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Report on the Verification of the Performance of MON 87769 and MON 89788 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack MON 87769 x MON 89788 Soybean

European Union Reference Laboratory for Genetically Modified Food and Feed

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Joint Research Centre

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European Commission
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27 April 2015

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Monsanto Company to request the authorisation of genetically modified stack (GM stack) MON 87769 X MON 89788 soybean, for food and feed uses, and import and processing, in accordance with articles 5 and 17 of Regulation (EC) No 1829/2003 GM Food and GM Feed. The unique identifier assigned to GM stack MON 87769 X MON 89788 soybean is MON-87769-7 x MON-89788-1.

The GM stack MON 87769 X MON 89788 soybean has been obtained by conventional crossing between two genetically modified soybean events: MON 87769 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 MON 87769 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 MON 87769 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 MON 87769 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) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

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

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

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

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1. Introduction

The EU legislative system [1, 2] for genetically modified food and feed provides 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 MON 87769 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 [3] and to the validation results on the individual events.

2. Step 1 (dossier reception and acceptance)

Monsanto Company submitted the detection methods, data demonstrating their adequate performance, and the corresponding control sample DNA extracted from GM stack MON 87769 X MON 89788 soybean 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 %). Trueness (bias %), was calculated by the EURL GMFF based on the data and information

provided by the applicant for the two methods on the stack DNA; precision (expressed as RSDr %) was provided by the applicant. Means are the average of fifteen replicates obtained through one run performed with ABI 7500 real-time PCR equipment, performed in duplicate. Percentages are expressed as (GM DNA / total DNA) x 100.

Note: Numerical values presented in the tables of this report 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 generate 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 %) for the MON 87769 and MON 89788 methods applied to GM stack MON 87769 X MON 89788 soybean.

MON 87769			
Unknown sample* GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.085(**)	1.1	8.9
RSD_r (%)	13	6.1	5.3
Bias (%)	0	8	-11
MON 89788			
Unknown sample* GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.08	0.99	9.7
RSD_r (%)	13	5.7	5.1
Bias (%)	-5.9	-1.0	-2.7

* Unknown samples are DNA samples containing different levels of GM DNA (expressed in copy number) from stack material and non-GM DNA from conventional material.

** Value not rounded

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)].

A request of complementary information regarding the genetic background of the positive and negative control samples was 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 MON 87769 X MON 89788 soybean.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenised seeds of GM stack MON 87769 X MON 89788 soybean
- genomic DNA extracted from homogenized seeds of non GM soybean.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack MON 87769 X MON 89788 soybean and genomic DNA extracted from 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 MON 87769 and MON 89788 methods when applying them to genomic DNA extracted from the GM stack MON 87769 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 of MON 87769 and MON 89788 in MON 87769 X MON 89788 verification samples.

MON 87769 GM% (GM DNA / Non-GM DNA x 100)	**MON 89788 GM%** (GM DNA / Non-GM DNA x 100)
0.10	0.10
0.50	0.40
0.90	0.90
5.0	4.0
9.0	8.0

The *in-house* verification followed the protocols already published as validated methods for the individual MON 87769 and MON 89788 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/summaries/MON89788_Soya_DNAExtrSampl_report.pdf.

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 *lec* (*lectin gene*). 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 (MON 87769 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 GM%.

4.4 PCR methods

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack MON 87769 X MON 89788 soybean using the single detection methods previously validated for the respective single GM events MON 87769 and MON 89788.

For detection of GM soybean events MON 87769 and MON 89788, DNA fragments of 87-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 TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at their 3′-end.

For quantification of GM soybean events MON 87769 and MON 89788, a taxon-specific reference system amplifies a 74-bp fragment of *lectin* (*Lec)* soybean endogenous gene (GenBank: K00821.1), using two *Lec* gene-specific primers and a *Lec* gene-specific probe labelled with FAM and TAMRA.

For quantification of GM soybean events MON 87769 and MON 89788, standard curves are generated both for the MON 87769 and MON 89788, and for the *Lec* 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 MON 87769 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 MON 89788 and MON 87769.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R2) for the MON 87769 method on GM stack MON 87769 X MON 89788 soybean.

Run	MON 87769			*Lec*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.33	100	0.99	-3.30	101	1.00
2	-3.17	107	0.99	-3.31	101	1.00
3	-3.22	104	1.00	-3.30	101	1.00
4	-3.25	103	1.00	-3.30	101	1.00
5	-3.26	102	0.99	-3.38	98	0.99
6	-3.36	99	0.99	-3.19	106	0.99
7	-3.43	96	1.00	-3.31	100	1.00
8	-3.37	98	0.99	-3.23	104	0.99
Mean	-3.30	101	0.99	-3.29	101	1.00

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 89788 method on GM stack MON 87769 X MON 89788 soybean.

Run	MON 89788			*Lec*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.45	95	1.00	-3.24	104	0.99
2	-3.37	98	1.00	-3.33	100	1.00
3	-3.51	93	0.99	-3.21	105	0.99
4	-3.39	97	1.00	-3.28	102	0.99
5	-3.34	99	1.00	-3.23	104	0.99
6	-3.42	96	1.00	-3.29	101	0.99
7	-3.33	100	1.00	-3.38	98	1.00
8	-3.32	100	1.00	-3.24	103	0.99
Mean	-3.39	97	1.00	-3.27	102	0.99

The mean PCR efficiencies of the GM and species-specific systems were above 97% (101% for MON 87769 and 97% for MON 89788, 101% and 102% for the *Lec* systems, respectively). The calibration curves for all assays were linear as estimated by the coefficient of determination (R^2), giving a value ≥0.99 for all assays. The data presented in Tables 3 and 4 confirm the appropriate performance characteristics of the two methods when tested on GM stack MON 87769 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 MON 87769 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 MON 87769 method applied to genomic DNA extracted from GM stack MON 87769 X MON 89788 soybean.

MON 87769					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**0.9**	**5.0**	**9.0**
Mean	0.0976(*)	0.46	0.92	4.7	9.4
SD	0.01	0.06	0.09	0.25	1.1
RSD_r (%)	12	13	9.4	5.4	12
Bias (%)	-2.4	-8.3	1.8	-7.0	3.9

* Value not rounded

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 89788 method applied to genomic DNA extracted from GM stack MON 87769 X MON 89788 soybean.

MON 89788					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.4**	**0.9**	**4.0**	**8.0**
Mean	0.103(*)	0.42	0.95	4.0	8.0
SD	0.01	0.03	0.07	0.46	0.86
RSD_r (%)	12	8.0	7.7	12	11
Bias (%)	3.0	3.9	5.3	-0.88	-0.52

* Value not rounded

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 -8.3% to 3.9% for MON 87769 and from -0.88% to 5.3% 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 MON 87769 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 5.4% and 13% for MON 87769 and between 7.7% and 12% for MON 89788, the two methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack MON 87769 X MON 89788 soybean.

5. Comparison of method performance on MON 87769 X MON 89788 and on the single events

An indicative comparison of the performance (bias, RSDr %) of the two methods applied to GM stack MON 87769 X MON 89788 soybean and on the single-line events is shown in Tables 7 and 8. The performance of the methods on the single lines was previously validated through international collaborative trials (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

Note: the comparison of data generated in different testing conditions and different times is intended to be only of qualitative nature; differences in the figures reported are not necessarily statistically significant.

Table 7. Qualitative comparison of the performance of the MON 87769 detection method applied to genomic DNA extracted from GM stack MON 87769 X MON 89788 soybean and to genomic DNA extracted from the single line event MON 89788.

Trueness and repeatability of MON 87769 quantification on MON 87769 × MON 89788			**Trueness and repeatability of MON 87769 quantification on single event MON 87769***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	-2.4	12	**0.10**	-5.8	13
0.5	-8.3	13	**0.5**	-5.2	14
0.9	1.8	9.4	**0.9**	-2.2	9.7
5.0	-7.0	5.4	**5.0**	4.6	7.0
9.0	3.9	12	**9.0**	1.8	14

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx)

Table 8. Qualitative comparison of the performance of the MON 89788 detection method applied to genomic DNA extracted from GM stack MON 87769 X MON 89788 soybean and to genomic DNA extracted from the single line event MON 89788.

Trueness and repeatability of MON 89788 quantification on MON 87769 × MON 89788			**Trueness and repeatability of MON 89788 quantification on single event MON 89788***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.10	3.0	12	**0.10**	-14	16
0.40	3.9	8.0	**0.40**	-5.0	22
0.90	5.3	7.7	**0.90**	-0.88	15
4.0	-0.88	12	**4.0**	11	13
8.0	-0.52	11	**8.0**	2.8	12

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx)

6. Conclusions

The performance of the two event-specific methods for the detection and quantification of soybean events MON 87769 and MON 89788, when applied to genomic DNA extracted from GM stack MON 87769 X MON 89788 soybean, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant.

The method verification has demonstrated that the PCR efficiency, linearity, trueness and repeatability of the methods were within the limits established by the ENGL.

In conclusion, the verification study confirmed that the two methods are capable to detect, identify and quantify each of the GM events when applied to genomic DNA of suitable quality, extracted from GM stack MON 87769 X MON 89788 soybean.

Therefore these methods, developed and validated to detect and quantify the single events, can be equally applied for the detection and quantification of the respective events combined in GM stack MON 87769 X MON 89788 soybean.

7. 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 food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable 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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The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events MON 87769 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 MON 87769 x MON 89788 soybean.

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