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Report on the Verification of the Performance of 305423 and 40-3-2 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack 305423 x 40-3-2 Soybean

European Union Reference Laboratory for Genetically Modified Food and Feed

2016

Joint Research Centre

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This publication is a Validated Methods, Reference Methods and Measurements Report
by the Joint Research Centre of the European Commission.

JRC100172

Printed in Italy

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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18 March 2016

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Pioneer Hi-Bred International Inc., as represented by Pioneer Overseas Corporation, to request the authorisation of genetically modified stack (GM stack) 305423 x 40-3-2 soybean of 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 305423 x 40-3-2 (unique identifier) soybean is DP-3Ø5423-1 x MON-Ø4Ø32-6.

The GM stack 305423 x 40-3-2 soybean has been obtained from traditional breeding methods between progeny of the two genetically modified soybean single events: 305423 and 40-3-2, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events 305423 and 40-3-2 (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 DNA extracted from GM stack 305423 x 40-3-2 soybean.

The results of the *in-house* verification led to the conclusion that the individual methods meet the ENGL performance criteria also when applied to DNA extracted from the GM stack 305423 x 40-3-2 soybean.

This report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

Content

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: 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 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 305423 x 40-3-2 soybean.

Upon reception of methods, samples and related data (step 1), the EURL GMFF carried out the assessment of the documentation (step 2) and the *in-house* verification of the methods (step 3) according to the requirements of Regulation (EC) No 641/2004 (Annex I).

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)

Pioneer submitted the detection methods, data demonstrating their adequate performance when applied to DNA extracted from the stack, and the corresponding control samples of DNA extracted from the GM stack soybean 305423 x 40-3-2 and from non GM soybean.

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

3. Step 2 (dossier scientific assessment)

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL[3] and with regard to their documentation and reliability.

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSDr %) calculated by the applicant for the two methods applied to 305423 x 40-3-2 soybean DNA. Means are the average of 15 quantification results generated from triplicates performed with ABI 7500 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. The calculations in the MS Excel files however were done over not rounded data. This approach might create small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) provided by the applicant for the 305423 and 40-3-2 methods applied to GM stack 305423 x 40-3-2 soybean.

305423**					
Sample* GM%	**Expected value (GMO %)**				
	0.08	**0.5**	**0.9**	**2.0**	**5**
Mean	0.07*	0.46	0.81	1.80	4.45
RSD_r (%)	14.70	8.30	3.89	4.32	4.56
Bias (%)	-6.88	-8.81	-9.89	-9.81	-11.08
40-3-2**					
Sample* GM%	**Expected value (GMO %)**				
	0.08	**0.5**	**0.9**	**-**	**5**
Mean	0.08	0.50	0.92	-	5.33
RSD_r (%)	10.22	9.41	5.24	-	8.02
Bias (%)	0.56	0.78	1.99	-	6.63

* Unknown samples are DNA samples containing different levels of GM DNA from stack material and non-GM DNA from conventional material.

** 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)].

Requests for complementary information regarding the DNA extraction protocol, sampling plan, control samples and DNA sequences were addressed to the applicant. The EURL GMFF verified the data and the complementary information received and accepted the 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 305423 x 40-3-2 soybean.

4.1 Materials

The following control samples were provided by the applicant:

- flour from homogenised seeds of GM stack 305423 x 40-3-2 soybean
- DNA extracted from homogenized seeds of non GM soybean.

The EURL GMFF prepared test samples of different GMO concentrations by mixing DNA extracted from GM stack 305423 x 40-3-2 soybean with the non GM soybean DNA, in a constant amount of total soybean DNA. The same concentrations as in the validation of the methods for the single lines were prepared. Table 2 shows the five GM concentrations used in the verification of the 305423 and 40-3-2 methods when applying them to DNA extracted from the GM stack 305423 x 40-3-2 soybean.

Table 2. Percentage (GM %) of event 305423 and event 40-3-2 in 305423 x 40-3-2 stack DNA contained in the verification samples.

305423 GM% [(GM DNA / total soybean DNA) x 100]	**40-3-2 GM%** [(GM DNA / total soybean DNA) x 100]
0.09	0.10
0.50	0.40
0.90	0.90
2.0	4.0
5.0	8.0

The protocols described by the applicant were implemented precisely in the EURL GMFF laboratory and were in accordance with the protocols already published for the individual 305423 and 40-3-2 GM events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

4.2 DNA extraction

A method for DNA extraction from soybean seeds was previously evaluated by the EURL GMFF with regard to its performance characteristics and was considered valid, i.e. fit for the purpose of providing soybean DNA of appropriate quality and amount to be 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 were carried out for each method. In each run, samples were analysed in parallel with both the GM-specific system and the reference system *lectin* gene *(Le1).* 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 (305423 and 40-3-2), 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 tests on DNA extracted from GM stack 305423 x 40-3-2 soybean using the single detection methods previously validated for the respective single GM events 305423 and 40-3-2.

For detection of GM soybean events 305423 and 40-3-2, DNA fragments of 93-bp and 84-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: a) FAM (6-carboxyfluorescein) as reporter dye at the 5'-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3'-end for event 305423, and b) FAM as reporter at the 5'-end and MGBNFQ (minor groove binding non-fluorescent quencher) as quencher at its 3'-end.

For quantification of GM soybean events 305423 and 40-3-2, a taxon-specific reference system amplifies a 74-bp fragment of *lectin* (*Le1),* a soybean endogenous gene (GenBank Accession No K00821), using two *lectin* gene-specific primers and a gene-specific probe labelled with FAM and TAMRA.

For quantification of GM soybean events 305423 and 40-3-2, standard curves are generated both for the 305423 and 40-3-2, and for the *Le1* 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 305423 and 40-3-2 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.

4.5 Deviations from the validated methods

No deviations from the original validated methods were introduced.

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 soybean events 305423 and 40-3-2.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 305423 method on GM stack 305423 × 40-3-2 soybean.

Run	305423			*Le1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.45	95	1.00	-3.57	91	1.00
2	-3.45	95	1.00	-3.51	93	1.00
3	-3.42	96	1.00	-3.59	90	1.00
4	-3.46	95	1.00	-3.58	90	1.00
5	-3.46	95	1.00	-3.60	90	1.00
6	-3.42	96	1.00	-3.57	91	1.00
7	-3.54	92	1.00	-3.61	89	1.00
8	-3.44	95	1.00	-3.61	89	1.00
Mean	**-3.46**	**95**	**1.00**	**-3.58**	**90**	**1.00**

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 40-3-2 method on GM stack 305423 × 40-3-2 soybean.

Run	40-3-2			*Le1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.55	91	0.99	-3.49	93	0.99
2	-3.45	95	0.98	-3.47	94	1.00
3	-3.40	97	0.99	-3.58	90	1.00
4	-3.50	93	1.00	-3.47	94	1.00
5	-3.38	98	1.00	-3.52	92	1.00
6	-3.47	94	0.99	-3.49	93	1.00
7	-3.43	96	0.99	-3.59	90	1.00
8	-3.49	93	1.00	-3.38	98	1.00
Mean	**-3.46**	**95**	**0.99**	**-3.50**	**93**	**1.00**

The mean PCR efficiencies of the GM and species-specific systems were above 90% (95% for the two GM events and, 90% and 93% for the *Le1* system). The linearity of the methods (R^2) was 1.00 for 305423 and *Le1* methods, and 0.99 for 40-3-2 method. The data presented in

Tables 3 and 4 confirm the appropriate performance characteristics of the two methods when tested on GM stack 305423 × 40-3-2 soybean 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 305423 × 40-3-2 soybean (see tables 5 and 6).

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 305423 method applied to DNA extracted from GM stack 305423 × 40-3-2 soybean.

305423					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.5**	**0.9**	**2.0**	**5.0**
Mean	0.09	0.52	0.93	2.02	4.90
SD	0.01	0.04	0.09	0.12	0.18
RSD_r (%)	12	7.5	9.9	6.1	3.7
Bias (%)	0.6	3.1	2.8	1.2	-1.9

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 40-3-2 method applied to DNA extracted from GM stack 305423 × 40-3-2 soybean.

40-3-2					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.4**	**0.9**	**4.0**	**8.0**
Mean	0.11	0.42	1.00	4.28	9.38
SD	0.01	0.05	0.11	0.49	0.95
RSD_r (%)	11	12	11	11	10
Bias (%)	13	6.1	11	7.1	17

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 -1.9% to 3.1% for 305423 and from 6.1% to 17% for 40-3-2. Therefore, the two methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack 305423 x 40-3-2 soybean.

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 EURL GMFF requires RSD_r values to be below 25%. As the values range between 3.7% and 12% for 305423 and between 10% and 12% for 40-3-2, the two methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack 305423 x 40-3-2 soybean.

5. Comparison of method performance on GM stack 305423 x 40-3-2 DNA and on DNA from the single-line GM events

An indicative comparison of the performance (bias, RSDr %) of the two methods applied to GM stack 305423 x 40-3-2 soybean and on the single-line events is shown in Tables 7 and 8. 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 7. Qualitative comparison of the performance of the 305423 detection method applied to DNA extracted from GM stack 305423 x 40-3-2 soybean and to DNA extracted from the single line event 305423.

Trueness and repeatability of 305423 quantification on 305423 x 40-3-2			**Trueness and repeatability of 305423 quantification on single event 305423***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.09	0.6	12	**0.09**	-6.3	17
0.5	3.1	7.5	**0.5**	-6.8	14
0.9	2.8	9.9	**0.9**	8.4	12
2.0	1.2	6.1	**2.0**	-3.1	12
5.0	-1.9	3.7	**5.0**	2.1	11

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

Table 8. Qualitative comparison of the performance of the 40-3-2 detection method applied to DNA extracted from GM stack 305423 x 40-3-2 soybean and to DNA extracted from the single line event 40-3-2.

Trueness and repeatability of 40-3-2 quantification on 305423 × 40-3-2			**Trueness and repeatability of 40-3-2 quantification on single event 40-3-2***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	13	11	**0.1**	-5.7	29
0.4	6.1	12	**0.4**	-11	26
0.9	11	11	**0.9**	-4.2	22
4.0	7.1	11	**4.0**	0.32	28
8.0	17	10	**8.0**	14	29

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

6. Conclusions

The performance of the two event-specific methods for the detection and quantification of soybean single line events 305423 and 40-3-2, when applied to DNA extracted from GM stack 305423 x 40-3-2 soybean, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant.

Therefore these methods, developed and validated to detect and quantify the single soybean events 305423 and 40-3-2, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack 305423 x 40-3-2 soybean.

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. 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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2016 – 16 pp. – 21.0 x 29.7 cm

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