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Report on the Verification of the Performance of GHB614, T304-40 and GHB119 event-specific PCR-based Methods applied to genomic DNA extracted from GM Stack GHB614 x T304-40 x GHB119 Cotton

European Union Reference Laboratory for Genetically Modified Food and Feed

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Report on the Verification of the Performance of GHB614, T304-40 and GHB119 event-specific PCR-based Methods applied to genomic DNA extracted from GM Stack GHB614 x T304-40 x GHB119 Cotton

European Union Reference Laboratory for GM Food and Feed

21 June 2016

Executive Summary

An application was submitted by Bayer CropScience LP, represented by Bayer CropScience N. V. (Belgium) to request the authorisation of genetically modified stack (GM stack) GHB614 x T304-40 x GHB119 cotton (tolerant to the herbicides glyphosate, glufosinate ammonium and to certain lepidopteran pests) 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 GHB614 x T304-40 x GHB119 cotton is BCS-GHØØ2-5 x BCS-GHØØ4-7 x BCS-GHØØ5-8.

The GM stack GHB614 x T304-40 x GHB119 cotton has been obtained by conventional crossing between three genetically modified cotton events: GHB614, T304-40 and GHB119, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events GHB614, T304-40 and GHB119 (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/MPR%20Report%20Application%2020_10_2015.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 GHB614 x T304-40 x GHB119 cotton.

The results of the *in-house* verification led to the conclusion that the individual methods meet the ENGL performance criteria also when applied to genomic DNA extracted from the GM stack GHB614 x T304-40 x GHB119 cotton.

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/MPR%20Report%20Application%2020_10_2015.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 GHB614 x T304-40 x GHB119 cotton.

Upon reception of methods, samples and related data (step 1), the EURL GMFF carried out the assessment of the documentation (step 2) and the *in-house* verification of the methods (step 3) according to the requirements of Regulation (EU) No 503/2013 (Annex III).

The results of the *in-house* verification study were evaluated with reference to the ENGL method performance requirements and to the validation results of the individual events.

2. Step 1 (dossier reception and acceptance)

Bayer CropScience LP submitted the detection methods, the data demonstrating their adequate performance, and the corresponding control samples consisting of genomic DNA extracted from GM stack cotton GHB614 x T304-40 x GHB119 and from non-GM cotton.
The dossier was found to be complete and thus was moved to step 2.

3. Step 2 (dossier scientific assessment)

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

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSDr %) calculated by the applicant for the three methods applied to genomic stack DNA. Means are

the average of eighteen replicates, obtained through three runs for each event, performed with ABI 7900HT fast 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, RSDr %) reported by the applicant for the GHB614, T304-40 and GHB119 methods applied to GM stack GHB614 x T304-40 x GHB119.

GHB614*			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.90**	**4.5**
Mean	0.068	0.85	4.64
RSD_r (%)	14.33	10.95	7.80
Bias (%)	16.35	9.08	9.26
T304-40			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.90**	**4.5**
Mean	0.071	0.74	3.76
RSD_r (%)	15.58	6.85	6.28
Bias (%)	11.83	18.04	16.41
GHB119			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.90**	**4.5**
Mean	0.069	0.75	4.24
RSD_r (%)	16.93	6.12	8.27
Bias (%)	14.22	16.69	6.39

* Numbers are not rounded but are presented as reported by the applicant

The applicant expressed the relative method repeatability as a RSD (relative standard deviation): every set of replicates (triplicates) yielded a variance estimate (standard deviation) and their mean was used to calculate the RSDr. The applicant expressed the bias% as the absolute value of the mean deviation from the reference value.
The EURL GMFF verified the data and concluded that they seemed to confirm that the methods meet the ENGL performance criteria[3].
One request of complementary information was addressed to Bayer CropScience LP concerning the size of the amplicon for the reference gene. 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 three methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 cotton.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from leaves of cotton plants harboring the GHB614 x T304-40 x GHB119 stack (homozygous)
- genomic DNA extracted from leaves of non-GM cotton plants (conventional Coker 312).

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 and genomic DNA extracted from non-GM cotton in a constant amount of total genomic cotton DNA. Table 2 shows the five GM concentrations used in the verification of the GHB614, T304-40 and GHB119 methods when applying them to genomic DNA extracted from the GM stack GHB614 x T304-40 x GHB119 cotton. These are the same concentrations used in the validation of these methods for the parental single GM lines.

Table 2. Percentage of GHB614, T304-40 and GHB119 in GHB614 x T304-40 x GHB119 verification samples.

GHB614 GM% (GM DNA/total DNA x 100)	**T304-40 GM%** (GM DNA/total DNA x 100)	**GHB119 GM%** (GM DNA/total DNA x 100)
4.5	4.5	4.5
2.0	2.0	2.0
0.90	0.90	0.90
0.40	0.40	0.40
0.09	0.10	0.10

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 GHB614, GHB119 and T304-40 GM events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

4.2 DNA extraction

A method for DNA extraction from cotton was previously validated by the EURL GMFF with regard to its performance characteristics and was considered valid, i.e. fit the purpose of providing cotton DNA of appropriate quality and amount for being used in subsequent PCR experiments. The protocol for the DNA extraction method is available at http://gmo-crl.jrc.ec.europa.eu/summaries/LLCotton25_DNAExtr_report.pdf.

Consequently, the EURL GMFF did not verify the DNA extraction method proposed by the applicant.

4.3 Experimental design

Eight PCR runs for each method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the reference system, *alcoholdehydrogenase C (AdhC).* 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 (GHB614, T304-40 and GHB119), 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 the determination of the GM%.

4.4 PCR methods

During the verification study, the EURL GMFF carried out parallel tests on genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 using the detection methods previously validated for the respective single GM events GHB614, T304-40 and GHB119.

For detection of GM cotton events GHB614, T304-40 and GHB119, DNA fragments of 120-bp, 78-bp and 90-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 events GHB614, T304-40 and GHB119, a cotton-specific reference system amplifies a 73-bp fragment of *AdhC* gene (*alcoholdehydrogenase C*) a cotton endogenous gene (Accession number, GeneBank: AF036569 and AF403330) using *AdhC* gene-specific primers and an *AdhC* gene specific probe labelled with VIC as reporter dye at its 5' end, and TAMRA as quencher at its 3' end.

For quantification of GM cotton events GHB614, T304-40 and GHB119, standard curves are generated 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 Cq values of the unknown samples are measured and, by means of the regression formula, the relative amount of GHB614, T304-40 and GHB119 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 Results

Tables 3, 4 and 5 present the values of the slopes of the different standard curves generated by the EURL GMFF when using genomic 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 cotton events GHB614, T304-40 and GHB119. Slope and R^2 coefficient values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the GHB614 method on GM stack GHB614 x T304-40 x GHB119 cotton.

Run	GHB614			*AdhC*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.29	101	1.00	-3.31	101	1.00
2	-3.43	96	1.00	-3.29	102	1.00
3	-3.11	110	1.00	-3.29	101	1.00
4	-3.33	100	1.00	-3.36	99	1.00
5	-3.36	98	0.99	-3.39	97	1.00
6	-3.37	98	1.00	-3.30	101	1.00
7	-3.27	102	1.00	-3.29	101	1.00
8	-3.31	100	1.00	-3.27	102	1.00
Mean	-3.31	101	1.00	-3.31	100	1.00

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the T304-40 method on GM stack GHB614 x T304-40 x GHB119 cotton.

Run	T304-40			*AdhC*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.39	97	1.00	-3.34	99	1.00
2	-3.37	98	1.00	-3.30	101	1.00
3	-3.33	100	1.00	-3.34	99	1.00
4	-3.28	102	1.00	-3.38	98	1.00
5	-3.37	98	1.00	-3.25	103	1.00
6	-3.31	100	1.00	-3.31	100	1.00
7	-3.37	98	1.00	-3.29	101	1.00
8	-3.45	95	0.99	-3.27	102	1.00
Mean	-3.36	98	1.00	-3.31	101	1.00

Table 5. Values of standard curve slope, PCR efficiency and linearity (R^2) for the GHB119 method on GM stack GHB614 x T304-40 x GHB119 cotton.

Run	GHB119			*AdhC*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.24	104	0.99	-3.27	102	1.00
2	-3.22	105	1.00	-3.28	102	0.99
3	-3.27	102	1.00	-3.31	101	1.00
4	-3.33	100	1.00	-3.33	100	1.00
5	-3.24	104	1.00	-3.30	101	1.00
6	-3.25	103	1.00	-3.32	100	0.99
7	-3.25	103	0.99	-3.33	100	1.00
8	-3.34	99	1.00	-3.29	101	1.00
Mean	-3.27	102	1.00	-3.30	101	1.00

The mean PCR efficiencies were 101% for GHB614 and 100% for *AdhC* (Table 3); for event T304-40 the mean PCR efficiency was 98% and 102% for GHB119, with the *AdhC* at 101% in both cases (Tables 4 and 5) . The mean 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 GHB614 x T304-40 x GHB119 cotton in terms of PCR efficiency and linearity.

The EURL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) of the three methods applied to samples of genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 cotton (see Tables 6, 7 and 8).

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the GHB614 method applied to genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 cotton.

GHB614					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.40**	**0.90**	**2.0**	**4.5**
Mean	0.08	0.35	0.78	1.8	4.4
SD	0.01	0.03	0.05	0.15	0.33
RSD_r (%)	12	9.7	6.6	8.2	7.5
Bias (%)	-14	-13	-14	-9.7	-3.2

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSDr %) of the T304-40 method applied to genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 cotton.

T304-40					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**2.0**	**4.5**
Mean	0.09	0.34	0.77	1.8	4.1
SD	0.01	0.03	0.06	0.13	0.19
RSD_r (%)	15	8.4	8.0	7.4	4.5
Bias (%)	-9.6	-14	-14	-11	-8.4

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the GHB119 method applied to genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 cotton.

GHB119					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**2.0**	**4.5**
Mean	0.08	0.33	0.80	1.8	4.4
SD	0.01	0.03	0.07	0.11	0.21
RSD_r (%)	12	10	8.7	6.2	4.8
Bias (%)	-21	-17	-11	-11	-1.8

The trueness of the method is estimated using the measurements of the method bias for each GM level. According to the ENGL, 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 -3.2% to -14% for GHB614, from -8.4% to -14% for T304-40 and from -1.8% to -21% for GHB119. Therefore, the three methods satisfy the ENGL requirements throughout their respective dynamic ranges, also when applied to genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 cotton.

Tables 6, 7 and 8 also show the relative repeatability standard deviation (RSD_r) as estimated for each GM level. According to the ENGL the RSD_r values should be below 25%. As the values range between 6.6% and 12% for GHB614, between 4.5% and 15% for T304-40 and between 4.8% and 12% for GHB119, the three methods satisfy this requirement throughout their respective dynamic ranges when applied to genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 cotton.

6. Conclusions

The performance of the three event-specific methods for the detection and quantification of cotton events GHB614, T304-40 and GHB119, when applied to genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119, 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 cotton events GHB614, T304-40 and GHB119, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack GHB614 x T304-40 x GHB119 or any of its sub-combinations, supposed that sufficient genomic DNA of appropriate quality is available.

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 503/2013 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: Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing. 20 April 2015. http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf

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