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# Event-specific Method for the Quantification of Soybean DAS-68416-4 Using Real-time PCR

*Validation Report*

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# Validation Report

### 13 May 2014

### European Union Reference Laboratory for GM Food and Feed

## Executive Summary

In line with its mandate[1] the European Union Reference Laboratory for GM Food and Feed (EU-RL 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-68416-4 (unique identifier DAS-68416-4). The validation study was conducted according to the EU-RL GMFF validation procedure (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and internationally accepted guidelines [(1, 2, 3, 4, 5)].

In accordance with current EU legislation[2], Dow AgroSciences LLC provided the detection method and the positive and negative control samples (genomic DNA extracted from soybean kernels harbouring the DAS-68416-4 event as positive control DNA, genomic DNA extracted from conventional soybean kernels as negative control DNA). The EU-RL 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 EU-RL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EU-RL 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[3].

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

[2] Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003".

[3] 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 EU-RL 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 EU-RL GMFF quality system.

The EU-RL 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 EU-RL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection IHCP provided by CERMET.

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# Content

# 1. Timeline

In line with Regulation (EC) No 1829/2003, Dow AgroSciences LLC provided the EU-RL GMFF with a copy of the official application for authorisation of an event-specific method for the detection and quantification of soybean event DAS-68416-4 (unique identifier DAS-68416-4) together with genomic DNA as negative and positive control samples (December 2010).

In response to an early submission of the method, the EU-RL GMFF started its step-wise procedure with step 1: dossier reception, followed by step 2: dossier evaluation in December 2010, i.e. before EFSA declared the official dossier as complete and valid (September 2011).

The scientific dossier evaluation (step 2) focused on the reported method performance characteristics assessed against the ENGL method acceptance criteria[4] (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). The assessment was positively concluded in April 2012.

In step 3 of the procedure (experimental testing), the EU-RL 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 blinded GM levels within the range of 0.1%-5% on a copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy 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 EU-RL 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 June 2012 with the conclusion that the method could be submitted to collaborative study (step 4).

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

The collaborative study (step 4) took place in July-August 2012. It demonstrated that the method is well suited for analysing and identifying DNA of GM soybean DAS-68416-4 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 acceptance) and step 2 (scientific dossier assessment)

Documentation and the data provided by the applicant were evaluated by the EU-RL GMFF for completeness (step 1) and compliance with the ENGL method acceptance criteria (step 2).

The specificity of the event-specific assay was assessed by the applicant in real-time PCR using genomic DNA samples (100 ng) containing 1% of the GMO, extracted from DAS-68416-4 (positive control sample) and from maize DAS 40278-9, DAS-40474-7, GA21, Bt176, Bt11, NK603, MON863, TC1507, MIR604, 59122, T25, MON810, MON88017, 3272, MON89034; sugar beet H7-1; oilseed rape Ms8, Rf3, T45, RT73; cotton MON531, MON15985, MON1445, GHB614, LLcotton25, 281-24-236x3006-210-23; potato EH92-527-1; soybean DAS-64209-9, A2704-12, DP305423, DP356043, GTS 40-3-2, MON89788, A5547-127; rice LLRICE62 and conventional soybean, maize, oilseed rape, rice, cotton, wheat, potato and sugar beet.

Specificity was also verified and confirmed by the EU-RL 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 because the *Le1* assay had been previously validated by the EU-RL GMFF. For details see http://gmo-crl.jrc.ec.europa.eu/summaries/DP356043_validated_Method_correctedversion1.pdf).

The parameters of the calibration curves (slope, $R^2$ coefficient) were appropriately 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-68416-4** | | ***Le1*** | |
|---|---|---|---|---|
| | **Slope** | **$R^2$** | **Slope** | **$R^2$** |
| **Run 1** | -3.42 | 1.00 | -3.42 | 1.00 |
| **Run 2** | -3.48 | 1.00 | -3.38 | 1.00 |
| **Run 3** | -3.56 | 1.00 | -3.36 | 1.00 |
| **Run 4** | -3.41 | 1.00 | -3.43 | 1.00 |
| **Run 5** | -3.50 | 1.00 | -3.42 | 1.00 |
| **Run 6** | -3.64 | 1.00 | -3.40 | 1.00 |
| **Run 7** | -3.51 | 1.00 | -3.36 | 1.00 |
| **Run 8** | -3.44 | 1.00 | -3.39 | 1.00 |
| Mean | -3.49 | 1.00 | -3.39 | 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 -3.49 and -3.39 for the DAS-68416-4 and for the *Le1* methods, respectively, and that the mean $R^2$ coefficient for the DAS-68416 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, which is fully in line with EU-RL GMFF guidance. Both parameters were established as being within the ENGL acceptance criteria (trueness ± 25%, RSDr ≤ 25% across the entire dynamic range).

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

| | **Test results** | | | | |
|---|---|---|---|---|---|
| **Expected GMO %** | **0.08** | **0.5** | **0.9** | **2.0** | **5.0** |
| Measured mean % | 0.075 | 0.46 | 0.88 | 2.2 | 5.0 |
| Precision (RSDr %) | 10 | 4.8 | 5.6 | 6.8 | 6.4 |
| Trueness (bias %) | -6.3 | -8.0 | -2.2 | 10 | 0.0 |

# 3. Step 3 (experimental testing of samples and methods)

## *3.1 DNA extraction*

Genomic DNA was isolated by the applicant from DAS-68416-4 and non GM soybean grains using the "CTAB-Anion-Exchange" method. That DNA extraction method has been in-house verified by

the EU-RL GMFF in the context of this application and was found to function suitably for the assessed matrix. The assessment report is published at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

## *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/statusofdoss.htm and in Annex 1 to this report) and subsequently validated by the EU-RL GMFF is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event DAS-68416-4 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean *Le1* (*lectin*) specific assay and the target assay (DAS-68416-4) are performed in separate wells.

For the detection of GM event DAS-68416-4, a 130-bp fragment of the region spanning the 3' plant-to-insert junction in soybean DAS-68416-4 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 a minor groove binding non fluorescent quencher (MGBNFQ) at its 3' end.

For the relative quantification of GM event DAS-68416-4, 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 (6-carboxytetramethylrhodamine) as quencher at its 3' end.

Standard curves are generated for both the DAS-68416-4 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-68416-4 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-68416-4/*Le1* x 100).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (expressed in picograms) by the published average 1C value for the soybean genome (1.13 pg) (Arumuganathan & Earle, 1991) [(6)]. The copy number values used in the quantification and the total DNA quantity used in the PCR reactions are reported 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) | 120 | 40 | 13.3 | 3.33 | 0.83 |
| Target taxon *Le1* copies | 106195 | 35398 | 11799 | 2950 | 737 |
| DAS-68416-4 soybean GM copies | 10620 | 3540 | 1180 | 295 | 74 |

## *3.3 EU-RL GMFF experimental testing (step 3)*

### 3.3.1 Determination of the zygosity ratio in the positive control sample

Annex II of Regulation (EU) No 619/2011 requires that "when results are primarily expressed as GM DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EU-RL GMFF." In order to satisfy this requirement, the EU-RL GMFF conducted an assessment of 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-68416-4 and *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).

Ten microliters (3.8 µg) of genomic DNA were digested at 37 ºC overnight with 45 units of the six-base cutter restriction enzyme EcoRI. The latter does not cleave within the annealing sites of the primers for the DAS-68416-4 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 240 nanograms of digested and 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 (no UNG, Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probe at the final concentrations of 550 nM/µL and 150 nM/µL respectively and 1 µL of DNA at a concentration of 0.5 ng/µL. This concentration avoids panel saturation after analysis (optimal between 200<positive partitions<700). The amplification reaction mixtures are further detailed in the corresponding Validated Method (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

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 partitions (or chambers) constituting one panel. The analysis was repeated three times; five replicates of the same dilution were loaded in five panels 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 1). Data analysis and copy number calculation was performed using the BioMark digital PCR Analysis software, the range of Ct retention was from 20 to 43.

Calculations of means and variances 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[5].

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

The method performance characteristics were verified by quantifying on a copy number basis five blinded test samples containing a range of 5%-0.1% GM levels. The experiments were performed under repeatability conditions first on an ABI 7900 real-time platform and then repeated on the Roche LC480 equipment, and followed the protocol described in the material and method section. Test samples with GM levels 5.0%, 2.0%, 0.9% and 0.4% 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 Regulation (EU) No 619/2011, the EU-RL GMFF estimated, based on 15 replicates, also the method precision (RSDr) at 0.1% GM level in mass fraction (m/m).

## *3.4 International collaborative study (step 4)*

The international collaborative study (step 4) involved twelve laboratories, all being National reference laboratories, assisting the EU-RL GMFF 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:

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

1. The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) [1]
2. ISO 5725 "Accuracy (trueness and precision) of measurement methods and results", Part 1 and Part 2 (ISO, 1994); ISO 5725-1:1994/Cor 1 (ISO 1998) and ISO 5725-2:1994/Cor 1 (ISO, 2002) [2, 3, 4, 5]

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

### 3.4.1 List of participating laboratories

The 12 participants in the DAS-68416-4 validation study where randomly selected from the 30 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories to strictly follow 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 international collaborative validation study of the detection method for soybean DAS-68416-4.

| Laboratory | Country |
|---|---|
| Agricultural Institute of Slovenia | SI |
| Bavarian Health and Food Safety Authority | DE |
| Center for Agricultural Technology Augustenberg | DE |
| Central Agricultural Office, Food and Feed Safety Directorate - Laboratory for GMO | HU |
| Crop Research Institute – Refer. Lab for GMO Detection and DNA fingerprinting | CZ |
| Federal Institute for Risk Assessment | DE |
| Institute for National Investigation for the Health and Veterinarian Nature Saxonia | DE |
| Laboratory Agro alimentary of the Spanish Ministry of Agriculture | ES |
| Laboratory of DNA analysis - Department of Gene Technology | EE |
| Landeslabor Schleswig-Holstein - Food, Veterinary and Environmental Diagnostic | DE |
| National Centre for Food, Spanish Food Safety Agency and Nutrition | ES |
| National Institute of Biology | SI |

### 3.4.2 Real-time PCR equipment used in the study

The laboratories involved in the collaborative study used a range of real-time PCR equipment: five laboratories used the ABI 7500, four used the ABI 7900, one used the Roche LC480, one used the Stratagene Mx3005P and one used ABI 9700.

The variability of equipment, with its known potential influence on PCR results, reflects the real situation in the control laboratories and the fact that in this case it did not significantly influence the performance of the method provides additional assurance that the method is 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 provided by the EU-RL GMFF to the participating laboratories. They where derived from:

*i)* genomic DNA extracted by the applicant from homozygous soybean seeds harbouring the event DAS-68416-4, and

*ii)* genomic DNA extracted by the applicant from conventional soybean seeds genetically similar to those harbouring the DAS-68416-4 event.

The control samples were prepared by the EU-RL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11[6].

These positive and negative control samples were also used by the EU-RL GMFF to prepare standards (of known GMO content) and test samples (of undisclosed GM content = blinded samples) by mixing DAS-68416-4 soybean DNA and non-GM soybean DNA.

The calibration sample S1 was prepared as 10% GM solution. Calibration samples S2 and S3 were prepared by serial three-fold dilution from the S1 sample and samples S4S5 were prepared by serial four-fold dilution from the S3 sample.

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 20 ng/uL) labelled from U1 to U20, representing 5 GM levels (Table 5).

[6] 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).

Table 5. DAS-68416-4 GM contents in genome copy number

| **DAS-68416-4 GM%**<br>GM copy number/soybean genome copy number x 100 |
|---|
| 5.00 |
| 2.00 |
| 0.90 |
| 0.40 |
| 0.10 |

- ✓ Reaction reagents:
  - TaqMan® Universal PCR Master Mix no UNG (2x), two vials: 5 mL
  - AmpliTaqGold (5U/µL), one vial: 64 µL
  - sterile distilled water, one vial: 5 mL

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

  *Le1* taxon-specific method
  - Lec for2 (10 µM): 520 µL
  - GMO3-126 Rev (10 µM): 500 µL
  - Lec probe (10 µM): 150 µL

  DAS-68416-4 method
  - DAS-68416-4-3f5 (10 µM): 440 µL
  - DAS-68416-4-3r3 (10 µM): 440 µL
  - DAS-68416-4-3p3 (10 µM): 120 µL

### 3.4.4 Design of the collaborative study

Participating laboratories received a detailed validation protocol that included the exact design of the PCR plates, ensuring that on each PCR plate all samples were analysed for the DAS-68416-4 specific system and for the *Le1* taxon-specific system in parallel. In total, two plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for the DAS-68416-4 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 EU-RL GMFF on an Excel sheet that was designed, validated and distributed by the EU-RL GMFF. All data are stored by the EU-RL GMFF on a dedicated and protected server.

The EU-RL 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

Eleven laboratories reported no deviations from the protocol.
One laboratory performed the PCR reactions in a total volume of 20 µL (instead of 25) because a 384-well plate configuration of the ABI 7900HT instrument was used. However, the final concentration of the PCR reagents and the volume of DNA loaded per reaction remained unchanged and the data of the laboratory were retained for analysis.

# 4. Results

## *4.1 EU-RL 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-68416-4 and *Le1* targets in the positive control sample.

| | |
|---|---|
| Mean ratio (DAS-68416-4/*Le1*) | 1.0 |
| Standard deviation | 0.069 |
| $RSD_r$ (%) | 6.8 |
| Standard error of the mean | 0.018 |
| Upper 95% CI of the mean | 1.1 |
| Lower 95% CI of the mean | 0.98 |

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 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 0.4% were tested by the EU-RL GMFF in two real-time PCR runs (run A and B on ABI 7900 and run D and E on Roche LC480) with two replicates for each GM-level on each plate (total of four replicates per GM-level). The sample with a 0.1% GM level was tested in 15 replicates in one run (run C on ABI 7900 and run F on Roche LC480). The corresponding standard curve parameters are shown in Tables 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-68416-4 method** | | | ***Le1* reference method** | | |
|---|---|---|---|---|---|---|
| | Slope | PCR efficiency* | $R^2$ | Slope | PCR efficiency* | $R^2$ |
| Run A | -3.45 | 95 | 0.99 | -3.31 | 101 | 1.00 |
| Run B | -3.42 | 96 | 1.00 | -3.33 | 99 | 1.00 |
| Run C | -3.49 | 93 | 1.00 | -3.31 | 101 | 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-68416-4 method** | | | ***Le1* reference method** | | |
|---|---|---|---|---|---|---|
| | Slope | PCR efficiency* | $R^2$ | Slope | PCR efficiency* | $R^2$ |
| Run D | -3.42 | 96 | 1.00 | -3.44 | 95 | 1.00 |
| Run E | -3.40 | 97 | 1.00 | -3.39 | 97 | 1.00 |
| Run F | -3.54 | 92 | 1.00 | -3.42 | 96 | 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 $\geq 0.98$.
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 | 4.9 | -1.5 | 6.9 |
| 2.0 | 1.8 | -7.9 | 19 |
| 0.9 | 0.81 | -9.8 | 6.8 |
| 0.4 | 0.34 | -15 | 14 |
| 0.1 | 0.09 | -14 | 19 |

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 | 5.0 | -0.66 | 3.5 |
| 2.0 | 1.8 | -9.5 | 1.1 |
| 0.9 | 0.81 | -10 | 4.7 |
| 0.4 | 0.37 | -8.6 | 6.8 |
| 0.1 | 0.10 | -3.1 | 11 |

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 Results of the international collaborative study*

### 4.2.1 PCR efficiency and linearity

The PCR efficiency (%) and $R^2$ values (expressing the linearity of the regression) for the standard curves, reported by participating laboratories and displayed in Table 9. The PCR efficiency (%) was 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-68416-4 system ranges from 86 to 96 and the linearity from 0.99 to 1.00; the amplification efficiency for the soybean specific system ranges from 90% to 99% and the linearity from 0.99 to 1.00. The mean PCR efficiency is 92% for the DAS-68416-4 assay and 94% 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-68416-4 and *Le1* assays.

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

| | | DAS-68416-4 | | | *Le1* | | |
|---|---|---|---|---|---|---|---|
| **Lab** | **Plate** | **Slope** | **PCR Efficiency (%)** | **$R^2$** | **Slope** | **PCR Efficiency (%)** | **$R^2$** |
| **1** | A | -3.48 | 94 | 1.00 | -3.43 | 96 | 1.00 |
| | B | -3.49 | 94 | 1.00 | -3.47 | 94 | 1.00 |
| **2** | A | -3.66 | 88 | 1.00 | -3.58 | 90 | 1.00 |
| | B | -3.70 | 86 | 1.00 | -3.58 | 90 | 1.00 |
| **3** | A | -3.73 | 86 | 1.00 | -3.53 | 92 | 1.00 |
| | B | -3.45 | 95 | 1.00 | -3.55 | 91 | 1.00 |
| **4** | A | -3.57 | 91 | 0.99 | -3.46 | 95 | 1.00 |
| | B | -3.43 | 96 | 1.00 | -3.53 | 92 | 1.00 |
| **5** | A | -3.53 | 92 | 0.99 | -3.41 | 97 | 1.00 |
| | B | -3.49 | 93 | 1.00 | -3.41 | 96 | 1.00 |
| **6** | A | -3.54 | 92 | 0.99 | -3.51 | 93 | 1.00 |
| | B | -3.45 | 95 | 1.00 | -3.43 | 96 | 1.00 |
| **7** | A | -3.67 | 87 | 1.00 | -3.45 | 95 | 1.00 |
| | B | -3.52 | 92 | 1.00 | -3.50 | 93 | 1.00 |
| **8** | A | -3.57 | 91 | 1.00 | -3.53 | 92 | 1.00 |
| | B | -3.53 | 92 | 1.00 | -3.47 | 94 | 1.00 |
| **9** | A | -3.66 | 88 | 0.99 | -3.52 | 92 | 1.00 |
| | B | -3.59 | 90 | 0.99 | -3.41 | 96 | 1.00 |
| **10** | A | -3.42 | 96 | 1.00 | -3.41 | 97 | 0.99 |
| | B | -3.46 | 95 | 1.00 | -3.42 | 96 | 0.99 |
| **11** | A | -3.49 | 93 | 1.00 | -3.48 | 94 | 1.00 |
| | B | -3.53 | 92 | 1.00 | -3.53 | 92 | 1.00 |
| **12** | A | -3.51 | 93 | 0.99 | -3.34 | 99 | 1.00 |
| | B | -3.44 | 95 | 1.00 | -3.39 | 97 | 1.00 |
| | **Mean** | **-3.54** | **92** | **1.00** | **-3.47** | **94** | **1.00** |

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

### 4.2.2 GMO quantification

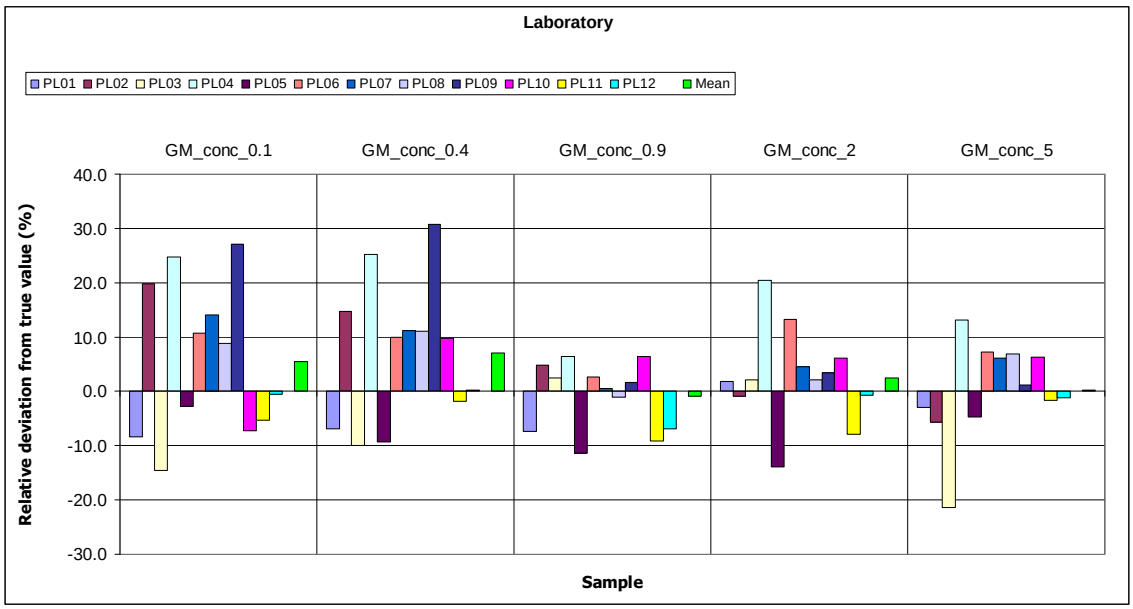
Table 10 reports the values of the four replicates for each GM level as provided by all laboratories. The % GM content is expressed in terms of GM DNA copy numbers in relation to target taxon-specific DNA copy numbers (copy/copy). This is equivalent to the % GM expressed in terms of mass fraction of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

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

| | 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** | 0.08 | 0.10 | 0.10 | 0.10 | 0.40 | 0.38 | 0.36 | 0.35 | 0.84 | 0.92 | 0.73 | 0.84 | 1.85 | 2.23 | 2.04 | 2.03 | 4.74 | - | 5.16 | 4.66 |
| **2** | 0.11 | 0.13 | 0.12 | 0.12 | 0.44 | 0.44 | 0.45 | 0.50 | 0.97 | 0.91 | 0.93 | 0.96 | 2.00 | 2.02 | 2.00 | 1.90 | 4.53 | 4.66 | 4.70 | 4.97 |
| **3** | 0.08 | 0.00 | 0.12 | 0.14 | 0.45 | 0.00 | 0.50 | 0.49 | 0.94 | 0.88 | 0.90 | 0.97 | 2.04 | 2.00 | 1.99 | 2.13 | 5.30 | 0.00 | 5.13 | 5.28 |
| **4** | 0.14 | 0.12 | 0.12 | 0.12 | 0.49 | 0.53 | 0.47 | 0.52 | 0.97 | 0.93 | 0.97 | 0.96 | 2.46 | 2.54 | 2.56 | 2.08 | 5.45 | 6.46 | 5.15 | 5.57 |
| **5** | 0.09 | 0.10 | 0.09 | 0.11 | 0.39 | 0.35 | 0.37 | 0.34 | 0.81 | 0.73 | 0.80 | 0.86 | 1.59 | 1.97 | 1.51 | 1.81 | 5.17 | 4.82 | 4.68 | 4.41 |
| **6** | 0.10 | 0.11 | 0.11 | 0.12 | 0.41 | 0.45 | 0.44 | 0.47 | 0.96 | 0.89 | 0.89 | 0.95 | 2.49 | 2.10 | 2.29 | 2.18 | 5.34 | 5.57 | 5.10 | 5.43 |
| **7** | 0.11 | 0.10 | 0.12 | 0.13 | 0.39 | 0.44 | 0.44 | 0.50 | 0.89 | 0.84 | 0.99 | 0.91 | 2.25 | 2.11 | 2.00 | 2.00 | 5.33 | 5.65 | 4.91 | 5.34 |
| **8** | 0.11 | 0.11 | 0.09 | 0.11 | 0.44 | 0.48 | 0.43 | 0.42 | 0.87 | 0.90 | 0.91 | 0.88 | 2.05 | 2.02 | 1.96 | 2.15 | 5.40 | 5.67 | 5.16 | 5.16 |
| **9** | 0.11 | 0.15 | 0.11 | 0.14 | 0.45 | 0.57 | 0.51 | 0.56 | 0.84 | 0.85 | 0.99 | 0.98 | 2.16 | 1.95 | 2.11 | 2.06 | 4.91 | 5.54 | 4.95 | 4.84 |
| **10** | 0.07 | 0.10 | 0.09 | 0.11 | 0.52 | 0.38 | 0.39 | 0.46 | 0.92 | 1.09 | 0.93 | 0.89 | 2.40 | 1.96 | 2.12 | 1.99 | 5.34 | 5.64 | 4.87 | 5.41 |
| **11** | 0.10 | 0.10 | 0.09 | 0.09 | 0.37 | 0.43 | 0.37 | 0.40 | 0.84 | 0.82 | 0.83 | 0.78 | 1.83 | 1.92 | 1.85 | 1.75 | 4.85 | 5.23 | 4.85 | 4.74 |
| **12** | 0.10 | 0.11 | 0.09 | 0.10 | 0.37 | 0.42 | 0.39 | 0.42 | 0.82 | 0.76 | 0.89 | 0.88 | 2.05 | 1.96 | 2.07 | 1.87 | 4.87 | 5.17 | 4.72 | 5.02 |

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 GM level measured by the respective laboratory in % of the true GM level; the green 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-68416-4 for all laboratories*




*PL12 at GM level 0.4% had very small relative deviation from the true value and the corresponding histogram does not show up in Figure 1. PL: participating laboratory.

A trend can be observed to overestimate the GM content at the lowest levels. One laboratory overestimated the GM content of sample 0.1% by 27% and of sample 0.4% by 30%.

All data were retained to feed the statistical analysis reported 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 EU-RL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), repeatability and reproducibility are 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.

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. Summary of validation results for the DAS-68416-4 detection and quantification 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 | 1 | 1 | - | - | 1 |
| Reason for exclusion | C | C | - | - | C |
| Mean value (%) | 0.11 | 0.44 | 0.89 | 2.1 | 5.1 |
| Relative repeatability standard deviation, $RSD_r$ (%) | 11 | 8.5 | 6.1 | 6.9 | 5.9 |
| Repeatability standard deviation | 0.01 | 0.04 | 0.05 | 0.14 | 0.30 |
| Relative reproducibility standard deviation, $RSD_R$ (%) | 15 | 14 | 8.2 | 10 | 7.8 |
| Reproducibility standard deviation | 0.02 | 0.06 | 0.07 | 0.22 | 0.40 |
| Bias (absolute value) | 0.01 | 0.04 | -0.01 | 0.05 | 0.11 |
| Bias (%) | 7.4 | 8.6 | -0.94 | 2.5 | 2.3 |

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

Table 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 EU-RL GMFF requires the $RSD_r$ value to be 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 relative repeatability standard deviation is below 25% at all GM levels, with the highest value of 11% at the 0.1% GM level. This confirms the information provided by the applicant concerning the RSDr, and verified by the EU-RL GMFF during step 3 of the method assessment

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 8.6% at the 0.4% GM level.

# 5. Compliance of the method for detection of GM event DAS-68416-4 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:

- at step 2 of the validation process (scientific assessment of the dossier), the EU-RL GMFF concluded that it could accept the applicant's data on method performance. Indeed, the RSDr at the level of 0.08% in terms of GM DNA copy numbers in relation to target taxon specific DNA copy numbers resulted to be 10% calculated from 16 replicates (Table 2), hence below the acceptance criteria of 25%;

- at step 3 of the validation process (in-house verification of the method), the EU-RL GMFF determined the RSDr % value at the level of 0.1% in mass fraction of GM material. The experiments were carried out under repeatability conditions on fifteen replicates. The RSDr resulted to be 19% when the method was tested on the ABI 7900 and 11% 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 study), the EU-RL GMFF analysed the data generated by the twelve participating laboratories for determining the method performance parameters. The RSDr of the method at the level of 0.1% of mass fraction of GM material was 11%, therefore below the limit of 25%.

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

Table 12. Precision of the event-specific method for quantitative detection of DAS-68416-4 at or around 0.1% level related to mass fractions of GM material

| **Source** | **RSDr %** | | **GM %** |
|---|---|---|---|
| Applicant's method optimisation* | 10 % | | 0.08 % |
| EU-RL GMFF in-house verification | 19 %[a] | 11%[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 EU-RL GMFF in-house verification and of the international collaborative study, it is concluded that the method RSDr is below 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

A method for detection, identification and quantification of GM event DAS-68416-4 was provided by the applicant. It is described in detail under 3.2 (and available as the "Validated Method" in Annex 1 and at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). The method has been fully validated in accordance to the EU-RL 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 I-2.C.2 to Regulation (EC) No 641/2004 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. 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. Arumuganathan K. and Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9, 208-218.

# Annex 1: Event-specific Method for the Quantification of Soybean DAS-68416-4 Using Real-time PCR

# Validated Method

**Joint Research Centre**
**Institute for Health and Consumer Protection**
**Molecular Biology and Genomics Unit**

**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-68416-4 (unique identifier DAS-68416-4) 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-68416-4, a 130-bp fragment of the region spanning the 3' insert-to-plant junction in soybean DAS-68416-4 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 compound MGBNFQ (Minor Groove Binding Non-Fluorescent Quencher) as a quencher dye at its 3' end.

For the relative quantification of soybean event DAS-68416-4 DNA, a soybean-specific reference system amplifies a 74-bp fragment of *lectin (Le1)*, a soybean endogenous gene (Accession number, GeneBank: K00821 and M30884), using *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with FAM as reporter dye at its 5' end, and TAMRA (6-carboxytetramethylrhodamine) 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-68416-4 DNA in a test sample, Ct values for the DAS-68416-4 and *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DAS-68416-4 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 (EU-RL GMFF).

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

## 2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.04% (related to mass fraction of GM material) in 100 ng of total soybean DNA. The relative LOD was not assessed by the EU-RL GMFF in the collaborative study.

## 2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.08% (related to mass fraction of GM material) in 100 ng of total soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1% (mass fraction of GM material).

## 2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 3' insert-to-plant junction in soybean DAS-68416-4; the sequence is specific to event DAS-68416-4 and thus imparts event-specificity to the method.

The specificity of the event-specific assay was assessed by the applicant in real-time PCR using genomic DNA samples (100 ng) containing 1% of the GMO, extracted from DAS-68416-4 (positive control sample) and from maize DAS 40278-9, DAS-40474-7, GA21, Bt176, Bt11, NK603, MON863, TC1507, MIR604, 59122, T25, MON810, MON88017, 3272, MON89034; sugar beet H7-1; oilseed rape Ms8, Rf3, T45, RT73; cotton MON531, MON15985, MON1445, GHB614, LLcotton25, 281-24-236x3006-210-23; potato EH92-527-1; soybean DAS-64209-9, A2704-12, DP305423, DP356043, GTS 40-3-2, MON89788, A5547-127; rice LLRICE62 and conventional soybean, maize, oilseed rape, rice, cotton, wheat, potato and sugarbeet.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the TaqMan® probe of the DAS-68416-4 event showed no amplification signals following quantitative PCR analysis (45 cycles).

The specificity of the soybean taxon-specific assay was not assessed by the method developer because the *Le1* assay had been previously validated by the EU-RL GMFF. For details see http://gmo-crl.jrc.ec.europa.eu/summaries/DP356043_validated_Method_correctedversion1.pdf).

Specificity was further verified and confirmed *in silico* by the EU-RL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

# 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-68416-4

### 3.2.1 General

The PCR set-up for the taxon-specific target sequence (*Le1*) and for the GMO (event DAS-68416-4) 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-68416-4 DNA in a total of 120 ng of soybean DNA (corresponding to approximately 106195 soybean genome copies and to 10619 copies of DAS-68416-4, with one genome assumed to correspond to 1.13 pg of haploid soybean genomic DNA) [(1)].

Standards S2 and S3 were prepared by serial 3-fold dilution of the S1 sample. Standard S4 and S5 were prepared by a 4-fold dilution of the standard S3.

The copy number values of the calibration samples and total DNA quantity used in PCR are reported in Table 1.

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

| **Sample** | S1 | S2 | S3 | S4 | S5 |
|---|---|---|---|---|---|
| Total amount of DNA in reaction (ng) | 120 | 40 | 13.3 | 3.35 | 0.85 |
| Target taxon *Le1* copies | 106195 | 35398 | 11799 | 2950 | 737 |
| DAS-68416-4 soybean GM copies | 10619 | 3540 | 1180 | 295 | 74 |

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.

### 3.2.3 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-68416-4 assay and one for the *Le1* assay) on ice and in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the DAS-68416-4 assay.

| Component | Final concentration | µL/reaction |
|---|---|---|
| TaqMan® Universal PCR Master Mix no AmpErase® UNG 2x | 1x | 12.5 |
| DAS-68416-4-3f5 (10 µM) | 550 nM | 1.375 |
| DAS-68416-4-3r3 (10 µM) | 550 nM | 1.375 |
| DAS-68416-4-3p3 (10 µM) | 150 nM | 0.375 |
| AmpliTaq Gold® DNA Polymerase (5U/µl) | 1U | 0.2 |
| Nuclease free water | # | 4.175 |
| DNA | # | 5 |
| 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 |
|---|---|---|
| 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 | 0.450 |
| Nuclease free water | # | 3.800 |
| DNA | # | 5 |
| Total reaction volume: | | 25 µL |

3. Mix well and centrifuge briefly.

4. Prepare two reaction tubes (one for the soybean DAS-68416-4 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-68416-4 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-68416-4/*Le1* methods.

| Step | Stage | | T (°C) | Time (s) | Acquisition | Cycles |
|---|---|---|---|---|---|---|
| 1 | Initial denaturation | | 95 | 600 | No | 1X |
| 2 | Amplification | Denaturation | 95 | 15 | No | 45X |
| | | 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-68416-4) 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-68416-4 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-68416-4 DNA in the unknown sample, the DAS-68416-4 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 (GM% = DAS-68416-4/*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 4326614.

- AmpliTaq Gold® DNA Polymerase, Applied Biosystems, Cat N8080248

### 4.3 Primers and Probes

| Oligonucleotides | Name | DNA Sequence (5′ to 3′) | Length (nt) |
|---|---|---|---|
| **DAS-68416-4** | | | |
| Forward primer | DAS-68416-4_3f5 | 5′ gTA CAT TAA AAA CgT CCg CAA TgT gT 3′ | 26 |
| Reverse primer | DAS-68416-4_3r3 | 5′ gTT TAA gAA TTA gTT CTT ACA gTT TAT TgT TAg 3′ | 33 |
| Probe | DAS-68416-4_3p3 | 5′-6FAM- TTA AgT TgT CTA AgC gTC AAT A –MGBNFQ-3′ | 22 |
| ***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: 6-carboxytetramethylrhodamine; MGBNFQ: Minor Groove Binding Non-Fluorescent Quencher

# 5. References

1. Arumuganathan K., Earle E.D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter,* 9: 208-218.

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European Commission
**EUR 26706 EN – Joint Research Centre – Institute for Health and Consumer Protection**

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**Abstract**

In line with its mandate the European Union Reference Laboratory for GM Food and Feed (EU RL 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-68416-4 (unique identifier DAS-68416-4). The validation study was conducted according to the EU-RL GMFF validation procedure (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and internationally accepted guidelines.

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 kernels harbouring the DAS-68416-4 event as positive control DNA, genomic DNA extracted from conventional soybean kernels as negative control DNA). The EU-RL GMFF 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 study confirms that the method meets the method performance requirements as established by the EU-RL 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 .

LB-NA- 26706-EN-N

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