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Report on the Verification of the Performance of MON 87751, MON 87701, MON 87708 and MON 89788 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean

European Union Reference Laboratory for Genetically Modified Food and Feed

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Institute for Health and Consumer Protection

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Report on the Verification of the Performance of MON 87751, MON 87701, MON 87708 and MON 89788 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean

17 June 2016

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Monsanto Company, as represented by Monsanto Europe S.A. to request the authorisation of genetically modified stack (GM stack) MON 87751 x MON 87701 x MON 87708 x MON 89788 (conferring insect protection, dicamba tolerance and glyphosate tolerance) 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) N° 1829/2003 GM Food and GM Feed. The unique identifier assigned to GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean is MON-87751-6 x MON-877Ø1-6 x MON-877Ø8-2 x MON-89788-1.

The GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean has been obtained by conventional crossing between the genetically modified soybean events: MON 87751, MON 87701, 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 87751, MON 87701, 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/MPR%20Report%20Application%2020_10_2015.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 87751 x MON 87701 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 genomic DNA extracted from the GM stack MON 87751 x MON 87701 x MON 87708 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.

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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 87751 x MON 87701 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 (EU) No 503/2013 (Annex III).

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)

Monsanto Company represented by Monsanto Europe S.A. submitted the detection methods, data demonstrating their adequate performance, and the corresponding control samples of DNA extracted from the GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean and from non GM soybean.

The dossier was found to be complete and was thus 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 four methods applied to stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean genomic DNA. Means are the average of fifteen replicates obtained through one run performed with ABI 7500 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, 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 87751, MON 87701, MON 87708 and MON 89788 methods applied to GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean.

MON 87751*			
Unknown sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.095	1.15	10.96
RSD_r (%)	10.89	4.53	4.38
Bias (%)	11.18	14.61	9.64
MON 87701*			
Unknown sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.095	1.14	9.78
RSD_r (%)	8.35	7.36	7.89
Bias (%)	12.08	14.39	-2.20
MON 87708*			
Unknown sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.088	1.04	9.24
RSD_r (%)	14.79	7.63	7.14
Bias (%)	3.18	4.39	-7.55
MON 89788*			
Unknown sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.096	1.08	10.42
RSD_r (%)	10.19	9.14	10.17
Bias (%)	13.16	7.66	4.19

*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)].

A request for complementary information regarding the methods, the control samples and the DNA sequences 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 four methods in its own laboratory and performed a verification of their performance when applied to DNA extracted from GM stack MON 87751 x MON 87701 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 87751 x MON 87701 x MON 87708 x MON 89788 soybean, homozygous for events MON 87751, MON 87701, MON 87708, and MON 89788, as positive control sample.
- Genomic DNA extracted from homogenised seeds of conventional (non GM) soybean, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack MON 87751 x MON 87701 x MON 87708 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 MON 87751, MON 87701**,** MON 87708 and MON 89788 methods when applying them to genomic DNA extracted from the GM stack MON 87751 x MON 87701 x MON 87708 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 MON 87751, MON 87701, MON 87708 and MON 89788 in MON 87751 x MON 87701 x MON 87708 x MON 89788 DNA of verification samples.

MON 87751 GM%* [(GM DNA / total soybean DNA x 100)]	**MON 87701 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	0.085	0.10	0.10
0.90	0.26	0.45	0.40
2.50	0.90	0.90	0.90
5.0	2.70	4.0	4.0
10.0	8.10	8.0	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 MON

87751**,** MON 87701**,** MON 87708 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.

Note: the EURL GMFF recommends that laboratories using a validated method always verify that the extracted genomic DNA is of sufficient quality and quantity, particularly when testing complex or difficult matrices.

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 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, each GM level in duplicate. PCR analysis was performed in triplicate for all samples. In total, for each method MON 87751, MON 87701, 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 the 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 MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean using the single detection methods previously validated for the respective single GM events MON 87751, MON 87701, MON 87708 and MON 89788.

For detection of GM soybean events MON 87751, MON 87701, MON 87708 and MON 89788, DNA fragments of 87 bp, 89 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 their 3'-end.

For quantification of GM soybean events MON 87751, MON 87701, 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* specific primers and a gene-specific probe labelled with FAM and TAMRA.

For relative quantification of GM soybean events MON 87751, MON 87701, MON 87708 and MON 89788 standard curves are generated both for the MON 87751, MON 87701, 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 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 MON 87751, MON 87701, 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.

4.5 Results

Tables 3, 4, 5 and 6 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 coefficient of determination (R^2) reported for all PCR systems in the eight runs, for GM soybean events MON 87751, MON 87701, MON 87708 and MON 89788. Slope and R^2 coefficient values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and coefficient of determination (R^2) for the MON 87751 method on GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean.

Run	MON 87751			lec		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.23	104	1.00	-3.20	105	1.00
2	-3.32	100	1.00	-3.34	99	1.00
3	-3.25	103	1.00	-3.26	103	1.00
4	-3.19	106	1.00	-3.27	102	1.00
5	-3.25	103	1.00	-3.25	103	1.00
6	-3.36	98	1.00	-3.27	102	1.00
7	-3.37	98	1.00	-3.29	101	1.00
8	-3.14	108	1.00	-3.25	103	1.00
Mean	-3.26	103	1.00	-3.27	102	1.00

Table 4. Values of standard curve slope, PCR efficiency and coefficient of determination (R^2) for the MON 87701 method on GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean.

Run	MON 87701			lec		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.33	100	1.00	-3.34	99	1.00
2	-3.47	94	1.00	-3.27	102	1.00
3	-3.29	102	1.00	-3.24	103	1.00
4	-3.44	95	0.99	-3.19	106	1.00
5	-3.32	100	1.00	-3.27	102	1.00
6	-3.42	96	1.00	-3.36	98	1.00
7	-3.37	98	1.00	-3.30	101	1.00
8	-3.25	103	1.00	-3.32	100	1.00
Mean	-3.36	98	1.00	-3.29	102	1.00

Table 5. Values of standard curve slope, PCR efficiency and coefficient of determination (R^2) for the MON 87708 method on GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean.

Run	MON 87708			lec		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.27	102	1.00	-3.30	101	1.00
2	-3.33	100	0.99	-3.39	97	1.00
3	-3.35	99	1.00	-3.28	102	1.00
4	-3.35	99	1.00	-3.25	103	1.00
5	-3.41	96	1.00	-3.35	99	1.00
6	-3.34	99	1.00	-3.36	99	1.00
7	-3.32	100	1.00	-3.32	100	1.00
8	-3.41	96	1.00	-3.32	100	1.00
Mean	-3.35	99	1.00	-3.32	100	1.00

Table 6. Values of standard curve slope, PCR efficiency and coefficient of determination (R^2) for the MON 89788 method on GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean.

Run	MON 89788			lec		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.23	104	1.00	-3.21	105	1.00
2	-3.27	102	0.99	-3.25	103	1.00
3	-3.23	104	0.99	-3.29	101	1.00
4	-3.30	101	1.00	-3.23	104	1.00
5	-3.21	105	1.00	-3.25	103	1.00
6	-3.21	105	1.00	-3.22	104	1.00
7	-3.22	104	1.00	-3.24	103	1.00
8	-3.32	100	1.00	-3.28	102	1.00
Mean	-3.25	103	1.00	-3.25	103	1.00

The mean PCR efficiencies of the GM and species-specific systems were 103% for MON 87751 method, 98% for MON 87701, 99% for MON 87708, 103% for MON 89788 and between 100% and 103% for the *lec* system). The mean coefficient of determination (R^2) was 1.00 for all systems in all cases. The data presented in Tables 3, 4, 5 and 6 confirm the appropriate performance characteristics of the four methods when tested on genomic DNA extracted from GM stack MON 87751 x MON 87701 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 four methods applied to samples of DNA extracted from GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean (see tables 7, 8, 9 and 10).

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 87751 method applied to genomic DNA extracted from GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean.

MON 87751					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.90**	**2.5**	**5.0**	**10.0**
Mean	0.10	0.84	2.59	4.86	11.0
SD	0.01	0.08	0.16	0.52	0.77
RSD_r (%)	13	9.7	6.1	11	7.1
Bias (%)	1.0	-6.8	3.4	-2.7	9.5

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 87701 method applied to genomic DNA extracted from GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean.

MON 87701					
Unknown sample GM%	**Expected value (GMO%)**				
	0.085	**0.26**	**0.90**	**2.7**	**8.1**
Mean	0.088	0.22	0.88	2.4	8.4
SD	0.01	0.02	0.05	0.29	0.77
RSD_r (%)	10	9.3	5.5	12	9.2
Bias (%)	3.2	-17	-2.1	-12	4.2

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 87708 method applied to genomic DNA extracted from GM stack MON 87751 x MON 87701 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	0.39	0.85	3.6	8.0
SD	0.01	0.04	0.08	0.44	0.77
RSD_r (%)	9.9	11	9.0	12	9.6
Bias (%)	-14	-14	-5.7	-11	0.59

Table 10. 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 87751 x MON 87701 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	0.35	0.88	3.6	8.6
SD	0.01	0.03	0.05	0.36	0.40
RSD_r (%)	8.0	9.2	5.9	9.9	4.6
Bias (%)	-11	-12	-2.5	-8.9	8.1

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 7, 8, 9 and 10, the values range from -6.8% to 9.5% for MON 87751, from -17% to 4.2% for MON 87701, from -14% to 0.59% for MON 87708, and from -12% to 8.1% for MON 89788. Therefore, the four methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean.

Tables 7, 8, 9 and 10 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 equal or below 25%. As the values range between 6.1% and 13% for MON 87751, between 5.5% and 12% for MON 87701, between 9.0% and 12% for MON 87708 and between 4.6% and 9.9% for MON 89788, the four methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean.

5. Conclusions

The performance of the four event-specific methods for the detection and quantification of soybean single line events MON 87751, MON87701, MON 87708 and MON 89788, when applied to genomic DNA extracted from GM stack MON 87751 x MON 87701 x MON 87708 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 MON 87751, MON87701, MON 87708 and MON 89788, can be equally applied for the detection and quantification of the respective events in genomic DNA extracted from the GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean or any of its sub-combinations, supposed that sufficient genomic DNA of appropriate quality is available.

This statement is valid for all types of food and feed products that could contain the GM stack MON 87751 x MON 87701 x MON 87708 x MON 89788 soybean or any of its sub-combinations.

6. References

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