



JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of GHB614, T304-40, GHB119 and COT102 event-specific PCR-based Methods applied to DNA extracted from GM Stack GHB614 x T304-40 x GHB119 x COT102 cotton

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Report on the Verification of the Performance of GHB614, T304-40, GHB119 and COT102 event-specific PCR-based Methods applied to DNA extracted from GM Stack GHB614 x T304- 40 x GHB119 x COT102 cotton

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European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Bayer CropScience AG (now BASF Agricultural Solutions), to request the authorisation of genetically modified stack (GM stack) GHB614 x T304-40 x GHB119 x COT102 cotton (resistance to certain lepidopteran pests and tolerance to glufosinate ammonium and glyphosate herbicides), 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 GHB614 x T304-40 x GHB119 x COT102 cotton is BCS-GHØØ2-5 x BCS-GHØØ4-7 x BCS-GHØØ5-8 x SYN-IR1Ø2-7.

The GM stack GHB614 x T304-40 x GHB119 x COT102 cotton has been obtained by conventional crossing between the genetically modified cotton events: GHB614, T304-40, GHB119 and COT102, 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, GHB119 and COT102 (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 GHB614 x T304-40 x GHB119 x COT102 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 x COT102 cotton.

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

Content

EXECUTIVE SUMMARY	1
1. INTRODUCTION	4
2. STEP 1 (DOSSIER RECEPTION AND ACCEPTANCE)	4
3. STEP 2 (DOSSIER SCIENTIFIC ASSESSMENT)	5
4. STEP 3 (EURL GMFF EXPERIMENTAL TESTING)	6
4.1 MATERIALS	6
4.2 DNA EXTRACTION	7
4.3 EXPERIMENTAL DESIGN	7
4.4 PCR METHODS	8
4.4.1 <i>Deviations from the validated methods</i>	8
4.5 RESULTS	9
5. CONCLUSIONS	12
6. REFERENCES	13

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 GHB614 x T304-40 x GHB119 x COT102 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) 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 CropScience AG (now BASF Agricultural Solutions) 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 cotton GHB614 x T304-40 x GHB119 x COT102 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 RSD_r %) calculated by the applicant for the four methods applied to GHB614 x T304-40 x GHB119 x COT102 cotton genomic DNA. Means are the average of eighteen replicates obtained through three runs performed with 7900HT Fast Real-Time PCR system 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 GHB614, T304-40, GHB119 and COT102 methods applied to GM stack GHB614 x T304-40 x GHB119 x COT102 cotton.

GHB614 *				T304-40 *			
Sample GM %	Expected value (GMO %)			Sample GM %	Expected value (GMO %)		
	0.08	0.9	4.5		0.08	0.9	4.5
Mean	0.072	0.83	3.80	Mean	0.072	0.90	4.65
RSD _r (%)	15.38	9.82	11.98	RSD _r (%)	16.40	10.96	8.93
Bias (%)	9.92	7.70	15.44	Bias (%)	10.17	0.02	3.35
GHB119 *				COT102 *			
Sample GM %	Expected value (GMO %)			Sample GM %	Expected value (GMO %)		
	0.08	0.9	4.5		0.08	0.9	4.5
Mean	0.073	0.89	4.36	Mean	0.089	0.97	4.90
RSD _r (%)	14.82	5.90	8.12	RSD _r (%)	24.16	11.20	17.00
Bias (%)	9.31	0.95	3.17	Bias (%)	11.38	7.86	8.96

* 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 of complementary information regarding the purity and zygosity status of the inserts in control samples, and one performance parameter of method COT102, 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 four 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 x COT102 cotton.

4.1 Materials

The following control samples were provided by the applicant:

- Genomic DNA genomic extracted from leaves of GM stack GHB614 x T304-40 x GHB119 x COT102 cotton, homozygous for the loci, as positive control sample.
- genomic DNA from leaves plant of conventional (non-GM) cotton whose genetic background is FM966, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 x COT102 cotton with the non-GM cotton genomic DNA, in a constant amount of total cotton 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 GHB614, T304-40, GHB119 and COT102 methods when applying them to genomic DNA extracted from the GM stack GHB614 x T304-40 x GHB119 x COT102 cotton.

Table 2. Percentage (GM %) of GHB614, T304-40, GHB119 and COT102 in GHB614 x T304-40 x GHB119 x COT102 stack genomic DNA contained in the verification samples.

GHB614 GM %* [(GM DNA / total cotton DNA x 100)]	T304-40 GM %* [(GM DNA / total cotton DNA x 100)]
0.09	0.10
0.40	0.40
0.90	0.90
2.0	2.0
4.5	4.5
GHB119 GM %* [(GM DNA / total cotton DNA x 100)]	COT102 GM %* [(GM DNA / total cotton DNA x 100)]
0.10	0.10
0.40	0.50
0.90	0.90
2.0	2.0
4.5	5.0

* percentage expressed in copy number 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 GHB614, T304-40, GHB119 and COT102 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 evaluated 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.

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 protocol can be used for extraction of DNA from seeds and grains ground to powder using a Waring™ blender or any other appropriate seed crushing device"*.

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 the "CTAB/Genomic-tip 20" (CRLVL13/04XP) method available at http://gmo-crl.jrc.ec.europa.eu/summaries/GHB614_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 were carried out for each method. In each run, samples were analysed in parallel with both the GM-specific system and the reference system AdhC, alcohol dehydrogenase C gene, or the cotton putative Sinapis Arabidopsis Homolog 7 gene, SAH7. 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 GHB614, T304-40, GHB119 and COT102, 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 GHB614 x T304-40 x GHB119 x COT102 cotton using the single detection methods previously validated for the respective single GM events GHB614, T304-40, GHB119 and COT102.

For detection of GM cotton events GHB614, T304-40, GHB119 and COT102, DNA fragments of 120-bp, 78-bp, 90-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 and TAMRA (carboxytetramethylrhodamine) as a quencher dye at their 3'-end for all four events.

For quantification of GM cotton events GHB614, T304-40 and GHB119 a taxon-specific reference system amplifies a 73-bp fragment of AdhC, alcoholdehydrogenase C gene, a cotton endogenous gene (GenBank AF036569), using two AdhC gene-specific primers and a gene-specific probe labelled with VIC[®] and TAMRA. For quantification of the GM cotton event COT102 a taxon-specific reference system amplifies a 115-bp or 123-bp fragment (depending on the amplification of the A- or D-subgenome) of sah7 (GenBank AY117067, AY117068), putative Sinapis Arabidopsis Homolog 7 gene, a cotton endogenous gene, using two sah7 gene-specific primers and a gene-specific probe labelled with VIC[®] and TAMRA.

For the relative quantification of GM cotton events GHB614, T304-40, GHB119 and COT102 standard curves are generated both for the GHB614, T304-40, GHB119 and COT102 and for the AdhC and Sah7 specific systems by plotting C_q values of the calibration standards against the logarithm of the DNA amount and by fitting a linear regression into these data. Thereafter, the C_q values of the unknown samples are measured and, by means of the regression formula, the relative amount of GHB614, T304-40, GHB119 and COT102 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>.

4.4.1 Deviations from the validated methods

No deviations from the original validated methods were introduced.

4.5 Results

Tables 3 to 6 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 cotton events GHB614, T304-40, GHB119 and COT102. Slope and R^2 values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and R^2 coefficient for the GHB614 method on GM stack GHB614 x T304-40 x GHB119 x COT102 cotton.

Run	GHB614			AdhC		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.16	107	1.00	-3.29	101	1.00
2	-3.21	105	1.00	-3.18	106	1.00
3	-3.28	102	1.00	-3.25	103	1.00
4	-3.29	101	1.00	-3.26	103	1.00
5	-3.21	105	0.99	-3.17	107	1.00
6	-3.00	116	0.99	-3.11	109	0.99
7	-3.08	111	0.99	-3.17	107	1.00
8	-3.21	105	0.99	-3.21	105	1.00
Mean	-3.18	107	0.99	-3.21	105	1.00

Table 4. Values of standard curve slope, PCR efficiency and R^2 coefficient for the T304-40 method on GM stack GHB614 x T304-40 x GHB119 x COT102 cotton.

Run	T304-40			AdhC		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.31	101	1.00	-3.12	109	0.99
2	-3.29	101	1.00	-3.07	112	1.00
3	-3.26	103	0.99	-3.09	111	1.00
4	-3.41	96	1.00	-3.21	105	1.00
5	-3.21	105	1.00	-3.15	108	1.00
6	-3.32	100	0.97	-3.23	104	0.99
7	-3.27	102	1.00	-3.12	109	1.00
8	-3.29	101	1.00	-3.08	111	1.00
Mean	-3.29	101	0.99	-3.13	109	1.00

Table 5. Values of standard curve slope, PCR efficiency and R^2 coefficient for the GHB119 method on GM stack GHB614 x T304-40 x GHB119 x COT102 cotton.

Run	GHB119			AdhC		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.31	100	1.00	-3.17	107	0.99
2	-3.27	102	1.00	-3.19	106	1.00
3	-3.31	100	1.00	-3.18	106	1.00
4	-3.32	100	1.00	-3.23	104	1.00
5	-3.22	104	1.00	-3.12	109	1.00
6	-3.27	102	1.00	-3.14	108	0.99
7	-3.33	100	1.00	-3.12	109	1.00
8	-3.39	97	1.00	-3.25	103	0.99
Mean	-3.30	101	1.00	-3.17	107	1.00

Table 6. Values of standard curve slope, PCR efficiency and R^2 coefficient for the COT102 method on GM stack GHB614 x T304-40 x GHB119 x COT102 cotton.

Run	COT102			Sah7		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.34	99	1.00	-3.38	97	1.00
2	-3.22	105	1.00	-3.44	95	1.00
3	-3.36	99	1.00	-3.31	101	1.00
4	-3.35	99	0.99	-3.52	93	0.99
5	-3.21	105	1.00	-3.54	92	1.00
6	-3.33	100	0.99	-3.43	96	1.00
7	-3.19	106	1.00	-3.49	94	1.00
8	-3.39	97	1.00	-3.49	94	1.00
Mean	-3.30	101	1.00	-3.45	95	1.00

The mean PCR efficiencies of the GM and species-specific systems were above 90 % (107 and 105 % for GHB614 and AdhC, 101 and 109 % for T304-40 and AdhC, 101 and 107 % for GHB119 and AdhC, 101 and 95 % for COT102 and Sah7, respectively). The mean R^2 coefficient of the methods was 0.99 or 1.00 for all systems in all cases. The data presented in Tables 3-6 confirm the appropriate performance characteristics of the four methods when tested on GM stack GHB614 x T304-40 x GHB119 x COT102 cotton 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 four methods applied

to samples of DNA extracted from GM stack GHB614 x T304-40 x GHB119 x COT102 cotton see Tables 7-10.

Table 7. 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 x COT102 cotton.

GHB614					
Unknown sample GM %	Expected value (GMO %)				
	0.09	0.40	0.90	2.0	4.5
Mean	0.08	0.38	0.89	1.9	4.5
SD	0.01	0.07	0.07	0.13	0.47
RSD_r (%)	18	18	8.2	6.9	10
Bias (%)	-7.5	-6.1	-0.83	-4.2	0.84

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the T304-40 method applied to genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 x COT102 cotton.

T304-40					
Unknown sample GM %	Expected value (GMO %)				
	0.10	0.40	0.90	2.0	4.5
Mean	0.09	0.38	0.87	2.0	4.5
SD	0.02	0.05	0.09	0.22	0.52
RSD_r (%)	18	13	10	11	12
Bias (%)	-9.8	-4.7	-3.8	-1.2	-0.81

Table 9. 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 x COT102 cotton.

GHB119					
Unknown sample GM %	Expected value (GMO %)				
	0.10	0.40	0.90	2.0	4.5
Mean	0.09	0.36	0.81	1.8	4.4
SD	0.02	0.04	0.06	0.12	0.54
RSD_r (%)	18	12	6.8	6.6	12
Bias (%)	-8.7	-9.1	-9.5	-10	-2.4

Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the COT102 method applied to genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 x COT102 cotton.

COT102					
Unknown sample GM %	Expected value (GMO %)				
	0.10	0.50	0.90	2.0	5.0
Mean	0.11	0.43	0.83	2.1	5.5
SD	0.03	0.03	0.07	0.39	0.98
RSD_r (%)	24	6.9	8.7	18	18
Bias (%)	12	-14	-7.8	6.2	11

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 7-10, the values range from -7.5 % to 0.84 % for GHB614, from -9.8 % to -0.81 % for T304-40, from -10 % to -2.4 % for GHB119 and from -14 % to 12 % for COT102. Therefore, the four methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack GHB614 x T304-40 x GHB119 x COT102 cotton.

Tables 7-10 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 6.9 % and 18 % for GHB614, between 10 % and 18 % for T304-40, between 6.6 % and 18 % for GHB119 and between 6.9 % and 24 % for COT102, the four methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack GHB614 x T304-40 x GHB119 x COT102 cotton.

5. Conclusions

The performance of the four event-specific methods for the detection and quantification of cotton single line events GHB614, T304-40, GHB119 and COT102, when applied to genomic DNA extracted from GM stack GHB614 x T304-40 x GHB119 x COT102 cotton, 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, GHB119 and COT102, 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 x COT102 cotton, 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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