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Report on the Verification of the Performance of MON 87708, MON 89788 and A5547-127 event-specific PCR-based Methods applied to DNA extracted from GM Stack MON 87708 x MON 89788 x A5547-127 soybean

European Union Reference Laboratory for
Genetically Modified Food and Feed

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Report on the Verification of the Performance of MON 87708, MON 89788 and A5547-127 event-specific PCR-based Methods applied to DNA extracted from GM Stack MON 87708 x MON 89788 x A5547-127 soybean

18/10/2017

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Monsanto Company, represented by Monsanto Europe S.A., to request the authorisation of genetically modified stack (GM stack) MON 87708 x MON 89788 x A5547-127 soybean (tolerance to dicamba, glyphosate and glufosinate), 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[(1)]. The unique identifier assigned to GM stack MON 87708 x MON 89788 x A5547-127 soybean is MON-877Ø8-9 x MON-89788-1 x ACS-GMØØ6-4.

The GM stack MON 87708 x MON 89788 x A5547-127 soybean was obtained by conventional crossing with the genetically modified soybean events: MON 87708, MON 89788 and A5547-127, 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 87708, MON 89788 and A5547-127 (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 an *in-house* verification of the performance of each validated method when applied to genomic DNA extracted from GM stack MON 87708 x MON 89788 x A5547-127 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 87708 x MON 89788 x A5547-127 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: 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.

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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, the EURL GMFF carries out an *in-house* verification of the performance of each event-specific method if this method has previously been validated for the parental single-line event and these events have been combined by conventional crossing. These criteria are met for the GM stack MON 87708 x MON 89788 x A5547-127 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)[2].

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 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 MON 87708 x MON 89788 x A5547-127 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 %) calculated by the applicant for the three methods applied to MON 87708 x MON 89788 x A5547-127 soybean genomic DNA. Means are the average of fifteen replicates obtained in two runs, one for the GM-specific assay and one for the taxon-specific reference assay, performed with ABI 7500 real-time PCR equipment. 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, RSDr %) provided by the applicant for the MON 87708, MON 89788 and A5547-127 methods applied to GM stack MON 87708 x MON 89788 x A5547-127 soybean.

MON 87708*			
Sample* GM%	Expected value (GMO %)		
	0.085	1	10
Mean	0.086	0.95	9.02
RSD_r (%)	18.78	9.63	13.98
Bias (%)	0.82	-4.74	-9.78
MON 89788			
Sample GM%	Expected value (GMO %)		
	0.085	1	10
Mean	0.067	1.03	9.62
RSD_r (%)	16.44	10.24	8.78
Bias (%)	-20.59	3.07	-3.82
A5547-127			
Sample GM%	Expected value (GMO %)		
	0.085	1	10
Mean	0.079	1.05	11.66
RSD_r (%)	12.22	7.43	7.66
Bias (%)	-6.72	4.67	16.60

* Values 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 regarding the tests conducted on the stack GM soybean 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 three methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack MON 87708 x MON 89788 x A5547-127 soybean.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenised seeds of MON 87708 x MON 89788 x A5547-127 soybean homozygous for the three inserts, as positive control sample.
- genomic DNA extracted from homogenized 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 87708 x MON 89788 x A5547-127 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 obtained. Table 2 shows the five GM concentrations used in the verification of MON 87708, MON 89788 and A5547-127 methods when applying them to genomic DNA extracted from the GM stack MON 87708 x MON 89788 x A5547-127 soybean.

Table 2. Percentage (GM %) of MON 87708 x MON 89788 x A5547-127 soybean genomic DNA contained in the verification samples.

MON 87708 GM%* [(GM DNA / total[plant] DNA x 100)]	**MON 89788 GM%*** [(GM DNA / total[plant] DNA x 100)]	**A5547-127 GM%*** [(GM DNA / total[plant] DNA x 100)]
0.10	0.10	0.08
0.45	0.40	0.40
0.90	0.90	0.90
4.0	4.0	4.0
8.0	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 87708, MON 89788 and A5547-127 soybean (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

4.2 DNA extraction

A method for DNA extraction from soybean 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.

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 87708, MON 89788 and A5547-127 soybean depends on the isolation of sufficient quantity and quality of purified DNA. The detection method for MON 87708, MON 89788, and A5547-127 should work as far as good quality and intact DNA can be extracted from processed food and feed materials.

The method provided is intended for extraction of genomic DNA from seeds, which results in 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 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]. Each of these steps can influence the quality and intactness of DNA contained in the final processed soybean products [7-8]. After extraction of DNA from certain of these processed matrices, the DNA may need additional rounds of processing in order to eliminate PCR inhibitors and clean-up the DNA, to achieve a quality of genomic DNA that is suitable for PCR [9-10]. The applicant also informed that, regardless of the DNA extraction method employed, studies have shown that the processing steps for soybean result in significant degradation of high molecular weight DNA and failure to amplify products greater than a few 100 base pairs [7-8]; in addition, random DNA fragmentation is known to lead to variability in quantitating DNA by qPCR [11], thus affecting the ability to accurately quantify the presence of the GM event in processed fractions.

Hence, 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/CRLVL_0805_XP.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 respective validated reference systems from the soybean endogenous 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 87708, MON 89788 and A5547-127, 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 MON 87708 x MON 89788 x A5547-127 soybean using the single detection methods previously validated for the respective single GM events MON 87708, MON 89788 and A5547-127 soybean.

For detection of GM soybean events MON 87708, MON 89788 and A5547-127, DNA fragments of *91*-bp, 139-bp and 75-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* (carboxytetramethylrhodamine) as a quencher dye at their 3′-end for all *three* events.

For quantification of GM soybean events MON 87708, MON 89788 and A5547-127, two taxon-specific reference systems were used based on lectin, a soybean endogenous gene (GenBank K00821). For the relative quantification of events MON 87708 and MON 89788 a taxon-specific reference system amplifies a 74-bp fragment of lectin using gene-specific primers and probe labelled with *FAM* and *TAMRA*; For the relative quantification of event A5547-127 a taxon-specific reference system amplifies a 102-bp fragment of lectin using gene-specific primers and probe labelled with *VIC®* and *TAMRA*.

For the relative quantification of GM soybean events MON 87708, MON 89788 and A5547-127 standard curves are generated for MON 87708, MON 89788 and A5547-127 and for lectin by plotting Cq values of the calibration standards against the logarithm of the target copy number 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 87708, MON 89788 and A5547-127 copies are 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 and 5 report the values of the slopes of the different standard curves generated by the EURL GMFF when using DNA extracted from the GM stack. PCR efficiency is calculated using the formula $[10^{(-1/slope)} - 1] \times 100$, the coefficient of determination (R^2) is reported for all PCR systems in the eight runs, for GM soybean events MON 87708, MON 89788 and A5547-127 soybean. Slope values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 87708 method on GM stack MON 87708 x MON 89788 x A5547-127 soybean.

Run	*MON 87708*			*lectin*		
	Slope	**PCR Efficiency (%)**	**R^2 coefficient**	**Slope**	**PCR Efficiency (%)**	**R^2 coefficient**
1	-3.45	95	1.00	-3.46	94	1.00
2	-3.43	96	1.00	-3.45	95	1.00
3	-3.46	95	1.00	-3.42	96	1.00
4	-3.53	92	1.00	-3.42	96	1.00
5	-3.42	96	1.00	-3.33	100	1.00
6	-3.45	95	1.00	-3.49	93	1.00
7	-3.29	101	1.00	-3.41	97	1.00
8	-3.41	96	1.00	-3.38	98	1.00
Mean	**-3.43**	**96**	**1.00**	**-3.42**	**96**	**1.00**

Table 4. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 89788 method on GM stack MON 87708 x MON 89788 x A5547-127 soybean.

Run	*MON 89788*			*lectin*		
	Slope	**PCR Efficiency (%)**	**R^2 coefficient**	**Slope**	**PCR Efficiency (%)**	**R^2 coefficient**
1	-3.27	102	0.99	-3.33	100	1.00
2	-3.36	99	0.99	-3.41	96	1.00
3	-3.25	103	0.99	-3.43	96	1.00
4	-3.41	96	0.99	-3.38	98	1.00
5	-3.28	102	0.99	-3.30	101	1.00
6	-3.35	99	0.99	-3.44	95	1.00
7	-3.40	97	0.99	-3.43	96	1.00
8	-3.38	98	0.99	-3.38	98	1.00
Mean	**-3.34**	**99**	**0.99**	**-3.39**	**97**	**1.00**

Table 5. Values of standard curve slope, PCR efficiency and R^2 coefficient for the A5547-127 method on GM stack MON 87708 x MON 89788 x A5547-127 soybean.

Run	*A5547-127*			*lectin*		
	Slope	**PCR Efficiency (%)**	**R^2 coefficient**	**Slope**	**PCR Efficiency (%)**	**R^2 coefficient**
1	-3.22	104	1.00	-3.18	106	1.00
2	-3.25	103	1.00	-3.16	107	0.99
3	-3.34	99	1.00	-3.15	108	0.99
4	-3.21	105	1.00	-3.17	107	1.00
5	-3.28	102	1.00	-3.16	107	0.99
6	-3.24	103	1.00	-3.22	105	0.99
7	-3.33	100	1.00	-3.15	108	0.99
8	-3.25	103	1.00	-3.08	111	1.00
Mean	**-3.27**	**102**	**1.00**	**-3.16**	**107**	**0.99**

The mean PCR efficiencies of the GM and species-specific systems were between 110% and 90%. The mean efficiency was 96% for MON 87708, 99% for MON 89788 and 102% for A5547-127, respectively and 96%, 97% and 107% respectively for the lectin systems used for relative quantification.

The mean R^2 coefficient of the methods was from 0.99 to 1.00 in all cases. The data presented in Tables 3, 4 and 5 confirm the appropriate performance characteristics of the three methods when tested on GM stack MON 87708 x MON 89788 x A5547-127 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, RSDr %) of the three methods applied to samples of DNA extracted from GM stack MON 87708 x MON 89788 x A5547-127 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 87708 method applied to genomic DNA extracted from GM stack MON 87708 x MON 89788 x A5547-127 soybean.

MON 87708					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.45**	**0.90**	**4.0**	**8.0**
Mean	0.12	0.42	0.91	3.7	8.1
SD	0.02	0.05	0.09	0.34	0.83
RSD_r (%)	14	11	9.3	9.1	10
Bias (%)	18	-6.4	1.4	-8.1	0.64

Table 7. 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 87708 x MON 89788 x A5547-127 soybean.

MON 89788					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**4.0**	**8.0**
Mean	0.09	0.33	0.85	3.6	8.5
SD	0.01	0.04	0.08	0.43	0.77
RSD_r (%)	12	13	9.3	12	9.0
Bias (%)	-11	-17	-5.2	-9.0	5.8

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the A5547-127 method applied to genomic DNA extracted from GM stack MON 87708 x MON 89788 x A5547-127 soybean.

A5547-127					
Unknown sample GM%	**Expected value (GMO%)**				
	0.08	**0.40**	**0.90**	**4.0**	**8.0**
Mean	0.07	0.35	0.85	3.8	7.7
SD	0.01	0.03	0.06	0.32	0.50
RSD_r (%)	11	7.6	6.5	8.5	6.5
Bias (%)	-18	-12	-6.0	-4.4	-4.0

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 6, 7 and 8, the values range from -8.1 % to 18 % for MON 87708, from -17 % to 5.8 % for MON 89788 and from -18 % to -4.0 % for A5547-127. Therefore, the three methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack MON 87708 x MON 89788 x A5547-127 soybean.

Tables 6, 7 and 8 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 9.1 % and 14 % for MON 87708, between 9.0% and 13 % for MON 89788 and between 6.5 % and 11% for A5547-127, the three methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack MON 87708 x MON 89788 x A5547-127 soybean.

5. Conclusions

The performance of the three event-specific methods for the detection and quantification of soybean single line events MON 87708, MON 89788 and A5547-127, when applied to genomic DNA extracted from GM stack MON 87708 x MON 89788 x A5547-127 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 87708, MON 89788 and A5547-127, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack MON 87708 x MON 89788 x A5547-127 soybean, supposed that sufficient genomic DNA of appropriate quality is available.

6. References

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