European Commission

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of MON 87705, MON 87708 and MON 89788 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack MON 87705 x MON 87708 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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Abstract

An application was submitted by Monsanto Company represented by Monsanto Europe S.A., to request the authorisation of genetically modified stack (GM stack) MON 87705 x MON 87708 x MON 89788 soybean and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, import and processing, in accordance with articles 5 and 17 of Regulation (EC) No 1829/2003 on GM Food and Feed. The unique identifier assigned to GM stack MON 87705 x MON 87708 x MON 89788 soybean is MON-87705-6 x MON-87708-9 x MON-89788-1.

The GM stack MON 87705 x MON 87708 x MON 89788 soybean has been obtained from traditional breeding methods between progeny of the three genetically modified soybean single events MON 87705, MON 87708 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 87705, MON 87708 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 DNA extracted from GM stack MON 87705 x MON 87708 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 DNA extracted from the GM stack MON 87705 x MON 87708 x MON 89788 soybean.

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9 June 2016

European Union Reference Laboratory for GM Food and Feed

Executive Summary

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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: 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 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 MON 87705 x MON 87708 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) and its amendment as laid down in Regulation (EU) No 503/2013 (Annex III).

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

2. Step 1 (dossier reception and acceptance)

Monsanto Company represented by Monsanto Europe S.A. 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 MON 87705 x MON 87708 x MON 89788 and from conventional 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 three methods applied to MON 87705 x MON 87708 x MON 89788 soybean genomic DNA. Means are the average of 15 quantification results. Percentages are expressed as GM DNA / total DNA x 100.

Note: Numerical values presented in the following tables (except data from applicant) 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 MON 87705, MON 87708 and MON 89788 methods applied to GM stack MON 87705 x MON 87708 x MON 89788 soybean.

MON 87705			
Sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.09	1.10	10.49
RSD_r (%)	10.24	6.60	4.57
Bias (%)	5.29	10.27	4.89
MON 87708			
Sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.09	1.08	10.61
RSD_r (%)	11.34	6.47	6.07
Bias (%)	8.21	8.44	6.15
MON 89788			
Sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.09	1.07	11.44
RSD_r (%)	8.99	5.45	5.12
Bias (%)	10.21	7.03	14.38

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)].

Requests for complementary information regarding the experimental design, data analysis and control samples were addressed to the applicant. The EURL GMFF verified the data and the complementary information received and accepted the 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 three methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack MON 87705 x MON 87708 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 87705 x MON 87708 x MON 89788 soybean homozygous for events MON 87705, MON 87708 and MON 89788;
- Genomic DNA extracted from homogenized seeds of conventional (non-GM) soybean.

The EURL GMFF prepared test samples of different GMO concentrations by mixing DNA extracted from GM stack MON 87705 x MON 87708 x MON 89788 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 prepared. Table 2 shows the five GM concentrations used in the verification of the MON 87705, MON 87708 and MON 89788 methods when applying them to genomic DNA extracted from the GM stack MON 87705 x MON 87708 x MON 89788 soybean.

Table 2. Percentage (GM %) of event MON 87705, event MON 87708 and event MON 89788 in MON 87705 x MON 87708 x MON 89788 stack genomic DNA contained in the verification samples.

MON 87705 GM% [(GM DNA / total soybean DNA) x 100]	**MON 87708 GM%** [(GM DNA / total soybean DNA) x 100]	**MON 89788 GM%** [(GM DNA / total soybean DNA) x 100]
0.10	0.10	0.10
0.50	0.45	0.40
0.90	0.90	0.90
5.0	4.0	4.0
8.0	8.0	8.0

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 MON 87705, MON 87708 and MON 89788 GM events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx) with the deviations reported in § 4.4.1.

4.2 DNA extraction

A method for DNA extraction from plant tissues (e.g. seeds, leaf) of soybean was previously evaluated by the EURL GMFF with regard to its performance characteristics and was

considered valid, i.e. fit for the purpose of providing DNA from soybean seeds of appropriate quality and amount to be used in subsequent PCR experiments.

Annex III to Reg. (EU) No 503/2013 [2] requires the applicant to discuss the validity and limitations of the detection methods in the various types of foods and feeds (matrices) that are expected to be placed on the market. To this regard the applicant stated that the applicability of the quantitative real-time PCR methods developed for MON 87705, MON 87708 and MON 89788 depends on the isolation of sufficient quantity and quality of purified DNA. The provided DNA extraction method is intended for extraction of genomic DNA from seeds, which results in primarily high molecular weight DNA. The applicant also informed the EURL GMFF that during the processing of soybean seeds into food and feed ingredients a number of steps are typically followed, which can influence the quality and intactness of the DNA contained in the final processed soybean products [4,5,6]. DNA extraction from certain of these processed matrices may require additional rounds of purification in order to achieve the quality standards needed for quantitative real-time PCR [7,8].
On a general note 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.

The protocol for the DNA extraction method is available at http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-05-06-XP-Corrected-version-1.pdf.

Consequently, the EURL GMFF did not verify the DNA extraction method proposed by the applicant.

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 *lec* (*lectin* gene). 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 (MON 87705, MON 87708 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 tests on genomic DNA extracted from GM stack MON 87705 x MON 87708 x MON 89788 soybean using the single detection methods previously validated for the respective single GM events MON 87705, MON 87708 and MON 89788.

For detection of GM soybean events MON 87705, MON 87708 and MON 89788, DNA fragments of 86-bp, 91-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 the 5'-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3'-end for all three events.

For quantification of GM soybean events MON 87705, MON 87708 and MON 89788, a taxon-specific reference system amplifies a 74-bp fragment of *lectin* (*lec*), a soybean endogenous gene (GenBank Accession No K00821.1), using two *lectin* gene-specific primers and a gene-specific probe labelled with FAM and TAMRA.

For quantification of GM soybean events MON 87705, MON 87708 and MON 89788, standard curves are generated both for the MON 87705, MON 87708 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 87705, MON 87708 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.

4.4.1 Deviations from the validated methods

No deviations from the original validated methods were introduced, except for MON 89788 method. In the original validation of this method unknown samples were prepared at 50 ng/uL, like standard curve samples. For the verification of MON 89788 method on stacked material, samples were prepared at a concentration of 45 ng/uL, to ensure that the *lec* Cqs remain within the standard curve range.

4.5 Results

Tables 3, 4 and 5 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 the coefficient of determination (R^2) reported for all PCR systems in the eight runs, for GM soybean events MON 87705, MON 87708 and MON 89788. Slope values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and coefficient of determination (R^2) for the MON 87705 method on GM stack MON 87705 x MON 87708 x MON 89788 soybean.

Run	MON 87705			*lec*		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.19	106	1.00	-3.31	101	1.00
2	-3.31	101	1.00	-3.36	99	1.00
3	-3.30	101	1.00	-3.34	99	1.00
4	-3.33	99	1.00	-3.38	98	1.00
5	-3.31	101	1.00	-3.36	98	1.00
6	-3.35	99	1.00	-3.38	98	1.00
7	-3.29	101	1.00	-3.37	98	1.00
8	-3.25	103	1.00	-3.32	100	1.00
Mean	**-3.29**	**101**	**1.00**	**-3.35**	**99**	**1.00**

Table 4. Values of standard curve slope, PCR efficiency and coefficient of determination (R^2) for the MON 87708 method on GM stack MON 87705 x MON 87708 x MON 89788 soybean.

Run	MON 87708			*lec*		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.32	100	1.00	-3.29	101	0.99
2	-3.40	97	0.99	-3.42	96	1.00
3	-3.23	104	1.00	-3.35	99	1.00
4	-3.37	98	0.99	-3.34	99	1.00
5	-3.27	102	1.00	-3.27	102	1.00
6	-3.27	102	1.00	-3.23	104	1.00
7	-3.31	100	1.00	-3.31	100	1.00
8	-3.26	102	1.00	-3.30	101	1.00
Mean	**-3.30**	**101**	**1.00**	**-3.32**	**100**	**1.00**

Table 5. Values of standard curve slope, PCR efficiency and coefficient of determination (R^2) for the MON 89788 method on GM stack MON 87705 x MON 87708 x MON 89788 soybean.

Run	MON 89788			*lec*		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.28	102	1.00	-3.21	105	1.00
2	-3.26	103	0.99	-3.32	100	1.00
3	-3.30	101	1.00	-3.26	103	1.00
4	-3.28	102	1.00	-3.34	99	1.00
5	-3.30	101	1.00	-3.33	100	1.00
6	-3.29	101	0.99	-3.31	100	1.00
7	-3.31	100	1.00	-3.25	103	1.00
8	-3.23	104	1.00	-3.34	99	1.00
Mean	**-3.28**	**102**	**1.00**	**-3.29**	**101**	**1.00**

The mean PCR efficiencies of the GM and species-specific systems were 101% for MON 87705 and MON 87708 GM events, 102% for MON 89788 GM event and between 99% and 101% for the *lec* system. The mean coefficient of determination (R^2) was 1.00 for all methods. The data presented in Tables 3, 4 and 5 confirm the appropriate performance characteristics of the three methods when tested on GM stack MON 87705 x MON 87708 x MON 89788 soybean in terms of PCR efficiency and coefficient of determination.

The EURL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSD_r %) of the three methods applied to samples of DNA extracted from GM stack MON 87705 x MON 87708 x MON 89788 soybean (see tables 6, 7 and 8).

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 87705 method applied to DNA extracted from GM stack MON 87705 x MON 87708 x MON 89788 soybean.

MON 87705					
Unknown sample GM%	Expected value (GMO%)				
	0.10	**0.50**	**0.90**	**5.0**	**8.0**
Mean	0.10	0.44	1.0	5.0	8.5
SD	0.01	0.05	0.07	0.50	0.74
RSD_r (%)	8.1	12	7.2	10	8.7
Bias (%)	-1.9	-12	13	-0.62	6.2

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 87708 method applied to DNA extracted from GM stack MON 87705 x MON 87708 x MON 89788 soybean.

MON 87708					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.45**	**0.90**	**4.0**	**8.0**
Mean	0.09	0.41	0.93	3.7	8.5
SD	0.01	0.04	0.07	0.21	0.61
RSD_r (%)	14	8.7	8.0	5.6	7.1
Bias (%)	-10	-9.1	3.2	-7.7	6.1

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 89788 method applied to DNA extracted from GM stack MON 87705 x MON 87708 x MON 89788 soybean.

MON 89788					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**4.0**	**8.0**
Mean	0.09	0.33	0.91	3.7	8.7
SD	0.01	0.03	0.09	0.34	0.69
RSD_r (%)	12	7.7	9.8	9.0	7.9
Bias (%)	-14	-16	1.3	-6.3	8.2

The trueness of the method is estimated using the measurements of the method bias for each GM level. According to the ENGL the trueness of the method should be less or equal to ± 25% across the entire dynamic range. As shown in Tables 6, 7 and 8, the values range from -12% to 13% for MON 87705, from -10% to 6.1% for MON 87708 and from -16% to 8.2% for MON 89788. Therefore, the three methods satisfy the ENGL requirements throughout their respective dynamic ranges, also when applied to genomic DNA extracted from GM stack MON 87705 x MON 87708 x MON 89788 soybean.

Tables 6, 7 and 8 also show the relative repeatability standard deviation (RSD_r) estimated for each GM level. According to the ENGL the RSD_r values should be below 25%. As the values range between 7.2% and 12% for MON 87705, between 5.6% and 14% for MON 87708 and between 7.7% and 12% for MON 89788 the three methods satisfy this requirement throughout their respective dynamic ranges when applied to genomic DNA extracted from GM stack MON 87705 x MON 87708 x MON 89788 soybean.

5. Conclusions

The performance of the three event-specific methods for the detection and quantification of soybean single line events MON 87705, MON 87708 and MON 89788, when applied to the control samples provided by the applicant, i.e. genomic DNA extracted from GM stack MON 87705 x MON 87708 x MON 89788 soybean, meets the ENGL performance requirements.

Therefore these methods, developed and validated to detect and quantify the single soybean events MON 87705, MON 87708 and MON 89788, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack MON 87705 x MON 87708 x MON 89788 soybean or any of its sub-combinations, supposed that sufficient genomic DNA of appropriate quality is available.

6. References

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