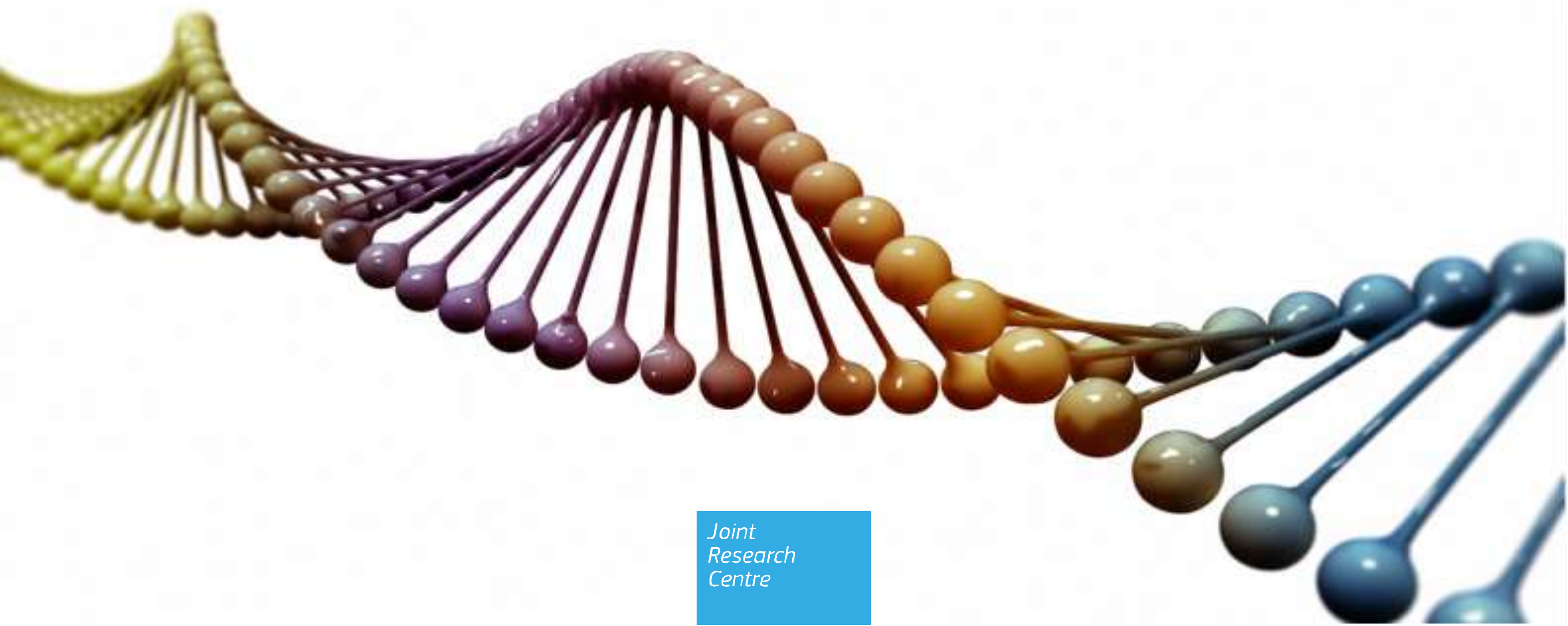
Ref. Ares(2024)5017402 - 11/07/2024

Report on the Verification of the Performance of MS11, RF3 and MON 88302 event-specific PCR-based Methods applied to DNA extracted from GM Stack MS11 x RF3 x MON 88302 oilseed rape

Validation Report

Zaoui, X., Maretti, M., Jacchia, S., Mazzara, M., Vincent, U.

2024



Joint
Research
Centre

This document is a publication by the Joint Research Centre (JRC), the European Commission's science and knowledge service. It aims to provide evidence-based scientific support to the European policymaking process. The contents of this publication do not necessarily reflect the position or opinion of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication. For information on the methodology and quality underlying the data used in this publication for which the source is neither Eurostat nor other Commission services, users should contact the referenced source. The designations employed and the presentation of material on the maps do not imply the expression of any opinion whatsoever on the part of the European Union concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

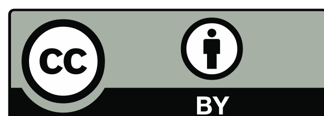
Contact information
European Commission
Directorate General Joint Research Centre
Directorate F – Health and Food
European Union Reference Laboratory for GM Food and Feed

EU Science Hub
https://joint-research-centre.ec.europa.eu

JRC138650

Ispra: European Commission, 2024

© European Union, 2024



The reuse policy of the European Commission documents is implemented by the Commission Decision 2011/833/EU of 12 December 2011 on the reuse of Commission documents (OJ L 330, 14.12.2011, p. 39). Unless otherwise noted, the reuse of this document is authorised under the Creative Commons Attribution 4.0 International (CC BY 4.0) licence (https://creativecommons.org/licenses/by/4.0/). This means that reuse is allowed provided appropriate credit is given and any changes are indicated.

For any use or reproduction of photos or other material that is not owned by the European Union, permission must be sought directly from the copyright holders. The European Union does not own the copyright in relation to the following elements:
- Cover page illustration, ©Jezper – stock.adobe.com

How to cite this report: European Commission, Joint Research Centre, Zaoui, X., Maretti, M., Jacchia, S., Mazzara, M. and Vincent, U., Report on the Verification of the Performance of MS11, RF3 and MON 88302 event-specific PCR-based Methods applied to DNA extracted from GM Stack MS11 x RF3 x MON 88302 oilseed rape, European Commission, Ispra, 2024, JRC138650.

Contents

Abstract 2
Quality assurance 3
1 Introduction 4
2 Dossier reception and acceptance (step 1) 5
3 Scientific assessment (step 2) 5
3.1 DNA extraction 5
3.2 PCR methods 6
3.2.1 Deviations from the validated methods introduced by the applicant 6
4 EURL GMFF experimental testing (step 3) 9
4.1 Materials 9
4.2 Experimental design 10
4.3 PCR methods 10
4.3.1 Deviations from the validated methods introduced by the EURL 10
4.3.2 Detection methods used by the EURL GMFF 11
4.4 Results 12
5 Conclusions 17
6 References 18
List of abbreviations and definitions 19
List of tables 20

Abstract

An application was submitted by BASF Agricultural Solutions Seed US LLC, represented in the EU by BASF SE, to request the authorisation of genetically modified stack (GM stack) MS11 x RF3 x MON 88302 oilseed rape 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 MS11 x RF3 x MON 88302 oilseed rape is BCS-BNØ12-7 x ACS-BNØØ3-6 x MON-883Ø2-9.

The GM stack MS11 x RF3 x MON 88302 oilseed rape has been obtained by conventional crossing between the genetically modified oilseed rape events: MS11, RF3 and MON 88302, 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, RF3 and MON 88302 (see https://gmo-crl.jrc.ec.europa.eu/method-validations). In line with the approach defined by the ENGL (https://gmo-crl.jrc.ec.europa.eu/doc/JRC125975_01.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 MS11 x RF3 x MON 88302 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 x MON 88302 oilseed rape.

This report is published at https://gmo-crl.jrc.ec.europa.eu/method-validations.

Quality assurance

The EURL GMFF is ISO/IEC 17025:2017 accredited [certificate number: BELAC 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) in food and feed by DNA extraction, DNA identification and Real-time PCR and for determination of Genetically Modified content in % (cp/cp) in food and feed by DNA extraction and digital PCR)].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

Report on the Verification of the Performance of MS11, RF3 and MON 88302 event-specific PCR-based Methods applied to DNA extracted from GM Stack MS11 x RF3 x MON 88302 oilseed rape

Validation Report

24 June 2024

European Union Reference Laboratory for GM Food and Feed

1 Introduction

The EU legislative framework (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 (see ENGL document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) 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 x MON 88302 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 (EC) 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 Dossier reception and acceptance (step 1)

BASF Agricultural Solutions Seed US LLC 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 x MON 88302 and from non-GM oilseed rape.

The dossier was found to be complete and was thus moved to step 2.

3 Scientific assessment (step 2)

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.

3.1 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, concerning the applicability of the quantitative real-time PCR methods developed for MS11, RF3 and MON 88302 the applicant stated that "the submitted method for DNA extraction is suitable for the isolation of genomic DNA from a wide variety of matrices (e.g. oilseeds seed, grain and other oilseeds tissues). The suitability of isolated DNA as an analyte for PCR-based detection of GMOs is dependent on the quality, purity, and quantity of the DNA. Although the DNA extraction method can be applied to different food and feed matrices, the application of the method to certain complex and difficult processed matrices may require adaptation. In fact, food processes can influence the quality and intactness of the DNA contained in the final processed products (4-7). Other challenges of working with processed food and feed matrices is the presence of PCR inhibitors, which can reduce the efficiency and/or reproducibility of PCR and thus may contribute to inaccurate PCR results (8, 9). Therefore, 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 (8, 9)."

On a general note, the EURL GMFF recommends that laboratories using this validated method for testing complex or difficult matrices verify that the extracted genomic DNA is of sufficient quality.

The protocol for the DNA extraction method is available at https://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.

3.2 PCR methods

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSD_r %) calculated by the applicant for the three methods applied to MS11 x RF3 x MON 88302 oilseed rape genomic DNA. Means are the average of eighteen replicates obtained through three runs performed with ABI 7900 HT real-time PCR equipment. Percentages are expressed as GM-oilseed rape copy numbers relative to haploid oilseed rape genome copy numbers.

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSD_r %) provided by the applicant (not rounded) for the MS11, RF3 and MON 88302 methods applied to GM stack MS11 x RF3 x MON 88302 oilseed rape.

MS11				
Sample GM %	Expected value (GMO %)			
	0.08	0.9	2.0	4.5
Mean	0.09	0.93	2.21	5.01
RSD_r (%)	16.3	5.9	9.4	6.8
Bias (%)	13.6	3.3	10.7	11.4
RF3				
Sample GM %	Expected value (GMO %)			
	0.08	0.9	2.0	4.5
Mean	0.07	0.93	2.05	4.77
RSD_r (%)	21.9	4.9	12.9	9.2
Bias (%)	-8.4	3.9	2.4	6.0
MON 88302				
Sample GM %	Expected value (GMO %)			
	0.08	0.9	2.0	4.5
Mean	0.07	0.81	1.84	4.22
RSD_r (%)	21.5	10.1	7.3	7.4
Bias (%)	-12.2	-9.8	-7.8	-6.2

3.2.1 Deviations from the validated methods introduced by the applicant

The applicant applied the following modifications to the validated methods:

- For the relative quantification of events MS11, RF3 and MON 88302 in the stacked GMO MS11 x RF3 x MON 88302 oilseed rape, the reference method acyl-ACP-thioesterase *FatA(A)* was used, modified from the one validated in the context of DP-073496-4 event (EURL-VL-02/12, https://gmo-

crl.jrc.ec.europa.eu/StatusOfDossiers.aspx), instead of *Ccf* validated in the single line MON 88302, and *CruA* validated in the single line for both MS11 and RF3.

- In relation to the validated *FatA(A)* method, the final concentrations of the forward primer, reverse primer and the probe were set at 200 nM each (originally 300 nM, 900 nM and 150 nM, respectively). In addition, the *TaqMan® Universal PCR Master Mix (2x) No UNG* was replaced by *Universal PCR Master Mix*, to include an initial decontamination of carry-over PCR products (UNG) step in the cycling program (at 50°C for 2 minutes). Finally, the reaction volume was modified from 20 µL to 25 µL;
- The dynamic range of all the methods was tested from 4.5 % to 0.08 % (related to mass fraction of GM material) instead of: 4.5 % - 0.05 % for the MS11 method; 3.6 % - 0.10 % for the RF3 method; 9 % - 0.05 % for the MON 88302 method (related to copy number ratio calculated in terms of haploid genomes for all three events);
- For RF3, a two-standard-curves method was used instead of the validated ΔCt method; the quencher of the RF3 probe was changed from TAMRA to BHQ1, and 40 cycles were run instead of 45;
- For the MON 88302 method, the volume of reaction was scaled down to 25 uL instead of 50 uL, and the concentration of both primers was changed to 400 nM, instead of 450 nM as in the validated method.

The EURL GMFF assessed the data and concluded that they were reliable and seemed to confirm that the methods meet the ENGL performance criteria (3).

However, a bridging study was requested to the applicant to demonstrate that, compared with the originally-validated protocols, the methods performed in compliance with the MPR-Part1[1] with regard to trueness and precision of the test samples. In this context:

- For the RF3 and MS11 detection methods, the applicant compared the modified methods to the validated MS11 x RF3 stack methods (EURL-VL-03/17, https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VR-VL0317-MS11xRf3.pdf), which used the *CruA* as reference. The applicant changed the reporter/quencher dye of the *CruA* probe from VIC/TAMRA to JOE/BHQ1. Therefore, the applicant tested the following combinations:
    - The modified RF3 and MS11 methods in relation to the *FatA(A)* as modified in this submission (EURL-VL-02/20, detailed above),
    - The modified RF3 and MS11 methods in relation to the *FatA(A)* reference validated with a ΔCt approach (EURL-VL-02/12, https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-02-12VR-EFSA-Corr1.pdf),
    - The RF3 and MS11 methods in relation to the *CruA* reference gene as used in EURL-VL-03/17 (link above).

- For the MON 88302 detection method, the applicant compared the modified method to the validated one (EURL-VL-09/11, https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-09-11-VM-MON88302.pdf), which used on the *Ccf* reference and the ΔCt approach. Therefore, the applicant tested the following combinations:
    - MON 88302 validated method (EURL-VL-09/11, ΔCt) with *FatA(A* EURL-VL-02/20, *FatA(A)* EURL-VL-02/12 and *Ccf* EURL-VL-09/11.

[1] European Network of GMO Laboratories (ENGL), 'Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing', 2015.

- MON 88302 modified method (EURL-VL-02/20) with *FatA(A)* EURL-VL-02/20, *FatA(A)* EURL-VL-02/12 and *Ccf* EURL-VL-09/11.

Test samples contained a total of 200 ng per reaction. Results, in terms of bias and repeatability, averaged over eighteen replicates, are shown in Tables 2-5.

Table 2. Values of bias and precision (RSDr) provided by the applicant for the MS11 method applied to GM stack MS11 x RF3 x MON 88302 oilseed rape.

	MS11/*FatA(A)* [1]				MS11/*FatA(A)* [2]				MS11/*CruA* [3]			
Sample GM %	Expected value (GMO %)				Expected value (GMO %)				Expected value (GMO %)			
	0.08	0.9	2.0	4.5	0.08	0.9	2.0	4.5	0.08	0.9	2.0	4.5
Mean	0.088	0.999	2.106	4.680	0.091	1.047	2.150	4.919	0.081	0.940	1.970	4.720
RSD_r (%)	14.57	8.26	7.68	7.64	16.78	7.58	11.28	8.59	10.67	5.24	9.38	6.93
Bias (%)	10.59	11.00	5.3	4.00	14.15	16.31	7.52	9.31	1.07	4.39	-1.48	4.88

[1] EURL-VL-02/20; [2] EURL-VL-02/12 (ΔCt); [3] EURL-VL-03/17

Table 3 Values of bias and precision (RSDr) provided by the applicant for the RF3 method applied to GM stack MS11 x RF3 x MON 88302 oilseed rape.

	RF3/*FatA(A)* [1]				RF3/*FatA(A)* [2]				RF3/*CruA* [3]			
Sample GM %	Expected value (GMO %)				Expected value (GMO %)				Expected value (GMO %)			
	0.08	0.9	2.0	4.5	0.08	0.9	2.0	4.5	0.08	0.9	2.0	4.5
Mean	0.069	0.836	2.066	4.406	0.070	0.875	2.100	4.627	0.064	0.786	1.942	4.444
RSD_r (%)	15.57	9.83	9.35	9.25	11.54	8.08	8.96	9.16	16.62	7.51	9.26	8.75
Bias (%)	-13.58	-7.14	3.29	-2.1	-12.11	-2.77	4.99	2.83	-20.55	-12.65	-2.94	-1.25

[1] EURL-VL-02/20; [2] EURL-VL-02/12 (ΔCt); [3] EURL-VL-03/17

Table 4. Values of bias and precision (RSDr) provided by the applicant for the validated MON 88302 method (EURL-VL-09/11, **ΔCt**) applied to GM stack MS11 x RF3 x MON 88302 oilseed rape.

	MON 88302/*FatA(A)* [1]				MON 88302/*FatA(A)* [2]				MON 88302/*Ccf* [3]			
Sample GM %	Expected value (GMO %)				Expected value (GMO %)				Expected value (GMO %)			
	0.08	0.9	2.0	4.5	0.08	0.9	2.0	4.5	0.08	0.9	2.0	4.5
Mean	0.080	0.886	2.135	4.667	0.083	0.929	2.218	4.909	0.077	0.865	2.058	4.725
RSD_r (%)	14.98	8.76	7.17	9.37	13.33	8.8	8.98	11.02	11.45	7.81	8.71	8.14
Bias (%)	0.43	-1.56	6.73	3.70	3.24	3.19	10.90	9.09	-4.20	-3.87	2.92	5.00

[1] EURL-VL-02/20; [2] EURL-VL-02/12 (ΔCt); [3] EURL-VL-09/11 (ΔCt)

Table 5. Values of bias and precision (RSDr) provided by the applicant for the submitted MON 88302 method (EURL-VL-02/20) applied to GM stack MS11 x RF3 x MON 88302 oilseed rape.

	MON 88302/*FatA(A)* [1]				MON 88302/*FatA(A)* [2]				MON 88302/*Ccf*			
Sample GM %	Expected value (GMO %)				Expected value (GMO %)				Expected value (GMO %)			
	0.08	0.9	2.0	4.5	0.08	0.9	2.0	4.5	0.08	0.9	2.0	4.5
Mean	0.079	0.872	2.188	4.452	0.082	0.917	2.223	4.677	0.075	0.853	2.134	4.500
RSD_r (%)	15.44	7.22	14.88	9.50	17.25	11.13	11.31	9.70	17.30	8.26	14.82	5.83
Bias (%)	-1.12	-3.12	9.40	-1.08	4.42	1.89	11.13	3.93	-5.89	-5.25	6.69	0.00

[1] EURL-VL-02/20; [2] EURL-VL-02/12 (ΔCt); [3] EURL-VL-09/11 (ΔCt)

Results showed that the deviations from validated methods introduced by the applicant did not negatively affect the performances of the modified methods for the detection of GM stack MS11 x RF3 x MON 88302 (in terms of trueness and precision), which were within the acceptance criteria established by the ENGL.

The dossier was therefore moved to step 3.

4 EURL GMFF experimental testing (step 3)

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 MS11 x RF3 x MON 88302 oilseed rape.

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.

4.1 Materials

The following control samples were provided by the applicant:

- Genomic DNA extracted from leaves of GM stack MS11 x RF3 x MON 88302 oilseed rape, hemizygous for all three events, as positive control sample.
- Genomic DNA extracted from leaves of conventional (non-GM) oilseed rape whose genetic background is near isogenic, 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 x MON 88302 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 6 shows the five GM concentrations used in the verification of the MS11, RF3 and MON 88302 methods when applying them to genomic DNA extracted from the GM stack MS11 x RF3 x MON 88302 oilseed rape.

Table 6. Percentage (GM %) of MS11, RF3 and MON 88302 in MS11 x RF3 x MON 88302 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)	MON 88302 GM %* (GM DNA / total oilseed rape DNA x 100)
0.05	0.10	0.05
0.40	0.40	0.40
0.90	0.90	0.90
2.0	1.8	4.5
4.5	3.6	9.0

* percentage expressed in mass ratio.

The protocols described by the applicant were implemented in the EURL GMFF laboratory and were in accordance with the protocols already published for the individual MS11, RF3 and MON 88302 GM events (available at https://gmo-crl.jrc.ec.europa.eu/method-validations). Nevertheless, deviations were introduced and described in §4.3.1.

4.2 Experimental design

Eight PCR runs were carried out for each method. In each run, samples were analysed in parallel with both the GM-specific and the reference method *FatA(A)* for MS11, RF3 events and MON 88302. 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, RF3 and MON 88302, 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 %.

4.3 PCR methods

4.3.1 Deviations from the validated methods introduced by the EURL

Originally, the RF3 method for quantification was validated as ratio of GM DNA copy numbers to target taxon-specific DNA copy number (CRL-VL-07/04), as the information available was that the taxon-specific target *CruA* was present as single copy gene in the genome. However, it later became evident that the *CruA* target was present in two copies per haploid genome. Therefore, in the current report all quantification data are reported as mass fractions of GM DNA in the oilseed rape stacked event MS11 x RF3 x MON 88302 in comparison to the mass fraction of the total oilseed rape DNA in the sample.

The GM% values of test samples tested at the EURL GMFF followed those of validated methods for each single event: 4.5 % to 0.05 % for MS11; 3.6 % to 0.10 % for RF3; 9 % to 0.05 % for MON 88302.

Similarly to the applicant, the EURL GMFF conducted a bridging study to verify that the relative quantification of the events MS11, RF3 and MON 88302 in combination with the taxon-specific method *FatA(A)* performed in accordance with the minimum acceptance criteria established in the MPR-Part 1 with regards to parameters of the calibration curve (amplification efficiency and R^2) and with regards to the trueness and precision of quantification of the test samples. In this context, the EURL GMFF tested all three methods with respect to *FatA(A)* and to the reference genes used in their respectively validated methods.

4.3.2 Detection methods used by the EURL GMFF

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack MS11 x RF3 x MON 88302 oilseed rape using the event-specific methods previously validated for the respective single GM events MS11, RF3 and MON 88302. Notably, when tested in relation to *FatA(A)*, the primers and probe final concentrations used for all GM events were the same as the ones used by the applicant: 200 nM for the primers and probe of *FatA(A)*; 400 nM for the primers and 200 nM for the probe of events MS11, RF3 and MON 88302.

For the detection of GM oilseed rape events MS11, RF3 and MON 88302, DNA fragments of 124-bp, 139-bp and 101-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 for all three events, MGB (minor groove binder) as a quencher dye at their 3′-end for the MS11 event and TAMRA (carboxytetramethylrhodamine) for RF3 and MON 88302 events.

For the relative quantification of GM oilseed rape events MS11, RF3 and MON 88302, a taxon-specific method amplifies a 126-bp fragment of acyl-ACP-thioesterase (*FatA(A)*), an oilseed rape endogenous gene (GenBank: X87842.1) using two *FatA(A)* gene-specific primers and a gene-specific probe labelled with FAM as reporter dye at its 5' end and MGB (minor groove binder) as a quencher dye at their 3′-end.

For the relative quantification of GM oilseed rape events MS11 and RF3, another taxon-specific method is also used, amplifying a 101-bp fragment of *Cruciferin A* (*CruA*), an oilseed rape endogenous gene (GenBank: X14555), using two *Cruciferin A* gene-specific primers and a gene-specific probe labelled with JOE as reporter dye at its 5' end and BHQ-1 (Black Hole Quencher® 1) for the MS11 event and with VIC® and TAMRA for the RF3 event. For the quantification of event MON 88302, another taxon-specific method is also used, amplifying a 78-bp fragment of *cruciferin* (*Ccf*), an oilseed rape endogenous gene (GenBank: X59294), using two *cruciferin* gene-specific primers and a gene-specific probe labelled with VIC and TAMRA.

For the relative quantification of GM oilseed rape events MS11 and MON 88302, standard curves are generated both for MS11 and MON 88302, as well as for the *FatA(A), CruA* and *Ccf*, respectively, 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, RF3 and MON 88302 DNA is estimated.

Following the RF3 validated method, for relative quantification of GM oilseed rape event RF3 DNA in a test sample associated to the *CruA*, 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 https://gmo-crl.jrc.ec.europa.eu/method-validations.

4.4 Results

Tables 7-12 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 in the eight runs for GM events MS11, RF3 and MON 88302.

Table 7. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MS11 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *FatA(A)*.

Run	MS11			*FatA(A)*		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.5	93	1.00	-3.4	96	1.00
2	-3.4	97	1.00	-3.5	94	1.00
3	-3.6	91	1.00	-3.4	95	1.00
4	-3.5	92	1.00	-3.5	95	1.00
5	-3.6	91	1.00	-3.4	95	1.00
6	-3.5	91	1.00	-3.4	95	1.00
7	-3.4	96	1.00	-3.4	96	1.00
8	-3.5	95	1.00	-3.4	97	1.00
Mean	-3.5	93	1.00	-3.4	95	1.00

Table 8. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MS11 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *CruA*.

Run	MS11			*CruA*		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.4	98	1.00	-3.3	99	1.00
2	-3.6	90	1.00	-3.3	99	1.00
3	-3.6	90	0.98	-3.3	100	1.00
4	-3.5	94	1.00	-3.3	100	1.00
5	-3.5	95	0.99	-3.3	100	1.00

6	-3.4	98	0.99	-3.3	100	1.00
7	-3.6	90	1.00	-3.3	100	1.00
8	-3.6	90	0.99	-3.3	99	1.00
Mean	-3.5	93	0.99	-3.3	100	1.00

Table 9. Values of standard curve slope, PCR efficiency and R^2 coefficient for the RF3 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *FatA(A)*.

Run	RF3			*FatA(A)*		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.4	99	1.00	-3.4	96	1.00
2	-3.4	96	1.00	-3.5	94	1.00
3	-3.3	99	1.00	-3.5	95	1.00
4	-3.4	96	1.00	-3.5	94	1.00
5	-3.3	100	1.00	-3.5	94	1.00
6	-3.4	95	1.00	-3.5	95	1.00
7	-3.4	95	1.00	-3.5	94	1.00
8	-3.4	98	1.00	-3.5	95	1.00
Mean	-3.4	97	1.00	-3.5	95	1.00

Table 10. Values of standard curve slope, PCR efficiency and R^2 coefficient for the RF3 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *CruA* and the ΔCt method.

Run	RF3		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.5	95	1.00
2	-3.5	93	1.00
3	-3.6	90	1.00
4	-3.4	97	1.00
5	-3.6	90	1.00

6	-3.5	95	1.00
7	-3.4	95	1.00
8	-3.5	92	1.00
Mean	-3.5	93	1.00

Table 11. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 88302 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *FatA(A)*.

Run	MON 88302			*FatA(A)*		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.4	98	1.00	-3.5	94	1.00
2	-3.5	93	1.00	-3.5	93	1.00
3	-3.3	99	1.00	-3.5	94	1.00
4	-3.3	99	1.00	-3.5	94	1.00
5	-3.5	95	1.00	-3.5	93	1.00
6	-3.4	98	1.00	-3.5	93	1.00
7	-3.5	92	1.00	-3.5	94	1.00
8	-3.5	94	1.00	-3.5	94	1.00
Mean	-3.4	96	1.00	-3.5	94	1.00

Table 12. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 88302 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *Ccf*.

Run	MON 88302			*Ccf*		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.4	96	1.00	-3.4	95	1.00
2	-3.4	97	1.00	-3.4	95	1.00
3	-3.5	95	1.00	-3.4	96	1.00
4	-3.4	96	1.00	-3.4	95	1.00
5	-3.5	94	1.00	-3.4	96	1.00

6	-3.4	98	1.00	-3.5	95	1.00
7	-3.5	94	1.00	-3.4	95	1.00
8	-3.3	100	1.00	-3.4	95	1.00
Mean	-3.4	96	1.00	-3.4	95	1.00

The mean PCR efficiencies of the GM and taxon-specific methods were above 90 % (93 % for MS11, 97 % for RF3-*FatA(A)*, 93 % for RF3-*CruA* and 96 % for MON 88302, respectively). The mean R^2 coefficient of the methods was 1.00 for all methods, except for MS11 (*CruA*) with 0.99. The data presented in Tables 7-12 confirm the appropriate performance characteristics of all methods when tested on GM stack MS11 x RF3 x MON 88302 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 all methods applied to samples of DNA extracted from GM stack MS11 x RF3 x MON 88302 oilseed rape (see Tables 13-18).

Table 13. 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 x MON 88302 oilseed rape, using *FatA(A)*.

MS11 (*FatA(A)*)					
Unknown sample GM %	Expected value (GMO %)				
	0.05	0.40	0.90	2.0	4.5
Mean	0.05	0.39	0.85	1.93	4.15
SD	0.00	0.02	0.03	0.05	0.10
RSD_r (%)	9.9	6.1	3.3	2.3	2.5
Bias (%)	-4.7	-2.3	-5.0	-3.4	-7.9

Table 14. 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 x MON 88302 oilseed rape, using *CruA*.

MS11 (*CruA*)					
Unknown sample GM %	Expected value (GMO %)				
	0.05	0.40	0.90	2.0	4.5
Mean	0.05	0.39	0.83	2.0	4.2
SD	0.01	0.02	0.02	0.03	0.20
RSD_r (%)	11	4.3	2.1	1.4	4.7
Bias (%)	-5.0	-3.0	-7.9	-2.1	-7.1

Table 15. 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 x MON 88302 oilseed rape, using *FatA(A)*.

RF3 (*FatA(A)*)					
Unknown sample GM %	Expected value (GMO %)				
	0.10	0.40	0.90	1.8	3.6
Mean	0.09	0.36	0.83	1.7	3.5
SD	0.01	0.02	0.03	0.04	0.08
RSD_r (%)	11	5.3	3.8	2.4	2.4
Bias (%)	-14	-10	-7.6	-3.7	-3.6

Table 16. 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 x MON 88302 oilseed rape, using *CruA*.

RF3 (*CruA*)					
Unknown sample GM %	Expected value (GMO %)				
	0.10	0.40	0.90	1.8	3.6
Mean	0.10	0.42	0.91	1.8	3.7
SD	0.01	0.02	0.03	0.08	0.10
RSD_r (%)	12	5.6	3.2	4.1	2.6
Bias (%)	0.89	3.8	1.2	2.2	2.1

Table 17. 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 MS11 x RF3 x MON 88302 oilseed rape, using *FatA(A)*.

MON 88302 (*FatA(A)*)					
Unknown sample GM %	Expected value (GMO %)				
	0.05	0.40	0.90	4.5	9.0
Mean	0.05	0.37	0.86	4.4	8.7
SD	0.01	0.02	0.03	0.04	0.12
RSD_r (%)	12	4.8	3.4	0.93	1.3
Bias (%)	-8.3	-7.5	-4.7	-2.1	-3.0

Table 18. 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 MS11 x RF3 x MON 88302 oilseed rape, using *Ccf*.

MON 88302 (*Ccf*)					
Unknown sample GM %	Expected value (GMO %)				
	0.05	0.40	0.90	4.5	9.0
Mean	0.04	0.36	0.88	4.4	9.1
SD	0.00	0.02	0.02	0.08	0.12
RSD_r (%)	11	4.4	2.4	1.8	1.4
Bias (%)	-13	-11	-2.1	-2.4	0.57

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 13-18, the values range from -2.3 % to -7.9 % for MS11 (*FatA(A)*), from -2.1 % to -7.9 % for MS11 (*CruA*), from -3.6 % to -14 % for RF3 (*FatA(A)*), from 0.89 % to 3.8 % for RF3 (*CruA*), from -2.1 % to -8.3 % for MON 88302 (*FatA(A)*) and from 0.57 % to -13 % for MON 88302 (*Ccf*). Therefore, all methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack MS11 x RF3 x MON 88302 oilseed rape.

Tables 13-18 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 2.3 % and 9.9 % for MS11 (*FatA(A)*), between 1.4 % and 11 % for MS11 (*CruA*), between 2.4 % and 11 % for RF3 (*FatA(A)*), between 2.6 % and 12 % for RF3 (*CruA*), between 0.93 % and 12 % for MON 88302 (*FatA(A)*) and between 1.4 % and 11 % for MON 88302 (*Ccf*), all methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack MS11 x RF3 x MON 88302 oilseed rape.

Overall, results showed that the modifications did not influence the performance of the methods in respect to the MON 88032 original method (EURL-VL-09/11VR), nor to the MS11 and RF3 as submitted and approved in the application for the stack MS11 x RF3 (EURL-VL-03/17VR).

5 Conclusions

The performance of the three event-specific methods for the detection and quantification of oilseed rape single line events MS11, RF3 and MON 88302, when applied to genomic DNA extracted from GM stack MS11 x RF3 x MON 88302 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, RF3 and MON 88302, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack MS11 x RF3 x MON 88302 oilseed rape or any of its sub-combinations, 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.

2. Commission Implementing 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. Gatto F., Savini C., Baillie C.K., Broothaerts W., Burns M., Dagand E., Debode F., Dobnik D. Grantina-Ievina L., Grohmann L., Lieske K., Marchesi U., Marien A., Mazzara M., Papazova N., Van der Berg J.P., Verginelli D., Zdenkova K. Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing part 2, Publications Office of the European Union, Luxembourg 2023, doi:10.2760/63656, JRC 125975. https://gmo-crl.jrc.ec.europa.eu/guidance-documents

4. Bauer T. *et al.*, 2003. The effect of processing parameters on DNA degradation in food. Eur. Food Res. Technol., 217: 338-343.

5. Chen Y. *et al.*, 2005. Degradation of endogenous and exogenous genes of Roundup Ready soybean during food processing. J. Agric. Food Chem., 53: 10239-10243.

6. Lipp M., Shillito R., Giroux R., Spiegelhalter F., Charlton S., Pinero D., Song P., 2005. Polymerase chain reaction technology as analytical tool in agricultural biotechnology. J AOAC Int 88 (1):136–155

7. Murray S. R. *et al.*, 2007. Use of quantitative real-time PCR to estimate maize endogenous DNA degradation after cooking and extrusion or in food products. J. Agric. Food Chem., 55: 2231-2239.

8. Demeke T., Jenkins G. R., 2010. Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. Anal. and Bioanal. Chem., 396: 1977-1990.

9. Peano C. *et al.*, 2004. Qualitative and quantitative evaluation of the genomic DNA extracted from GMO and non-GMO foodstuffs with four different extraction methods. J. Agric. Food Chem., 52: 6962, 6968.

List of abbreviations and definitions

EURL GMFF	European Union Reference Laboratory for GM Food and Feed
PCR	Polymerase chain reaction
ENGL	European Network of GMO Laboratories

List of tables

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSD_r %) provided by the applicant (not rounded) for the MS11, RF3 and MON 88302 methods applied to GM stack MS11 x RF3 x MON 88302 oilseed rape. 6

Table 2. Values of bias and precision (RSDr) provided by the applicant for the MS11 method applied to GM stack MS11 x RF3 x MON 88302 oilseed rape. 8

Table 3 Values of bias and precision (RSDr) provided by the applicant for the RF3 method applied to GM stack MS11 x RF3 x MON 88302 oilseed rape. 8

Table 4. Values of bias and precision (RSDr) provided by the applicant for the validated MON 88302 method (EURL-VL-09/11, ΔCt) applied to GM stack MS11 x RF3 x MON 88302 oilseed rape. 8

Table 5. Values of bias and precision (RSDr) provided by the applicant for the submitted MON 88302 method (EURL-VL-02/20) applied to GM stack MS11 x RF3 x MON 88302 oilseed rape. 8

Table 6. Percentage (GM %) of MS11, RF3 and MON 88302 in MS11 x RF3 x MON 88302 stack genomic DNA contained in the verification samples. 10

Table 7. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MS11 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *FatA(A)*. 12

Table 8. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MS11 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *CruA*. 12

Table 9. Values of standard curve slope, PCR efficiency and R^2 coefficient for the RF3 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *FatA(A)*. 13

Table 10. Values of standard curve slope, PCR efficiency and R^2 coefficient for the RF3 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *CruA* and the ΔCt method. 13

Table 11. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 88302 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *FatA(A)*. 14

Table 12. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 88302 method on GM stack MS11 x RF3 x MON 88302 oilseed rape, using *Ccf*. 14

Table 13. 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 x MON 88302 oilseed rape, using *FatA(A)*. 15

Table 14. 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 x MON 88302 oilseed rape, using *CruA*. 15

Table 15. 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 x MON 88302 oilseed rape, using *FatA(A)*. 16

Table 16. 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 x MON 88302 oilseed rape, using *CruA*. 16

Table 17. 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 MS11 x RF3 x MON 88302 oilseed rape, using *FatA(A)*. 16

Table 18. 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 MS11 x RF3 x MON 88302 oilseed rape, using *Ccf*. 17

Getting in touch with the EU

In person

All over the European Union there are hundreds of Europe Direct centres. You can find the address of the centre nearest you online (european-union.europa.eu/contact-eu/meet-us_en).

On the phone or in writing

Europe Direct is a service that answers your questions about the European Union. You can contact this service:

— by freephone: 00 800 6 7 8 9 10 11 (certain operators may charge for these calls),

— at the following standard number: +32 22999696,

— via the following form: european-union.europa.eu/contact-eu/write-us_en.

Finding information about the EU

Online

Information about the European Union in all the official languages of the EU is available on the Europa website (european-union.europa.eu).

EU publications

You can view or order EU publications at op.europa.eu/en/publications. Multiple copies of free publications can be obtained by contacting Europe Direct or your local documentation centre (european-union.europa.eu/contact-eu/meet-us_en).

EU law and related documents

For access to legal information from the EU, including all EU law since 1951 in all the official language versions, go to EUR-Lex (eur-lex.europa.eu).

EU open data

The portal data.europa.eu provides access to open datasets from the EU institutions, bodies and agencies. These can be downloaded and reused for free, for both commercial and non-commercial purposes. The portal also provides access to a wealth of datasets from European countries.

Science for policy

The Joint Research Centre (JRC) provides independent, evidence-based knowledge and science, supporting EU policies to positively impact society

EU Science Hub
joint-research-centre.ec.europa.eu