targets in the positive control sample were determined by digital PCR (dPCR), performed on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Three micrograms of genomic DNA were digested at 37 ºC overnight with 30 units of the six-base cutter restriction enzyme EcoRI. The latter does not cleave within the annealing sites for the primers of the DAS-44406-6 or *Le1* amplification systems. EcoRI restriction sites are located outside the respective targeted sequences. Further to digestion, the DNA was precipitated with ammonium acetate 2.5 M and two volumes of absolute ethanol. The outcome of enzymatic digestion was controlled by running approximately 200 nanograms of EcoRI digested and 200 nanograms of undigested DNA alongside DNA molecular markers in 1% agarose-gel electrophoresis. The digested template DNA was used in digital PCR experiments.

Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix with (no UNG, Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probe, at the reaction concentrations indicated in the corresponding Validated Method (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm), 1 µL of DNA at a concentration of

0.5 ng/µL, suitable 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 approximately 4.6 µL were distributed into the 765 chambers (or partitions) constituting one panel. The analysis was repeated three times, five replicates in five panels were run each time for both the GM- and reference-assay, with a total number of fifteen data sets for both targets. No template controls were included. Amplification conditions were as reported in the Validated Method (see Annex). Data analysis and copy number calculation was performed using the BioMark digital PCR Analysis software, the range of Ct retention was from 20 to 40.

Calculations of mean and variance were carried out according to the procedure outlined for random variables in Annex 4 of the ENGL guidance document 'Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods'[e].

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

The method performance characteristics were verified (EURL GMFF step 3) by quantifying on a copy number basis five blinded test samples with known GM levels, within the range of 0.1%-5%. The experiments were performed on an ABI 7900 and on Roche LC®480 under repeatability conditions and followed the protocol described in the material and method section. Test samples with GM-levels 5.0%, 2.0%, 0.9%, 0.4% and 0.1% were tested in two real-time PCR runs with two replicates for each GM-level on each plate (total of four replicates per GM-level). The test sample with GM-level 0.1% was tested in 15 replicates in an additional run. 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.

In order to assess the method compliance with Reg. (EU) No 619/2011, the EURL GMFF determined the zygosity of the GM-insert in the positive control sample and estimated, based on 15 replicates, the method precision (RSDr) at 0.1% GM level in mass fraction.

3.4 International collaborative study (step 4)

The international collaborative study (EURL GMFF step 4) involved twelve laboratories, all being "National reference laboratories, assisting the CRL for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 1981/2006. The study was carried out in accordance with the following internationally accepted guidelines:

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

- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) [1]
- ISO 5725 "Accuracy (trueness and precision) of measurement methods and results", Part 1 and Part 2 (ISO, 1994); ISO 5725-1:1994/Cor 1 (ISO 1998) and ISO 5725-2:1994/Cor 1 (ISO, 2002) [2-5]

The objective of the international collaborative study was to verify in experienced laboratories the trueness and precision of the PCR analytical method 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 participants in the DAS-44406-6 validation study where randomly selected from the 31 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories for strictly following the standard operational procedures that were provided for the execution of the protocol. The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the validation of the detection method for soybean DAS-44406-6

Laboratory	Country
Agricultural Institute of Slovenia	SI
Crop Research Institute - Reference Laboratory for GMO Detection and DNA	CZ
DTU-Food, National Food Institute	DK
Italian National Institute for Health	IT
Laboratory Agroalimentary of the Spanish Ministry of Agriculture	ES
Laboratory of DNA analysis - Department of Gene Technology - Tallinn University	EE
National Health Laboratory, Food Control Department	LU
National Institute of Biology	SI
National Research Institute of Animal Production, Lublin	PL
Office for Consumer Protection of the German Federal State Saarland-Saarbrücken	DE
Service Commun des Laboratoires du MINEFI - Laboratoire de Strasbourg	FR
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used a range of real-time PCR equipment: six laboratories used the ABI 7500, four used the ABI 7900, one laboratory used ABI 7700, one used Stratagene Mx 3005P.

The variability of equipment, with its known potential influence on PCR results, reflects the real 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 where provided by the EURL GMFF to the participating laboratories. They were derived from:

i) genomic DNA extracted by the applicant from homozygous soybean seeds harbouring the event DAS-44406-6.

ii) genomic DNA extracted by the applicant from conventional soybean seeds genetically similar to those harbouring the DAS-44406-6 event.

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

These positive and negative control samples were also used by the EURL GMFF to prepare standards (of known GMO content) and test samples (of undisclosed GM content = blinded samples) by mixing DAS-44406-6 soybean DNA and non-GM soybean DNA.

The calibration sample S1 was prepared by mixing the appropriate amount of DAS-44406-6 DNA with control non-GM soybean DNA to obtain a 10% GM solution (8850 GM copies). Calibration samples S2 to S5 were prepared by serial dilutions from S1 sample (Table 3).

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

- ✓ Five calibration samples with known concentrations of GM-event (175 µL of DNA solution each) labelled from S1 to S5 (Table 3).
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution each at 18ng/uL) labelled from U1 to U20, representing 5 GM levels (Table 5).

Table 5. DAS-44406-6 GM contents in genome copy number

DAS-44406-6 GM% GM copy number/soybean genome copy number x 100
5.00
2.00
0.90
0.40
0.10

[f] Control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample). Regulation (EC) No 1829/2003, Art. 2 (11).

- ✓ Reaction reagents:
  - TaqMan® Universal PCR Master Mix no UNG (2x), one vial: 8 mL
  - distilled sterile water, one vial: 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): 150 µL

  DAS-44406-6 assay
  - DAS-44406-5F (10 µM): 240 µL
  - DAS-44406-5R (10 µM): 240 µL
  - DAS-44406-6-5p1 (10 µM): 150 µL

3.4.4 Design of the collaborative study

Participant 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 DAS-44406-6 specific system and for the *Le1* taxon-specific system. In total, two plates were run per each participating laboratory.

The laboratories prepared the master-mixes for the DAS-44406-6 and *Le1* assays in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per determined plate lay-out.

The amplification reactions 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.

3.4.5 Deviations reported from the protocol

Ten laboratories reported no deviations from the method protocol.
One laboratory repeated one plate since efficiencies were lower than expected and one other laboratory reported amplification in a no-template sample possibly due to a pipetting error.

4. Results

4.1 Step 3: EURL GMFF experimental testing

4.1.1 Zygosity ratio in the positive control sample

The results of the tests to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Summary of dPCR analysis conducted on the DAS-44406-6 and *Le1* targets in the positive control sample.

Mean ratio (DAS-44406-6/*Le1*)	1.0*
Standard deviation	0.073
RSD_r (%)	7.2
Standard error of the mean	0.02
Upper 95% CI of the mean	1.1
Lower 95% CI of the mean	0.98

*Mean of fifteen datasets

The 95% confidence interval (CI) spans around 1 and therefore the mean ratio is not significantly different from an expected ratio of 1, assuming a GM homozygous 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%, 0.4% and 0.1% (copy/copy) were tested in two real-time PCR runs (run A and B on ABI 7900 and run D and E on Roche LC®480) with two replicates for each GM level on each plate (total of four replicates per GM-level).

The sample with GM level 0.1% mass/mass (equivalent to 0.1% copy/copy) was tested in 15 replicates in one run (run C on ABI 7900 and run F on Roche LC®480).

The corresponding standard curve parameters and the results of efficiency, linearity, trueness and precision are shown in Table 7a and 7b and in Tables 8a and 8b.

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

	DAS-44406-6 method			***Le1* reference method**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.49	93	1.00	-3.39	97	1.00
Run B	-3.44	95	1.00	-3.27	102	1.00
Run C	-3.40	97	1.00	-3.42	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 Roche LC®480

	DAS-44406-6 method			***Le1* reference method**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run D	-3.46	94	1.00	-3.47	94	1.00
Run E	-3.45	95	1.00	-3.44	95	1.00
Run F	-3.32	100	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 range from -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98.
Tables 7a and 7b document that that the slopes of the standard curves, and the R^2 coefficients were in all cases 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. Tests carried out on ABI 7900.

Target GM-level %	**Measured GM %**	**Bias %**	**Precision (RSDr %)**
5.0	4.5	-10	9.8
2.0	1.7	-13	5.3
0.9	0.91	0.95	3.5
0.4	0.42	3.9	13
0.1	0.09	-7.7	15

Table 8b. Outcome of the in-house tests, with regards to the quantification of the five test samples. Tests carried out on Roche LC®480.

Target GM-level %	Measured GM %	Bias %	Precision (RSDr %)
5.0	5.1	2.35	5.6
2.0	1.8	-11	2.4
0.9	0.89	-1.4	2.4
0.4	0.38	-5.3	3.9
0.1	0.09	-11	9.2

According to the ENGL method acceptance criteria the method's trueness, measured as bias %, should be within ±25% of the target value over the entire dynamic range. The method's precision, estimated as RSDr % (relative repeatability standard deviation) should be ≤25% over the dynamic range. Tables 8a and 8b document that trueness and precision of quantification were within the limits established by the ENGL for both PCR machines used.

4.2 Step 4: international collaborative study

4.2.1 PCR efficiency and linearity

The PCR efficiency (%) and R^2 values (expressing the linearity of the regression), reported by participating laboratories for the DAS-44406-6 and the *Le1* assays 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. Values of slope, PCR efficiency and R^2 obtained during the validation study

		DAS-44406-6			*Le1*		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.54	92	1.00	-3.49	93	1.00
	B	-3.63	88	1.00	-3.53	92	1.00
2	A	-3.60	90	1.00	-3.72	86	1.00
	B	-3.57	91	1.00	-3.64	88	1.00
3	A	-3.55	91	1.00	-3.37	98	0.99
	B	-3.47	94	1.00	-3.35	99	1.00
4	A	-3.62	89	1.00	-3.50	93	1.00
	B	-3.55	91	0.99	-3.50	93	1.00
5	A	-3.57	91	1.00	-3.42	96	1.00
	B	-3.62	89	1.00	-3.46	95	1.00
6	A	-3.34	99	0.98	-3.35	99	0.99
	B	-3.52	92	0.98	-3.43	96	1.00
7	A	-3.65	88	1.00	-3.56	91	1.00
	B	-3.55	91	1.00	-3.56	91	1.00
8	A	-3.78	84	1.00	-3.70	86	1.00
	B	-3.72	86	1.00	-3.66	88	0.99
9	A	-3.68	87	1.00	-3.55	91	1.00
	B	-3.67	87	1.00	-3.51	93	1.00
10	A	-3.63	88	1.00	-3.55	91	1.00
	B	-3.57	91	1.00	-3.49	94	1.00
11	A	-3.47	94	1.00	-3.36	98	1.00
	B	-3.37	98	1.00	-3.23	104	1.00
12	A	-3.64	88	1.00	-3.62	89	1.00
	B	-3.66	88	1.00	-3.66	88	1.00
	Mean	**-3.58**	**90**	**1.0**	**-3.51**	**93**	**1.0**

Table 9 indicates that the efficiency of amplification for the DAS-44406-6 system ranges from 84% to 99% and the linearity from 0.98 to 1.00; the amplification efficiency for the soybean-specific system ranges from 86% to 104% and the linearity is again from 0.98 to 1.00. Although some laboratories reported efficiencies slightly outside the acceptance (e.g. lab 8, 9 and 12), the mean PCR efficiency was 90% for the DAS-44406-6 assay and 93% for the *Le1* assay. Both values were within the ENGL acceptance criteria. The average R^2 of the methods was 1.00 for both the DAS-44406-6 and *Le1* assays.

These results confirm the appropriate performance of the methods tested in terms of efficiency and linearity.

4.2.2 GMO quantification

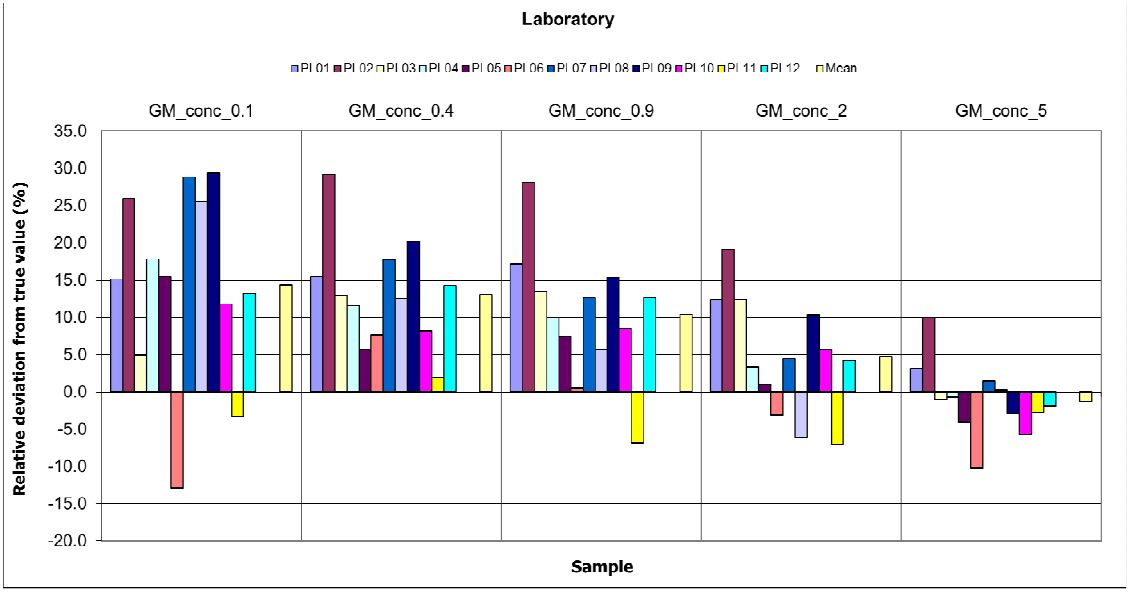
Table 10 reports the values of the four replicates for each GM level as provided by all laboratories. The % GM content is expressed in terms of GM DNA copy numbers in relation to target taxon-specific DNA copy numbers (copy/copy). This is equivalent to the % GM expressed in terms of mass fraction of GM materials (mass/mass).

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

	GMO content (%)																			
LAB	0.1				0.4				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	0.13	0.11	0.13	0.10	0.43	0.49	0.49	0.44	1.16	1.07	0.94	1.04	2.26	2.12	2.39	2.21	5.47	5.01	5.53	4.60
2	0.12	0.13	0.14	0.11	0.51	0.54	0.47	0.55	1.20	1.17	1.07	1.17	2.50	2.15	2.47	2.42	5.71	4.93	5.66	5.70
3	0.10	0.10	0.10	0.11	0.42	0.43	0.53	0.43	1.03	1.04	1.02	0.99	2.30	2.10	2.29	2.29	4.85	5.02	5.73	4.21
4	0.12	0.11	0.13	0.11	0.44	0.41	0.47	0.46	0.92	1.05	0.91	1.07	2.12	2.00	2.27	1.88	4.88	4.71	5.24	5.02
5	0.11	0.12	0.12	0.10	0.39	0.48	0.41	0.42	0.90	1.05	0.88	1.04	2.05	1.84	2.33	1.86	5.26	4.27	5.23	4.43
6	0.10	0.10	0.06	0.10	0.33	0.44	0.42	0.53	0.70	1.21	0.67	1.05	1.54	2.42	2.09	1.71	4.59	5.84	3.16	4.36
7	0.12	0.13	0.15	0.12	0.43	0.46	0.50	0.49	0.97	1.06	1.03	1.00	2.35	1.97	1.85	2.19	5.43	5.10	4.82	4.95
8	0.12	0.10	0.13	0.15	0.43	0.40	0.53	0.45	0.89	0.86	1.18	0.88	1.95	1.90	1.61	2.04	4.81	5.22	5.17	4.85
9	0.13	0.12	0.13	0.14	0.47	0.46	0.56	0.44	1.01	1.05	1.01	1.09	2.23	2.09	2.19	2.32	4.97	4.66	5.09	4.71
10	0.12	0.09	0.11	0.12	0.43	0.38	0.53	0.39	0.95	1.03	0.88	1.05	2.25	2.02	2.08	2.10	4.84	4.71	4.84	4.47
11	0.10	0.09	0.10	0.10	0.38	0.40	0.41	0.44	0.79	0.80	0.83	0.93	1.90	1.67	1.77	2.09	4.54	4.47	5.21	5.24
12	0.11	0.12	0.12	0.10	0.45	0.48	0.48	0.41	1.03	1.10	0.91	1.01	2.14	1.96	2.20	2.05	5.07	4.75	5.12	4.67

A graphical representation of the data reported in Table 10 is provided in Figure 1, where the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the deviation of the average GM level measured by the respective laboratory in % of the true GM level, the light yellow bar on the right represents the mean relative deviation over all twelve participating laboratories for each true GM level.

Figure 1. Relative deviation (%) from the true value of DAS-44406-6 for all laboratories*



* PL: participating laboratory.

Overall, most mean relative deviations from the true values were within a maximum of 25%. At GM-level 0.1% eight laboratories were within the limit, at GM-level 0.4% and 0.9% eleven laboratories were within the limit and at 2.0% and 5% GM-level all laboratories were within the limit. Four laboratories overestimated GM-level 0.1% by more than 25%, similarly, one laboratory overestimated GM-level 0.4% and 0.9% by more than 25%, with a trend for overestimation for all laboratories at all GM levels.

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

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

Table 11. Summary of validation results for the DAS-44406-6 method, expressed as GM DNA copy numbers in relation to target taxon specific DNA copy numbers

	Test Sample Expected GMO %				
	0.1	**0.4**	**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	0	0	1	0	1
Reason for exclusion	-	-	C	-	C
Mean value of measured GM-content	**0.11**	**0.45**	**1.0**	**2.1**	**5.0**
Relative repeatability standard deviation, RSD_r (%)	11	11	7.9	9.0	7.3
Repeatability standard deviation	0.013	0.048	0.079	0.189	0.365
Relative reproducibility standard deviation, RSD_R (%),	15	11	10	11	7.6
Reproducibility standard deviation	0.017	0.051	0.103	0.228	0.380
Bias (absolute value)	0.01	0.05	0.10	0.09	-0.02
Bias (%)	14	13	11	4.7	-0.4

C= Cochran's test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2 (3).
Bias is estimated according to ISO 5725 data analysis protocol.

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. Indeed, the highest value of RSD_R is 15% at the 0.1% GM level, thus within the acceptance criterion.

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 that the RSD_r value is below 25%, as indicated by the ENGL (see 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 11% at the 0.1% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. In this case, the method satisfies this requirement across the dynamic range tested, with the highest value of bias of 14% at the 0.1% GM level.

5. Compliance of the method for detection of event DAS-44406-6 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 12:

- at step 2 of the validation process (scientific assessment of the dossier) the EURL GMFF concluded that it could accept the applicant's data on method performance and therefore accepted that the RSDr at 0.085% (expressed as ratio of GM- DNA copy numbers to target taxon-specific DNA copy numbers) was 15% on 16 replicates (Table 2), hence below 25%;

- at step 3 of the validation process (experimental testing of samples and method) the EURL GMFF determined the RSDr at the level of 0.1% related to mass fraction of GM-material on the basis of fifteen replicates under repeatability conditions. The RSDr was 15% when the method was tested on the ABI 7900 and 9.2% when it was tested on the Roche LC®480 (Tables 8a and 8b, respectively), hence below 25%;

- further to the conclusion of step 4 of the validation process (collaborative trial), the EURL GMFF analysed the data generated by the 12 participating laboratories for determining the method performance parameters. The RSDr of the method at the level 0.1% of mass fraction of GM-material was 15%, therefore below the limit of 25%.

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

Table 12. Precision of the method for quantitative detection of DAS-44406-6, at or around 0.1% level related to mass fractions of GM material

Source	RSDr %	GM %
Applicant's method optimisation*	15 %	0.085 %
EURL GMFF tests	15%[a] and 9.2%[b]	0.1 %
Collaborative study	11 %	0.1 %

* GM- DNA copy numbers in relation to target taxon specific DNA copy numbers

[a] ABI 7900 real-time PCR

[b] Roche LC®480 real-time PCR

Based on the results of the EURL GMFF in-house verification and of the collaborative study, it is concluded that the method RSDr is equal or less 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.

6. Conclusions

The method provided by the applicant and described in detail under 3.2 (and available as at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1) has been validated in accordance to the EURL GMFF validation scheme, respecting all requirements of the relevant EU legislation and international standards for method validation.

The 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 III-3.C.2 to Regulation (EU) No 503/2013 and (EU) No 619/2011 and meets all method performance requirements established by the ENGL. The method is therefore valid to be used for regulatory purposes, including the quantification of low level presence [0.1% (m/m)] of the GM event in feed. It can be assumed that it is applicable to any appropriately extracted soybean DNA.

7. References

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

6. Plant DNA C-values Database, http://data.kew.org/cvalues/.Filzmoser P., Maronna R. and Werner M. Outlier identification in high dimensions, Computational Statistics and Data Analysis, 2008; 52: 1694–1711.

Annex 1: Event-specific Method for the Quantification of Soybean DAS-44406-6 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 event DAS-44406-6 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 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-44406-6, a 99-bp fragment of the region spanning the 5' insert-to-plant junction in soybean DAS-44406-6 event 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 the dye TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3' end.

For the relative quantification of soybean event DAS-44406-6 DNA, a soybean-specific reference system amplifies a 74-bp fragment of lectin *(Le1)* from *Glycine max*, a soybean 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 TAMRA as quencher at its 3' end.

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-44406-6 DNA in a test sample, Ct values for the DAS-44406-6 and *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DAS-44406-6 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 seeds. Precision and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF). The study was undertaken with twelve participating laboratories in October/November 2012.

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 0.04% GMO 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% GMO in 100 ng of total soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1%.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the junction between genomic DNA and the 5' end of the transgene insert. The sequence is specific to event DAS-44406-6 and thus imparts event-specificity to the method.

The event-specificity of the methods was assessed by the applicant both experimentally and by means of bioinformatics analysis. This 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 specificity of the soybean taxon-specific assay was not assessed by the method developer since a previously validated *lectin (le1)* system was used (http://gmo-crl.jrc.ec.europa.eu/summaries/356043-5_val_report.pdf).

Further details can be found in section 2 of "Event-specific Method for the Quantification of Soybean DAS-44406-6 by Real-time PCR - Validation Report" available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

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 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-44406-6

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*Le1*) and for the GMO (event DAS-44406-6) target sequence is to be 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 trial.

For the collaborative trial, the calibration curve was established on the basis of five samples. The first point of the calibration curve contained 10% soybean DAS-44406-6 DNA in a total of 100 ng of soybean DNA (corresponding to approximately 88496 soybean genome copies and to 8850 copies of DAS-44406-6, with one genome assumed to correspond to 1.13 pg of haploid soybean genomic DNA) [(1)].

The total amount of DNA/reaction and the GM% content of standards S1 to S5 are reported in Table 1 below.

Table 1. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	100	50	16.67	4.167	0.833
Target taxon *Le1* copies	88496	44248	14749	3687	737
DAS-44406-6 Soybean GM copies	8850	4425	1475	369	74

A calibration curve is to be produced by plotting the Ct values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available within the sequence detection system software of the method user.

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. To prepare the amplification reaction mixtures, add the following components (Table 2 and 3) in two reaction tubes (one for the DAS-44406-6 assay and one for the *Le1* assay) on ice and in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture, final volume/concentration per reaction well for the **DAS-44406-6** assay.

Component	Final concentration	µL/reaction
TaqMan®Universal PCR Master Mix No AmpErase® UNG 2X	1x	12.5
DAS-44406-5F (10 µM)	300 nM	0.75
DAS-44406-5R (10 µM)	300 nM	0.75
DAS-44406-6-5p1 (10 µM)	180 nM	0.45
Nuclease free water	#	5.55
DNA	#	5.00
Total reaction volume:		25 µL

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

Component	Final concentration	µL/reaction
TaqMan®Universal PCR Master Mix No AmpErase® UNG 2X	1x	12.5
Lec for2 (10 µM)	650 nM	1.625
GMO3-126 Rev (10 µM)	650 nM	1.625
Lec probe (10 µM)	180 nM	0.45
Nuclease free water	#	3.80
DNA	#	5.00
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.

4. Prepare two reaction tubes (one for the soybean DAS-44406-6 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-44406-6 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 to reduce to a minimum 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-44406-6/*Le1* methods.

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

3.3 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-44406-6) 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 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 Ct-values for each reaction.

The standard curves are generated both for the *Le1* and the DAS-44406-6 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 sample.

To obtain the percentage value of event DAS-44406-6 DNA in the unknown sample, the DAS-44406-6 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 (GM% = DAS-44406-6/*Le1* x 100).

4. 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 instrument (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

Oligonucleotides	Name	DNA Sequence (5' to 3')	Length (nt)
DAS-44406-6			
Forward primer	DAS-44406-5F	5'-TTA TTG TTC TTG TTG TTT CCT CTT TAG G–3'	28
Reverse primer	DAS-44406-5R	5'-CCT CAA TTG CGA GCT TTC TAA TTT-3'	24
Probe	DAS-44406-6-5p1	5'-6FAM-ATT CGG ACC TCC ATG ATG ACC TTA CCG TT-TAMRA-3'	29
Le1			
Forward primer	Lec for2	5'-CCA GCT TCG CCG CTT CCT TC-3'	20
Reverse primer	GMO3-126 Rev	5'-GAA GGC AAG CCC ATC TGC AAG CC-3'	23
Probe	Lec probe	5'-6FAM-CTT CAC CTT CTA TGC CCC TGA CAC-TAMRA-3'	24

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine

5. References

1. Plant DNA C-values Database, http://data.kew.org/cvalues/.Filzmoser P., Maronna R. and Werner M. Outlier identification in high dimensions, Computational Statistics and Data Analysis, 2008; 52: 1694–1711.

European Commission
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Title: **Event-specific Method for the Quantification of Soybean DAS-44406-6 by Real-time PCR**

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

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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 soybean event DAS-44406-6 (unique identifier DAS-44406-6). 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 (2-6).

In accordance with current EU legislation , Dow AgroSciences LLC provided the detection method and the positive and negative control samples (genomic DNA extracted from soybean seeds harbouring the DAS-44406-6 event as positive control DNA, genomic DNA extracted from conventional soybean seeds as negative control DNA). The EURL GMFF verified the performance data provided by the applicant, where necessary experimentally, prepared the validation samples (calibration samples and blind samples at different GM percentage [DNA/DNA]), organised an international collaborative study, and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL and according to Annex I-2.C.2 to Regulation (EC) No 641/2004 and it fulfils the analytical requirements of Regulation (EU) No 619/2011.

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JRC Mission

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