

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of MS11 and RF3 event- specific PCR-based Methods applied to DNA extracted from GM Stack MS11 x RF3 oilseed rape

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Report on the Verification of the Performance of MS11 and RF3 event-specific PCR-based Methods applied to DNA extracted from GM Stack MS11 x RF3 oilseed rape

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European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Bayer Crop Science to request the authorisation of genetically modified stack (GM stack) MS11 x RF3 oilseed rape (tolerance to glufosinate-ammonium, male sterility/fertility restorer system), 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 MS11 x RF3 oilseed rape is BCS-BNØ12-7 x ACS-BNØØ3-6.

The GM stack MS11 x RF3 oilseed rape has been obtained by conventional crossing between the genetically modified oilseed rape events: MS11 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 MS11 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 MS11 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 MS11 x RF3 oilseed rape.

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: 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 (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 event and these events have been stacked by conventional crossing. These criteria are met for the GM stack MS11 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 ⁽³⁾ and to the validation results on the individual events.

2. Step 1 (dossier reception and acceptance)

Bayer Crop Science 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 oilseed rape MS11 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 RSD_r %) calculated by the applicant for the two methods applied to MS11 x RF3 oilseed rape genomic DNA. Means are the average of eighteen replicates obtained through three runs performed with ABI Prism[®] 7900HT 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 MS11 and RF3 methods applied to GM stack MS11 x RF3 oilseed rape.

MS11 *			
Sample GM %	Expected value (GMO %)		
	0.08	0.9	4.5
Mean	0.077	0.85	4.61
RSD _r (%)	17.47	16.69	11.13
Bias (%)	-4.21	-5.71	2.53
RF3 *			
Sample GM %	Expected value (GMO %)		
	0.08	0.9	4.5
Mean	0.074	0.843	4.62
RSD _r (%)	20.09	16.00	16.77
Bias (%)	-7.49	-6.37	2.73

* 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⁾.

Two requests for complementary information regarding the method and 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 genomic DNA extracted from GM stack MS11 x RF3 oilseed rape.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from leaves of GM stack MS11 x RF3 oilseed rape, hemizygous for the GM-loci, as positive control sample.
- genomic DNA extracted from leaves of a near-isogenic conventional (non-GM) oilseed rape, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack MS11 x RF3 oilseed rape with the non-GM oilseed rape genomic DNA, in a constant amount of total oilseed rape genomic DNA. The same GM 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 MS11 and RF3 methods when applying them to genomic DNA extracted from the GM stack MS11 x RF3 oilseed rape.

Table 2. Percentage (GM %) of MS11 and RF3 in MS11 x RF3 stack genomic DNA contained in the verification samples.

MS11 GM %* [(GM DNA / total oilseed rape DNA x 100)]	RF3 GM %** [(GM DNA / total oilseed rape DNA x 100)]
0.05	0.10
0.40	0.40
0.90	0.90
2.0	1.8
4.5	3.6

* percentage expressed in haploid genome copy numbers

** percentage expressed in mass fractions of GM DNA

The protocols described by the applicant were implemented in the EURL GMFF laboratory and were in accordance with the protocols already validated and published for the individual MS11 and RF3 GM events (available at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). The applicant verified the RF3 method in the stacked GM material MS11 x RF3 with a two standard curve approach in line with current ENGL method performance requirements ⁽³⁾. Details of deviations are described in §4.5.

4.2 DNA extraction

A method for DNA extraction from oilseed rape was previously evaluated 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.

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 plant DNA extraction protocol which is derived from the publicly available 'Dellaporta' method submitted by Bayer CropScience, and validated and published on January 29th, 2007 by the EURL GMFF can be applied to extraction of DNA from flour of ground seeds and grain (CRLVL07/04XP Corrected Version 1).

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/OSR_DNAExtr_sampl_correctedversion1_CRL_VL_07_04.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 oilseed rape reference system CruA, *cruciferin A* 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 MS11 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 the GM %.

4.4 PCR methods

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack MS11 x RF3 oilseed rape using the single detection methods previously validated for the respective single GM events MS11 and RF3.

For detection of GM oilseed rape events MS11, a DNA fragments of 124-bp is 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 (minor groove binder nonfluorescent quencher) as a quencher dye at its 3'-end.

For detection of GM oilseed rape events RF3, a DNA fragment of 139-bp is 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 (5-Carboxytetramethylrhodamine) as a quencher dye its 3'-end.

For quantification of GM oilseed rape events MS11 and RF3, a taxon-specific reference system amplifies a 101-bp fragment of *CruA cruciferin A* gene an oilseed rape endogenous gene (GenBank X14555), using two *CruA* gene-specific primers and a gene-specific probe labelled with JOE (4,5-dichloro-dimethoxy-fluorescein) or VIC and BHQ-1 (black hole quencher 1) or TAMRA.

For the relative quantification of GM oilseed rape events MS11 standard curves are generated both for the MS11 and for the *CruA, cruciferin A*, 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 MS11 DNA is estimated.

For relative quantification of GM oilseed rape event RF3 DNA in a test sample, the ΔCq values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCq values against the logarithm of the relative amount of RF3 event DNA). The ΔCq values of the unknown samples are measured and, by means of the regression formula, the relative amount of RF3 event 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

The RF3 method used by the applicant to generate the data submitted the EURL were based on the 'two standard curves' approach (one calibration curve for the GM system and one for the reference system). This approach produced results well within the ENGL acceptance criteria. It is however different from the ΔC_q approach adopted by the EURL GMFF during the validation of the single line. The EURL GMFF performed the verification of the RF3 method applied to MS11 x RF3 DNA using the validated method with no modifications (available at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>).

When the RF3 method was originally validated, the information available was that the target taxon-specific target *CruA* was present as single copy gene in the genome. Recently it became evident that the *CruA* target is present in two copies per haploid genome. Since the RF3 control samples used was homozygous, the dynamic range reported in the validation report is correct when considered in mass fractions of GM DNA. Therefore the EURL GMFF verified the RF3 method on GM stack MS11 x RF3 DNA in mass fractions of GM DNA (§ Table 2).

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 $[10^{(-1/\text{slope})} - 1] \times 100$, and of the coefficient of determination (R^2) reported for all PCR systems in the eight runs, for GM oilseed rape events MS11 and RF3. Slope values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MS11 method on GM stack MS11 x RF3 oilseed rape.

Run	MS11			CruA		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.43	95	1.00	-3.41	97	1.00
2	-3.49	93	1.00	-3.44	95	1.00
3	-3.49	93	1.00	-3.41	96	1.00
4	-3.59	90	1.00	-3.42	96	1.00
5	-3.50	93	1.00	-3.38	98	1.00
6	-3.53	92	1.00	-3.43	96	1.00
7	-3.51	93	1.00	-3.42	96	1.00
8	-3.51	93	1.00	-3.42	96	1.00
Mean	-3.51	93	1.00	-3.42	96	1.00

Table 4. Values of standard curve slope, PCR efficiency and R^2 coefficient for the RF3 method on GM stack MS11 x RF3 oilseed rape.

Run	RF3		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.63	89	1.00
2	-3.63	89	1.00
3	-3.19	106	1.00
4	-3.27	102	0.99
5	-3.57	91	1.00
6	-3.70	86	1.00
7	-3.40	97	1.00
8	-3.51	93	1.00
Mean	-3.48	94	1.00

The mean PCR efficiencies of the GM and taxon-specific reference systems were above 90 % (93 % for MS11 and 96 % for CruA systems, and 94 % for the ΔCq RF3 regression curve, respectively). The mean R^2 coefficient of the methods was 1.00 for all systems in all cases. The data presented in Tables 3 and 4 confirm the appropriate performance characteristics of the two methods when tested on GM stack MS11 x RF3 oilseed rape 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, RSD_r %) of the two methods applied to samples of DNA extracted from GM stack MS11 x RF3 oilseed rape see Tables 5 and 6.

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MS11 method applied to genomic DNA extracted from GM stack MS11 x RF3 oilseed rape.

MS11					
Unknown sample GM %	Expected value (GMO %)				
	0.05	0.40	0.90	2.0	4.5
Mean	0.05	0.42	0.90	2.0	4.7
SD	0.00	0.01	0.02	0.06	0.14
RSD_r (%)	8.1	2.7	1.7	2.8	3.1
Bias (%)	0.16	4.8	0.15	0.82	3.7

Table 6. 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 MS11 x RF3 oilseed rape.

RF3					
Unknown sample GM %	Expected value (GMO %)				
	0.10	0.40	0.90	1.8	3.6
Mean	0.09	0.36	0.84	1.7	3.5
SD	0.02	0.04	0.04	0.06	0.17
RSD _r (%)	17	10	5.0	3.7	4.8
Bias (%)	-12	-11	-6.1	-5.8	-1.7

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 5 and 6, the values range from 0.16 % to 4.8 % for MS11 and from -12 % to -1.7 % for RF3. Therefore, the two methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack MS11 x RF3 oilseed rape.

Tables 5 and 6 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 1.7 % and 8.1 % for MS11 and between 3.7 % and 17 % for RF3, the two methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack MS11 x RF3 oilseed rape.

5. Conclusions

The performance of the two event-specific methods for the detection and quantification of oilseed rape single line events MS11 and RF3, when applied to genomic DNA extracted from GM stack MS11 x RF3 oilseed rape, 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 oilseed rape events MS11 and RF3, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack MS11 x RF3 oilseed, supposed 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. Regulation (EU) No 503/2013 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 (ENGL), 'Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing', 2015.

http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf.

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