European Commission

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of DAS-68416-4 and MON 89788 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack DAS-68416-4 x MON-89788 soybean

European Union Reference Laboratory for Genetically Modified Food and Feed

2016

Joint Research Centre

EURL European Union Reference Laboratory for GM Food & Feed

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Contact information
Molecular Biology and Genomics Unit
Address: Joint Research Centre, Via Enrico Fermi 2749, TP 201, 21027 Ispra (VA), Italy
E-mail: eurl-gmff@jrc.ec.europa.eu
Tel.: +39 0332 78 5165
Fax: +39 0332 78 9333

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https://ec.europa.eu/jrc

JRC102288

Ispra, Italy: European Commission, 2016

How to cite: European Union Reference Laboratory for GM Food and Feed; Report on the Verification of the Performance of DAS-68416-4 and MON 89788 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack DAS-68416-4 x MON-89788 soybean; JRC102288

EUROPEAN COMMISSION
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29/06/2016

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Dow Agrosciences LLC, represented by Dow Agrosciences Europe, to request the authorisation of genetically modified stack (GM stack) DAS-68416-4 x MON 89788 soybean (tolerant to application of 2,4-D, glufosinate-ammonium and glyphosate herbicides), for all uses as for any other soybean, excluding cultivation, in accordance with articles 5 and 17 of Regulation (EC) No 1829/2003 on GM Food and GM Feed. The unique identifier assigned to GM stack DAS-68416-4 x MON 89788 soybean is DAS-68416-4 x MON-89788-1.
The GM stack DAS-68416-4 x MON 89788 soybean has been obtained by conventional crossing between two genetically modified soybean events DAS-68416-4 and MON 89788, 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-68416-4 and MON 89788 (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-68416-4 x MON 89788 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-68416-4 x MON 89788 soybean.

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

Content

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172 (Flexible Scope for DNA extraction and qualitative/quantitative PCR).
The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

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

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

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit (MBG)
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, 21027 Ispra (VA) – Italy
Functional mailbox: eurl-gmff@jrc.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 method 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-68416-4 x MON 89788 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 (EC) No 641/2004 (Annex I).

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

2. Step 1 (dossier reception and acceptance)

Dow Agrosciences LLC submitted the detection methods, the data demonstrating their adequate performance, and the corresponding control samples of DNA extracted from the GM stack soybean DAS-68416-4 x MON 89788 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 RSDr %) calculated by the applicant for the two methods on the stack DNA. Means are the average of sixteen replicates obtained through eight runs performed with Agilent Mx3005P real-time PCR equipment. Percentages are expressed as GM DNA / total DNA 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. 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, RSDr %) provided by the applicant for DAS-68416-4 and MON 89788 methods applied to GM stack DAS-68416-4 x MON 89788 soybean.

DAS-68416-4**				
Unknown Sample* GM%	**Expected value (GMO %)**			
	0.08	**0.9**	**2.0**	**5.0**
Mean	0.072	1.014	2.09	6.04
RSD_r (%)	9.9	8.3	8.4	7.8
Bias (%)	-10	12.7	4.5	20.8
MON 89788				
Unknown sample GM%	**Expected value (GMO %)**			
	0.08	**0.9**	**5.0**	**8.0**
Mean	0.068	0.938	5.18	8.79
RSD_r (%)	9.1	9.8	8.6	11
Bias (%)	-15	4.2	3.6	9.9

* Unknown samples are DNA samples containing different levels of GM DNA from stack material and non-GM DNA from conventional material.

** 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)].

Two requests of complementary information regarding control samples, DNA sequences and flat rate payment were addressed to the applicant. 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 DNA extracted from GM stack DAS-68416-4 x MON 89788 soybean.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homozygous seeds of DAS-68416-4 x MON 89788 soybean
- genomic DNA extracted from homogenised seeds of non-GM soybean

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack DAS-68416-4 x MON 89788 soybean and non-GM soybean in a constant amount of total soybean DNA. The same 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-68416-4 and MON 89788 methods when applying them to genomic DNA extracted from the GM stack DAS-68416-4 x MON 89788 soybean. These are the same concentrations used in the validation of these methods for the parental single line GMOs.

Table 2. Percentage (GM%) of DAS-68416-4 and MON 89788 in DAS-68416-4 x MON 89788 soybean DNA of verification samples.

DAS-68416-4 GM%* (GM DNA/ total soybean DNA x 100)	**MON 89788 GM%*** (GM DNA/ total soybean DNA x 100)
0.10	0.10
0.40	0.40
0.90	0.90
2.0	4.0
5.0	8.0

*(percentage expressed in copy number ratio)

The protocols described by the applicant were implemented precisely in the EURL GMFF laboratory and were in accordance with the protocols already published for the individual DAS-68416-4 and MON 89788 GM events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

4.2 DNA extraction

A method for DNA extraction from soybean seeds was previously evaluated by the EURL GMFF with regard to its performance characteristics and was 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 is available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.
Consequently, the EURL GMFF did not verify the DNA extraction method proposed by the applicant.

4.3 Experimental design

Eight PCR runs for each method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the reference system lectin (*Le1),* a soybean endogenous gene (GenBank Accession No K00821). Five GM levels were examined per run, for each GM level in duplicate. PCR analysis was performed in triplicate for all samples. In total, for each method, DAS-68416-4 and MON 89788*,* 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-68416-4 x MON 89788 soybean using the single detection methods previously validated for the respective single GM events DAS-68416-4 and MON 89788*.*

For detection of GM soybean events DAS-68416-4 and MON 89788, DNA fragments of 130-bp and 139-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 MGB (minor groove binding non-fluorescent quencher) and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at their 3′-end for DAS-68416-4 and MON 89788, respectively.

For quantification of GM soybean events DAS-68416-4 and MON 89788, a taxon-specific reference system amplifies a 74-bp fragment of lectin (*Le1*) a soybean endogenous gene (GenBank Accession No K00821), using two lectin gene-specific primers and a lectin gene-specific probe labelled with FAM and TAMRA.

For quantification of GM soybean events DAS-68416-4 and MON 89788 standard curves are generated both for the DAS-68416-4 and MON 89788 and for the lectin (*Le1*) specific system by

plotting Cq values of the calibration standards against the logarithm of the DNA copy numbers and by fitting a linear regression into these data. Thereafter, the normalised Cq values of the unknown samples are measured and, by means of the regression formula, the relative amount of DAS-68416-4 and MON 89788 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.

4.5 Deviations from the validated methods

No deviations from the original validated methods were introduced.

4.6 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 $[10^{(-1/slope)} - 1] \times 100$, and of the R^2 (expressing the linearity of the regression) reported for all PCR systems in the eight runs, for GM soybean events DAS-68416-4 and MON 89788.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the DAS-68416-4 method on GM stack DAS-68416-4 x MON 89788 soybean.

Run	DAS-68416-4			*Le1*		
	Slope*	**PCR Efficiency (%)**	**Linearity (R^2)**	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**
1	-3.57	91	1.00	-3.38	97	1.00
2	-3.70	86	0.99	-3.34	99	1.00
3	-3.61	89	1.00	-3.39	97	1.00
4	-3.56	91	1.00	-3.46	94	1.00
5	-3.44	95	1.00	-3.39	97	1.00
6	-3.44	95	1.00	-3.29	101	1.00
7	-3.59	90	1.00	-3.40	97	1.00
8	-3.63	88	0.99	-3.43	96	1.00
Mean	-3.57	91	1.00	-3.39	97	1.00

*Slope values were rounded to two digits

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 89788 method on GM stack DAS-68416-4 x MON 89788 soybean.

Run	MON 89788			*Le1*		
	Slope	**PCR Efficiency (%)**	**Linearity (R^2)**	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**
1	-3.28	102	1.00	-3.30	101	1.00
2	-3.30	101	1.00	-3.37	98	1.00
3	-3.41	96	1.00	-3.35	99	1.00
4	-3.31	100	1.00	-3.38	98	1.00
5	-3.31	101	1.00	-3.29	101	1.00
6	-3.37	98	1.00	-3.38	98	1.00
7	-3.40	97	1.00	-3.39	97	1.00
8	-3.43	96	1.00	-3.32	100	1.00
Mean	-3.35	99	1.00	-3.35	99	1.00

The mean PCR efficiencies of the GM and species-specific systems were above 90% (91% for the DAS-68416-4 and 99% the MON 89788 systems, and 97% and 99% for *Le1*, respectively). The linearity of the methods (R^2) 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-68416-4 x MON 89788 soybean in terms of PCR efficiency and linearity.

The EURL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) of the two methods applied to samples of DNA extracted from GM stack DAS-68416-4 x MON 89788 soybean (see tables 5 and 6).

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the DAS-68416-4 method applied to genomic DNA extracted from GM stack DAS-68416-4 x MON 89788 soybean.

DAS-68416-4					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**2.0**	**5.0**
Mean	0.12	0.38	0.95	1.8	5.2
SD	0.01	0.05	0.09	0.27	0.32
RSD_r (%)	12	13	8.9	14	6.1
Bias (%)	17	-5.7	6.0	-8.1	3.2

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON89788 method applied to genomic DNA extracted from GM stack DAS-68416-4 x MON 89788 soybean.

MON 89788					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**4.0**	**8.0**
Mean	0.1	0.36	0.89	3.7	8.1
SD	0.01	0.03	0.06	0.28	0.70
RSD_r (%)	13	8.9	7.1	7.7	8.6
Bias (%)	-3.6	-9.9	-1.0	-8.5	1.7

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 ± 25% across the entire dynamic range. As shown in Tables 5 and 6, the values range from -8.1% to 17% for DAS-68416-4 and from -9.8% to 1.7% for MON 89788. Therefore, the two methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack DAS-68416-4 x MON 89788 soybean.

Tables 5 and 6 also show the relative repeatability standard deviation (RSD_r) as estimated for each GM level. According to the ENGL acceptance criteria and method performance requirements, the EURL GMFF requires RSD_r values to be below 25%. As the values range between 6.1% and 14% for DAS-68416-4 and between 7.1% and 13% for MON 89788, the two methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack DAS-68416-4 x MON 89788 soybean.

5. Conclusions

The performance of the two event-specific methods for the detection and quantification of soybean single line events DAS-68416-4 and MON 89788, when applied to genomic DNA extracted from GM stack DAS-68416-4 x MON 89788 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-68416-4 and MON 89788, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack DAS-68416-4 x MON 89788 soybean, supposed 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. Commission Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified foodand feed, the notification of existing products and adventitious or technicallyunavoidable presence of genetically modified material which has benefited from afavourable risk evaluation (Text with EEA relevance). OJ L 102, 7.4.2004, p. 14–25.

3. European Network of GMO Laboratories: Definition of minimum performance requirements for analytical methods of GMO testing. 13 October 2008. http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf.

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