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JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of MON 88913 and MON 15985 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack MON 88913 x MON 15985 Cotton

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 88913 × MON 15985 cotton (tolerant to glyphosate and protected against targeted lepidopteran insect pests) 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 88913 × MON 15985 cotton is MON-88913-8 x MON-15985-7.

The GM stack MON 88913 × MON 15985 cotton has been obtained by conventional crossing between two genetically modified cotton events: MON 88913 and MON 15985, 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 88913 and MON 15985 (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 88913 × MON 15985 cotton.

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 88913 × MON 15985 cotton.

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10 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.

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 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 88913 × MON 15985 cotton.

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 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 DNA extracted from GM stack cotton MON 88913 × MON 15985 and from non GM cotton.

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 %) based on the data provided by the applicant, and precision (expressed as RSDr %), calculated by the applicant for each method applied on the stack genomic DNA. Means are the average of fiftteen replicates obtained through one run performed with ABI PRISM 7700 sequence detection system. Percentages are expressed as GM DNA / total DNA x 100.

Note: Numerical values presented in the following tables (except performance data reported by the 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, RSDr %) for the MON 88913 and MON 15985 methods applied to GM stack MON 88913 x MON 15985 cotton.

MON 88913			
Unknown sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.082	1.10	10.83
RSD_r (%)	16.23	6.00	7.37
Bias (%)	-3.53	10	8.30
MON 15985			
Unknown sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.081	1.06	11.45
RSD_r (%)	17.88	10.86	6.63
Bias (%)	-4.71	6.00	14.50

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)].

Four requests of complementary information regarding scope, sequences, zygosity of the positive control, and genetic background of positive and negative control samples 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 MON 88913 × MON 15985 cotton.

4.1 Materials

The following control samples were provided by the applicant:

- Genomic DNA extracted from MON 88913 x MON 15985 cotton seeds (Lot GLP-0602-16860-S), homozygous for MON 88913 and MON 15985
- Genomic DNA extracted from conventional cotton seeds (Lot GLP-0602-16859-S)

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack MON 88913 × MON 15985 cotton and genomic DNA extracted from non GM cotton in a constant amount of total cotton 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 88913 and MON 15985 methods when applying them to genomic DNA extracted from the GM stack MON 88913 × MON 15985 cotton. These are the same concentrations used in the validation of these methods for the parental single line GMOs.

Table 2. Percentage of MON 88913 and MON 15985 in MON 88913 × MON 15985 in the verification samples.

MON 88913 GM% (GM DNA / total cotton DNA x 100)	**MON 15985 GM%** (GM DNA / total cotton DNA x 100)
0.09	0.10
0.30	0.40
0.90	0.90
3.0	2.5
8.0	6.0

The protocols validated for the individual GM events MON 88913 and MON 15985 (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx), were followed in the *in-house* verification with the deviation reported in § 4.4.1.

4.2 DNA extraction

A method for DNA extraction from cotton 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 cotton 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/MON88913_cotton_DNAExtr_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 *acp1* (*acyl carrier protein*). 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 88913 and MON 15985), 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 genomic DNA extracted from GM stack MON 88913 × MON 15985 cotton using the single detection methods previously validated for the respective single GM events MON 88913 and MON 15985.

For detection of GM cotton events MON 88913 and MON 15985, DNA fragments of 94-bp and 82-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 MGBNFQ (non-fluorescent quencher) or TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at the 3′-end for MON 88913 and MON 15985, respectively.

For quantification of GM cotton events MON 88913 and MON 15985, a taxon-specific reference system amplifies a 76-bp fragment *acp1 a* cotton endogenous gene using two *acp1* gene-specific primers and an *acp1* gene-specific probe labelled with FAM and TAMRA.

For quantification of GM cotton events MON 88913 and MON 15985, standard curves are generated both for the MON 88913 and MON 15985 and for the *acp1* 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 88913 and MON 15985 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.

4.1.1 Deviations from the validated methods

Updated bioinformatics analyses indicated that the *acp1* target is present in two copies per cotton haploid genome while the methods for quantification of MON 88913 and MON 15985 were originally validated as ratio of GM DNA copy numbers to target taxon-specific DNA copy number, assuming that the latter was only present in one copy per genome. Therefore, in the current report all quantification data are reported as mass fractions of GM DNA in the cotton stacked event MON 88913 x MON 15985 (§ 4.1) in comparison to the mass fraction of the total cotton DNA in the sample.

Note: the under-estimation of the copy number for the reference gene is only relevant if the measurement is done in copy numbers and then a wrong conversion factor is used to estimate the mass fraction. However, results should be expressed in the same unit of measurement of the calibrator. In most cases CRMs, certified in mass fractions of GM material, are used as calibrators; consequently, the copy number of the reference gene does not have an influence on the expression of results of quantification.

4.5 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 coefficient of determination (R^2) reported for all PCR systems in the runs, for GM cotton events MON 88913 and MON 15985. Slope values were rounded to two digits. Two additional curves were run for the method MON 88913 to test in sixteen replicates the level 0.9% in the stack cotton MON 88913 x MON 15985 (Table 3B).

Table 3A. Values of standard curve slope, PCR efficiency and R^2 for the MON 88913 method on GM stack MON 88913 × MON 15985 cotton.

Run	MON 88913			*acp1*		
	Slope	**PCR Efficiency (%)**	**Linearity (R^2)**	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**
1	-3.15	108	1.00	-3.18	106	0.99
2	-3.17	107	1.00	-3.25	103	0.99
3	-3.24	103	0.99	-3.17	107	1.00
4	-3.17	107	1.00	-3.13	108	1.00
5	-3.23	104	0.99	-3.21	105	1.00
6	-3.20	105	0.99	-3.21	105	1.00
7	-3.15	108	0.99	-3.27	102	0.99
8	-3.11	110	1.00	-3.17	107	1.00
Mean	-3.18	106	0.99	-3.20	106	1.00

Table 3B. Values of standard curve slope, PCR efficiency and R^2 for the MON 88913 method on GM stack MON 88913 × MON 15985 cotton at the 0.9% GM-level.

Run	MON 88913			*acp1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
9	-3.19	106	0.99	-3.11	110	0.99
10	-3.20	105	1.00	-3.14	108	1.00
Mean	-3.20	105	1.00	-3.12	109	1.00

Table 4. Values of standard curve slope, PCR efficiency and R^2 for the MON 15985 method on GM stack MON 88913 × MON 15985 cotton.

Run	MON 15985			*acp1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.10	110	1.00	-3.18	106	0.99
2	-3.16	107	1.00	-3.20	105	1.00
3	-3.09	111	1.00	-3.13	109	1.00
4	-3.16	107	1.00	-3.25	103	0.99
5	-3.10	110	1.00	-3.22	104	0.99
6	-3.10	110	0.99	-3.11	110	0.99
7	-3.08	111	0.99	-3.13	109	0.99
8	-3.03	114	0.99	-3.20	105	1.00
Mean	-3.10	110	1.00	-3.18	106	0.99

The mean PCR efficiencies of the GM and species-specific systems were above 100% (105-106% for the MON 88913, from 105% to 109% for the *acp1* system, and 110% for MON 15985, respectively). The coefficient of determination (R^2) of the methods was between 0.99 and 1.00 for all systems. The data presented in Tables 3 and 4 confirm the appropriate performance characteristics of the two methods when tested on GM stack MON 88913 × MON 15985 cotton 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 88913 × MON 15985 cotton, see Tables 5 and 6.

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 88913 method applied to genomic DNA extracted from GM stack MON 88913 × MON 15985 cotton.

MON 88913					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.30**	**0.90**	**3.0**	**8.0**
Mean	0.07	0.23	0.74	2.90	7.78
RSD_r (%)	11	9.8	4.7	10	8.7
Bias (%)	-24	-22	-17	-3.2	-2.8

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 15985 method applied to genomic DNA extracted from GM stack MON 88913 × MON 15985 cotton.

MON 15985					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**2.5**	**6.0**
Mean	0.08	0.32	0.73	2.37	5.97
RSD_r (%)	15	10	8.2	22	9.4
Bias (%)	-21	-20	-19	-5.2	-0.48

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 -2.8% to -24% for MON 88913 and from -0.48% to -21% for MON 15985. Therefore, the two methods satisfy the ENGL requirements throughout their respective dynamic ranges, also when applied to genomic DNA extracted from GM stack MON 88913 × MON 15985 cotton.

Tables 5 and 6 also show the relative repeatability standard deviation (RSD_r) as estimated for each GM level. According to the ENGL, the RSD_r values should be below 25%. As the values range between 4.7% and 11% for MON 88913 and between 8.2% and 22% for MON 15985, the two methods satisfy this requirement throughout their respective dynamic ranges when applied to genomic DNA extracted from GM stack MON 88913 × MON 15985 cotton.

5. Conclusions

The performance of the two event-specific methods for the detection and quantification of cotton events MON 88913 and MON 15985, when applied to the control samples provided by the applicant, i.e. genomic DNA extracted from GM stack MON 88913 × MON 15985 cotton, meets the ENGL performance requirements.

Therefore these methods, developed and validated to detect and quantify the single cotton events, can be equally applied for the detection and quantification of the respective events

combined in GM stack MON 88913 × MON 15985 cotton, provided 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 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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