



JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of DAS-81419-2 and DAS-44406-6 event-specific PCR-based Methods applied to DNA extracted from GM Stack DAS-81419-2 x DAS-44406-6 soybean

Savini C., Maretti M.,
Mazzara M., Emons H.

European Union Reference Laboratory for
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Contact information

Name: EURL GMFF

Email: JRC-EURL-GMFF@ec.europa.eu

JRC Science Hub

<https://ec.europa.eu/jrc>

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European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Dow AgroSciences LLC, as represented by European Development Center Dow AgroSciences Ltd to request the authorisation of genetically modified stack (GM stack) DAS-81419-2 x DAS-44406-6 soybean (resistant against certain lepidopteran insect pests and tolerant to 2,4-D, glufosinate-ammonium and glyphosate herbicides), for food and feed uses, import and processing, in accordance with articles 5 and 17 of Regulation (EC) N° 1829/2003 GM Food and GM Feed. The unique identifier assigned to GM stack DAS-81419-2 x DAS-44406-6 soybean is DAS-81419-2 x DAS-44406-6.

The GM stack DAS-81419-2 x DAS-44406-6 soybean has been obtained by conventional crossing between the genetically modified soybean events: DAS-81419-2 and DAS-44406-6, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events DAS-81419-2 and DAS-44406-6 (see <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EURL GMFF has carried out only an *in-house* verification of the performance of each validated method when applied to genomic DNA extracted from GM stack DAS-81419-2 x DAS-44406-6 soybean.

The results of the *in-house* verification led to the conclusion that the individual methods meet the ENGL performance criteria also when applied to genomic DNA extracted from the GM stack DAS-81419-2 x DAS-44406-6 soybean.

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

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Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: Belac 268 TEST (Flexible Scope for DNA extraction, DNA identification and real Time PCR)] and ISO 17043:2010 accredited (certificate number: Belac 268 PT, proficiency test provider).

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

Address of contact laboratory:

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

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

1. Introduction

The EU legislative system ^(1, 2) for genetically modified food and feed foresees that any GMO for food and feed use shall undergo the authorisation process before it can be placed on the market. This holds true also for a GMO containing more than one single GM event obtained by conventional crossing, co-transformation or re-transformation (genetically modified stack).

Consequently, the application for authorisation of a GM stack shall be accompanied, among others, by an event-specific method for detection, identification and quantification for each GM event composing the stack, and by samples of the stack and food and feed derived from it. The EURL GMFF shall validate the event specific methods of detection proposed by the applicant with regard to their performance when applied to DNA extracted from the stack, and shall report to the European Food Safety Authority, who will include the EURL GMFF report in the overall opinion concerning the risk assessment and potential authorisation of the assessed stack. In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EURL GMFF carries out an *in-house* verification of the performance of each event-specific methods if this method has previously been validated by the EURL GMFF for the parental single-line event and these events have been stacked by conventional crossing. These criteria are met for the GM stack DAS-81419-2 x DAS-44406-6 soybean.

Upon reception of methods, samples and related data (step 1), the EURL GMFF carried out the assessment of the documentation (step 2) and the *in-house* verification of the methods (step 3) according to the requirements of Regulation (EU) No 503/2013 (Annex III).

The results of the *in-house* verification study were evaluated with reference to ENGL method performance requirements ⁽³⁾ and to the validation results on the individual events.

2. Step 1 (dossier reception and acceptance)

Dow AgroSciences LLC submitted the detection methods, data demonstrating their adequate performance when applied to genomic DNA extracted from the stack, and the corresponding control samples of DNA extracted from the GM stack soybean DAS-81419-2 x DAS-44406-6 and from non GM soybean.

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

3. Step 2 (dossier scientific assessment)

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL⁽³⁾ and with regard to their documentation and reliability.

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSD_r %) calculated by the applicant for the two methods applied to DAS-81419-2 x DAS-44406-6 soybean genomic DNA. Means are the average of sixteen replicates obtained through eight runs per for each event-specific detection system performed with Agilent Mx3005P[®] real-time PCR equipment. Individual GM-values obtained by averaging triplicates. Percentages are expressed as GM copy number/ reference gene copy number x 100.

Note: Numerical values presented in the following tables were rounded keeping two digits for values ≤ 1 , one digit for values between 1 and 10 and no digit for values ≥ 10 , unless otherwise stated. The calculations in the MS Excel files however were done over not rounded data. This approach might create small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSD_r %) provided by the applicant for the DAS-81419-2 and DAS-44406-6 methods applied to GM stack DAS-81419-2 x DAS-44406-6 soybean.

DAS-81419-2 *				
Sample GM %	Expected value (GMO %)			
	0.085	0.900	2.0	5.0
Mean	0.082	0.892	2.23	5.12
RSD_r (%)	9.00	7.50	4.30	5.70
Bias (%)	-3.50	-0.90	11.50	2.40
DAS-44406-6 *				
Sample GM %	Expected value (GMO %)			
	0.085	0.900	2.0	5.0
Mean	0.090	0.900	2.26	5.12
RSD_r (%)	15.00	11.60	9.00	8.30
Bias (%)	5.90	0.00	13.00	2.40

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

The EURL GMFF verified the data and concluded that they were reliable and seemed to confirm that the methods meet the ENGL performance criteria⁽³⁾.

One request for complementary information was addressed to the applicant, regarding the method, the control samples and the file of the annotated sequences of the GM-inserts. The EURL GMFF verified the data and the complementary information received and accepted the received clarifications as satisfactory. The dossier was therefore moved to step 3.

4. Step 3 (EURL GMFF experimental testing)

In step 3 the EURL GMFF implemented the two methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack DAS-81419-2 x DAS-44406-6 soybean.

4.1 Materials

The following control samples were provided by the applicant as:

- genomic DNA extracted from seeds of GM stack DAS-81419-2 x DAS-44406-6 soybean, homozygous for the loci, as positive control sample.
- genomic DNA extracted from seeds of conventional (non-GM) soybean with comparable genetic background, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack DAS-81419-2 x DAS-44406-6 soybean with the non-GM soybean genomic DNA, in a constant amount of total soybean genomic DNA. The same GM concentrations as in the validation of the methods for the single lines were achieved. Table 2 shows the five GM concentrations used in the verification of the DAS-81419-2 and DAS-44406-6 methods when applying them to genomic DNA extracted from the GM stack DAS-81419-2 x DAS-44406-6 soybean.

Table 2. Percentage (GM %) of DAS-81419-2 and DAS-44406-6 in DAS-81419-2 x DAS-44406-6 stack genomic DNA contained in the verification samples.

DAS-81419-2 GM %* [(GM DNA / total soybean DNA x 100)]	DAS-44406-6 GM %* [(GM DNA / total soybean DNA x 100)]
0.10	0.10
0.50	0.40
0.90	0.90
2.0	2.0
5.0	5.0

* percentage expressed in copy number ratio.

The applicant performed 45 amplification cycles instead of 40 as indicated by the validated protocols. The protocols of event DAS-81419-2 and DAS-44406-6 were implemented in the EURL GMFF laboratory in accordance with the validated methods already published for the individual DAS-81419-2 and DAS-44406-6 GM events (available at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>); the two methods were run with 40 cycles.

4.2 DNA extraction

The applicant referred to a method for DNA extraction from soybean previously validated by the EURL GMFF with regard to its performance characteristics. The method is considered valid, i.e. fit the purpose of providing soybean DNA of appropriate quality and amount for being used in subsequent PCR experiments.

The protocol for the DNA extraction method from soybean grains validated in the context of the method for DAS-68416 is available at <http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-11-10-XP-Report-DNA-Ext.pdf>.

The EURL GMFF recommends that laboratories using this validated method for testing complex or difficult matrices always verify that the extracted genomic DNA is of sufficient quality.

4.3 Experimental design

Eight PCR runs were carried out for each method. In each run, samples were analysed in parallel with both the GM-specific system and the reference system *Le1* (lectin). Five GM levels were examined per run, each GM level in duplicate. PCR analysis was performed in triplicate for all samples. In total, for each method DAS-81419-2 and DAS-44406-6, the quantification of the five GM levels was performed as an average of sixteen replicates per GM level (8 runs x 2 replicated levels per run). An Excel spreadsheet was used for determination of the GM %.

4.4 *PCR methods*

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack DAS-81419-2 x DAS-44406-6 soybean using the single detection methods previously validated for the respective single GM events DAS-81419-2 and DAS-44406-6.

For detection of GM soybean events DAS-81419-2 and DAS-44406-6, DNA fragments of 105-bp and 99-bp respectively are amplified using specific primers. PCR products are measured during each cycle (real-time) by means of target-specific oligonucleotide probes labelled with two fluorescent dyes: FAM (6-carboxyfluorescein) as reporter dye at their 5'-end and TAMRA (5-carboxytetramethylrhodamine) as a quencher dye at their 3'-end for the two events.

For quantification of GM soybean events DAS-81419-2 and DAS-44406-6, a taxon-specific reference system amplifies a 74-bp fragment of *Le1* (lectin) a soybean endogenous gene (GenBank accession number K00821), using two gene-specific primers and a gene-specific probe labelled with FAM and TAMRA.

For the relative quantification of GM soybean events DAS-81419-2 and DAS-44406-6 standard curves are generated both for the DAS-81419-2 and DAS-44406-6 and for the *Le1* (lectin) specific system by plotting Cq values of the calibration standards against the logarithm of the DNA amount and by fitting a linear regression into these data. Thereafter, the Cq values of the unknown samples are measured and, by means of the regression formula, the relative amount of DAS-81419-2 and DAS-44406-6 DNA is estimated.

For detailed information on the preparation of the respective standard curve calibration samples please refer to the protocols of the validated methods at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

4.4.1 *Deviations from the validated methods*

No deviations from the original validated methods were introduced.

4.5 *Results*

Tables 3 and 4 present the values of the slopes of the different standard curves generated by the EURL GMFF when using DNA extracted from the GM stack, from which the PCR efficiency is calculated using the formula $[10^{(-1/\text{slope})} - 1] \times 100$, and of the coefficient of determination (R^2) reported for all PCR systems in the eight runs, for GM soybean events DAS-81419-2 and DAS-44406-6. Slope values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and R^2 coefficient for the DAS-81419-2 method on GM stack DAS-81419-2 x DAS-44406-6 soybean.

Run	DAS-81419-2			<i>Le1</i>		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.59	90	1.00	-3.38	97	1.00
2	-3.54	92	1.00	-3.37	98	1.00
3	-3.48	94	1.00	-3.34	99	1.00
4	-3.53	92	1.00	-3.35	99	1.00
5	-3.50	93	1.00	-3.39	97	1.00
6	-3.38	98	1.00	-3.39	97	1.00
7	-3.42	96	1.00	-3.35	99	1.00
8	-3.45	95	1.00	-3.39	97	1.00
Mean	-3.48	94	1.00	-3.37	98	1.00

Table 4. Values of standard curve slope, PCR efficiency and R^2 coefficient for the DAS-44406-6 method on GM stack DAS-81419-2 x DAS-44406-6 soybean.

Run	DAS-44406-6			<i>Le1</i>		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.54	92	1.00	-3.33	100	1.00
2	-3.63	89	0.99	-3.30	101	1.00
3	-3.48	94	1.00	-3.35	99	1.00
4	-3.59	90	1.00	-3.35	99	1.00
5	-3.45	95	0.99	-3.29	101	1.00
6	-3.58	90	0.99	-3.28	102	1.00
7	-3.26	103	0.99	-3.33	100	1.00
8	-3.57	90	1.00	-3.31	100	1.00
Mean	-3.51	93	1.00	-3.32	100	1.00

The mean PCR efficiencies of the GM and species-specific systems were above 90 % (94 % and 98 % for DAS-81419-2 and *Le1* and 93 % and 100 % for DAS-44406-6 and *Le1*, respectively). The mean R^2 coefficient of the methods was 1.00 for all systems in all cases. The data presented in Tables 3 and 4 confirm the appropriate performance characteristics of the two methods when tested on GM stack DAS-81419-2 x DAS-44406-6 soybean in terms of PCR efficiency and R^2 coefficient.

The EURL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSD_r %) of the two methods applied to samples of DNA extracted from GM stack DAS-81419-2 x DAS-44406-6 soybean see Tables 5 and 6.

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the DAS-81419-2 method applied to genomic DNA extracted from GM stack DAS-81419-2 x DAS-44406-6 soybean.

DAS-81419-2					
Unknown sample GM %	Expected value (GMO %)				
	0.10	0.50	0.90	2.0	5.0
Mean	0.10	0.45	0.88	1.8	5.0
SD	0.01	0.03	0.06	0.14	0.19
RSD_r (%)	9.4	6.2	6.6	7.7	3.7
Bias (%)	3.6	-10	-2.4	-9.9	0.48

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the DAS-44406-6 method applied to genomic DNA extracted from GM stack DAS-81419-2 x DAS-44406-6 soybean.

DAS-44406-6					
Unknown sample GM %	Expected value (GMO %)				
	0.10	0.40	0.90	2.0	5.0
Mean	0.10	0.35	0.89	1.8	5.3
SD	0.01	0.04	0.07	0.29	0.43
RSD_r (%)	14	10	7.3	16	8.1
Bias (%)	0.51	-12	-1.2	-7.6	6.9

The trueness of the method is estimated using the measurements of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method should be less or equal to ± 25 % across the entire dynamic range. As shown in Tables 5 and 6, the values range from -10 % to 3.6 % for DAS-81419-2 and from -12 % to 6.9 % for DAS-44406-6. Therefore, the two methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack DAS-81419-2 x DAS-44406-6 soybean.

Tables 5 and 6 also show the relative repeatability standard deviation (RSD_r) estimated for each GM level. According to the ENGL acceptance criteria and method performance requirements, the RSD_r values should be equal to or below 25 %. As the values range between 3.7 % and 9.4 % for DAS-81419-2 and between 7.3 % and 16 % for DAS-44406-6, the two methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack DAS-81419-2 x DAS-44406-6 soybean.

5. Conclusions

The performance of the two event-specific methods for the detection and quantification of soybean single line events DAS-81419-2 and DAS-44406-6, when applied to genomic DNA extracted from GM stack DAS-81419-2 x DAS-44406-6 soybean, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant.

Therefore these methods, developed and validated to detect and quantify the single soybean events DAS-81419-2 and DAS-44406-6, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack DAS-81419-2 x DAS-44406-6 soybean, provided that sufficient genomic DNA of appropriate quality is available.

6. References

1. Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed (Text with EEA relevance). OJ L 268, 18.10.2003, p. 1–23.
2. Regulation (EU) No 503/2013 of 3 April 2013 "on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006".
3. European Network of GMO Laboratories (ENGL), 'Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing', 2015. http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf.

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