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Report on the Verification of the Performance of MON 88302, Ms8 and Rf3 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack MON 88302 x Ms8 x Rf3

European Union Reference Laboratory for
Genetically Modified Food and Feed

2015

Joint Research Centre

EURL European Union Reference Laboratory for GM Food & Feed

European Commission
Joint Research Centre
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European Union Reference Laboratory for GM Food and Feed

11 November 2015

Executive Summary

An application was submitted by Monsanto Europe S.A. and Bayer CropScience AG to request the authorisation of genetically modified stack (GM stack) MON 88302 x Ms8 x Rf3 oilseed rape (tolerant to glyphosate and glufosinate-ammonium herbicide families) and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, and 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 88302 x Ms8 x Rf3 oilseed rape is MON-883Ø2-9 x ACS-BNØØ5-8 x ACS-BNØØ3-6.

The GM stack MON 88302 x Ms8 x Rf3 oilseed rape has been obtained by conventional crossing between three genetically modified oilseed rape events: MON 88302, Ms8 and Rf3, 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 88302, Ms8 and Rf3 (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 88302 x Ms8 x Rf3 oilseed rape.

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 88302 x Ms8 x Rf3 oilseed rape.

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 verify 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, that 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 events and if these events have been stacked by conventional crossing. These criteria are met for the GM stack MON 88302 x Ms8 x Rf3 oilseed rape.

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 of the individual events.

2. Step 1 (dossier reception and acceptance)

Monsanto Europe S.A. and Bayer CropScience AG submitted the detection methods, the data demonstrating their adequate performance, and the corresponding control samples consisting of DNA extracted from GM stack oilseed rape MON 88302 x Ms8 x Rf3 and from non-GM oilseed rape.

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 on the stack DNA. Means are the average of fifteen replicates, obtained through one run for each event, performed with ABI 7500 real-time PCR equipment. Percentages are expressed as GM DNA / total DNA x 100.

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) for the MON 88302, Ms8 and Rf3 methods applied to GM stack MON 88302 x Ms8 x Rf3.

MON 88302			
Unknown sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10.0**
Mean	0.104	1.12	9.85
RSD_r (%)	16.40	6.26	7.74
Bias (%)	22.68	12.13	-1.47
Ms8			
Unknown sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10.0**
Mean	0.092	0.96	8.83
RSD_r (%)	18.14	6.86	6.17
Bias (%)	8.80	-3.52	-11.73
Rf3			
Unknown sample GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10.0**
Mean	0.084	0.91	8.28
RSD_r (%)	13.08	7.46	7.59
Bias (%)	-1.01	-8.50	-17.24

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)].

Three requests of complementary information were addressed to Monsanto Europe S.A. and one to Bayer CropScience AG. They were related to the *in-house* verification, the deviations from the validated protocols for Ms8 and Rf3 events (listed in 4.5) and the DNA sequences of the stacked events. 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 DNA extracted from GM stack MON 88302 x Ms8 x Rf3.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenized seeds of MON 88302 x Ms8 x Rf3 oilseed rape
- genomic DNA extracted from homogenized non-GM oilseed rape seeds.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack MON 88302 x Ms8 x Rf3 and genomic DNA extracted from non-GM oilseed rape in a constant amount of total oilseed rape DNA. Table 2 shows the five GM concentrations used in the verification of the MON 88302, Ms8 and Rf3 methods when applying them to genomic DNA extracted from the GM stack MON 88302 x Ms8 x Rf3 oilseed rape. These are the same concentrations used in the validation of these methods for the parental single GM lines.

Table 2. Percentage of MON 88302, Ms8 and Rf3 in MON 88302 x Ms8 x Rf3 verification samples.

MON 88302 GM% (GM copies / Non-GM copies x 100)	**Ms8 GM%** (GM DNA mass/ Non-GM DNA mass x 100)	**Rf3 GM%** (GM DNA mass/ Non-GM DNA mass x 100)
9.00	3.60	3.60
4.50	1.80	1.80
0.90	0.90	0.90
0.40	0.40	0.40
0.05	0.10	0.10

The *in-house* verification followed the protocols (reagents, concentrations, primer/probe sequences) already published as validated methods for the individual MON 88302, Ms8 and Rf3 events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

4.2 DNA extraction

A method for DNA extraction from oilseed rape was previously validated by the EURL GMFF with regard to its performance characteristics and was considered valid, i.e. fit the purpose of providing oilseed rape 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/StatusOfDossiers.aspx

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; *cruciferin* was used as the reference gene in all three cases; for MON 88302, the *Ccf* reference system was used while for Ms8 and Rf3 the *CruA* reference system was used in accordance with the corresponding previously validated methods. 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 88302, Ms8 and Rf3), 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 88302 x Ms8 x Rf3 using the single detection methods previously validated for the respective single GM events MON 88302, Ms8 and Rf3.

For detection of GM oilseed rape events MON 88302, Ms8 and Rf3, DNA fragments of 101-bp, 130-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: 6-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 oilseed rape events MON 88302, Ms8 and Rf3, two taxon-specific reference systems targeting the same reference gene (*cruciferin*) were used. For MON 88302, a 78-bp fragment of oilseed rape-specific *cruciferin* storage proteins (*Ccf*) was used as the reference system (EMBL: X59294, UniprotKb: P33523), using *Ccf* gene-specific primers and a specific probe labelled with VIC as the reporter at its 5'-end and TAMRA as the quencher at its 3'-end. For Ms8 and Rf3 the *CruA* reference system (GenBank X14555) was used that amplifies a 101-bp fragment, using two *CruA* gene-specific primers and a *CruA* gene-specific probe labelled with VIC and TAMRA.

For quantification of GM oilseed rape event MON 88302 standard curves are generated both for the MON 88302 and for the *Ccf* 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 88302 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.

For quantification of GM oilseed rape events Ms8 and Rf3 DNA in a test sample, the normalised ΔCt values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCq values against the logarithm of the amount of Ms8 and Rf3 events DNA, respectively). The normalised ΔCq values of the unknown samples are measured and, by means of the regression formula, the relative amount of Ms8 and Rf3 events, respectively, is estimated.

4.5 Deviations from the validated methods

For Ms8 and Rf3 events, the methods used by the applicant to generate the data submitted to the EURL GMFF in this application were based on the 'two standard curves' approach (one calibration curve for the GM system and one for the reference system). This approach was not substantially affecting the method and produced results within the ENGL the acceptance criteria. It is however different from the ΔCt approach originally followed by the applicant and validated in the context of the single line applications. However, the EURL GMFF performed the verification of the Ms8 and Rf3 methods on Ms8 x Rf3 x GT73 DNA using the validated methods with no modifications (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

When Ms8 and Rf3 methods were originally validated, the information available was that the target taxon-specific target *CruA* was present as single copy gene in the genome. Recently, thanks to more advanced bioinformatics tools, it became evident that the *CruA* target is present in two copies per haploid genome; as a consequence, since Ms8 and Rf3 control samples used were homozygous, the dynamic range reported in the validation reports is correct when values are considered in mass fractions of GM DNA. Therefore the EURL GMFF verified the Ms8 and Rf3 methods on GM stack Ms8 x Rf3 x GT73 DNA in mass fractions of GM DNA.

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 oilseed rape events MON 88302, Ms8 and Rf3.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 88302 method on GM stack MON 88302 x Ms8 x Rf3 oilseed rape.

Run	MON 88302			*Ccf*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.37	98	1.00	-3.26	103	1.00
2	-3.34	99	1.00	-3.30	101	1.00
3	-3.36	99	1.00	-3.26	103	1.00
4	-3.29	101	0.99	-3.30	101	1.00
5	-3.30	101	1.00	-3.23	104	1.00
6	-3.32	100	1.00	-3.32	100	1.00
7	-3.34	99	1.00	-3.26	102	1.00
8	-3.29	101	1.00	-3.33	100	1.00
Mean	-3.33	100	1.00	-3.28	102	1.00

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the Ms8 method on GM stack MON 88302 x Ms8 x Rf3 oilseed rape.

Run	Ms8		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.45	95	1.00
2	-3.33	100	1.00
3	-3.40	97	1.00
4	-3.37	98	1.00
5	-3.51	93	1.00
6	-3.42	96	1.00
7	-3.37	98	1.00
8	-3.28	102	1.00
Mean	-3.39	97	1.00

Table 5. Values of standard curve slope, PCR efficiency and linearity (R^2) for the Rf3 method on GM stack MON 88302 x Ms8 x Rf3 oilseed rape.

Run	Rf3		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.44	95	1.00
2	-3.45	95	1.00
3	-3.60	90	1.00
4	-3.45	95	1.00
5	-3.39	97	1.00
6	-3.51	93	1.00
7	-3.75	85	1.00
8	-3.49	93	1.00
Mean	-3.51	93	1.00

The mean PCR efficiencies of the GM and species-specific systems were 100% for MON 88302 and 102% for *Ccf* system respectively; for event Ms8 the mean PCR efficiency was 97%, while for Rf3 it was 93%. The linearity of the methods (R2) was 1.00 for all systems 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 88302 x Ms8 x Rf3 oilseed rape 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 three methods applied to samples of DNA extracted from GM stack MON 88302 x Ms8 x Rf3 oilseed rape see Tables 6, 7 and 8.

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 88302 method applied to genomic DNA extracted from GM stack MON 88302 x Ms8 x Rf3 oilseed rape.

MON 88302					
Unknown sample GM%	Expected value (GMO%)				
	0.05	**0.40**	**0.90**	**4.5**	**9.0**
Mean	0.04	0.31	0.81	4.1	8.7
SD	0.00	0.02	0.06	0.35	0.57
RSD_r (%)	11	5.3	7.5	8.4	6.6
Bias (%)	-17	-24	-9.8	-9.1	-3.5

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSDr %) of the Ms8 method applied to genomic DNA extracted from GM stack MON 88302 x Ms8 x Rf3 oilseed rape.

Ms8					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**1.8**	**3.6**
Mean	0.11	0.39	0.87	1.7	3.6
SD	0.03	0.05	0.09	0.20	0.29
RSD_r (%)	24	13	10	12	7.9
Bias (%)	9.4	-1.8	-3.2	-5.2	1.1

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the Rf3 method applied to genomic DNA extracted from GM stack MON 88302 x Ms8 x Rf3 oilseed rape.

Rf3					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**1.8**	**3.6**
Mean	0.11	0.38	0.88	1.6	3.6
SD	0.01	0.05	0.07	0.12	0.23
RSD_r (%)	12	12	7.7	7.5	6.5
Bias (%)	9.9	-4.3	-2.3	-9.0	-0.18

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 6, 7 and 8, the values range from -3.5% to -24% for MON 88302, from -5.2% to 9.4% for Ms8 and from -9.0% to 9.9% for Rf3. Therefore, the three methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack MON 88302 x Ms8 x Rf3 oilseed rape.

Tables 6, 7 and 8 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 5.3% and 11% for MON 88302, between 7.9% and 24% for Ms8 and between 6.5% and 12% for Rf3, the three methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack MON 88302x Ms8 x Rf3 oilseed rape.

5. Comparison of method performance on MON 88302 x Ms8 x Rf3 and on the single events

An indicative comparison of the performance (bias, RSDr %) of the three methods applied to GM stack MON 88302x Ms8 x Rf3 and to the single-lines is shown in Tables 9, 10 and 11. 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 9. Qualitative comparison of the performance of the MON 88302 detection method applied to genomic DNA extracted from GM stack MON 88302 x Ms8 x Rf3 oilseed rape and to genomic DNA extracted from the single line event MON 88302.

Trueness and repeatability of MON 88302 quantification on MON 88302 x Ms8 x Rf3 oilseed rape			**Trueness and repeatability of MON 88302 quantification on single event MON 88302***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.05	-17	11	**0.05**	6.2	9.1
0.40	-24	5.3	**0.40**	0.56	11
0.90	-9.8	7.5	**0.90**	-3.3	6.4
4.5	-9.1	8.4	**4.5**	3.6	7.2
9.0	-3.5	6.6	**9.0**	-0.78	8.0

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

Table 10. Qualitative comparison of the performance of the Ms8 detection method applied to genomic DNA extracted from GM stack MON 88302 x Ms8 x Rf3 oilseed rape and to genomic DNA extracted from the single line event Ms8.

Trueness and repeatability of Ms8 quantification on MON 88302 x Ms8 x Rf3 oilseed rape			**Trueness and repeatability of Ms8 quantification on single event Ms8***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.10	9.40	24	**0.10**	7.4	22
0.40	-1.8	13	**0.40**	-3.5	18
0.90	-3.2	10	**0.90**	-1.0	14
1.80	-5.2	12	**1.80**	-1.0	17
3.60	1.1	7.9	**3.60**	-7.5	11

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

Table 11. Qualitative comparison of the performance of the Rf3 detection method applied to genomic DNA extracted from GM stack MON 88302 x Ms8 x Rf3 oilseed rape and to genomic DNA extracted from the single line event Rf3.

Trueness and repeatability of Rf3 quantification on MON 88302 x Ms8 x Rf3 oilseed rape			**Trueness and repeatability of Rf3 quantification on single event Rf3***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.10	9.9	12	**0.10**	6.9	13
0.40	-4.3	12	**0.40**	4.4	12
0.90	-2.3	7.7	**0.90**	4.5	14
1.80	-9.0	7.5	**1.80**	-2.5	12
3.60	-0.18	6.5	**3.60**	-5.2	13

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6. Conclusions

The performance of the three event-specific methods for the detection and quantification of oilseed rape events MON 88302, Ms8 and Rf3, when applied to genomic DNA extracted from GM stack MON 88302 x Ms8 x Rf3, 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 three 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 88302 x Ms8 x Rf3.

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 88302 x Ms8 x Rf3.

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. Regulation (EC) No 503/2003 of 3 April 2013 "on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006".

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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