



JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

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

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

17 November 2020

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Bayer CropScience Division (now BASF Agricultural Solutions Seed US LLC) to request the authorisation of genetically modified stack (GM stack) T304-40 x GHB119 x COT102 cotton (tolerant to the herbicide glufosinate ammonium and resistant to certain lepidopteran pests), 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 T304-40 x GHB119 x COT102 cotton is BCS-GHØØ4-7 x BCS-GHØØ5-8 x SYN-IR1Ø2-7.

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

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

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Quality assurance

The EURL GMFF is ISO 17025:2017 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)] 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 legislation ^(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 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) No 503/2013 (Annex III).

The results of the *in-house* verification study were evaluated with reference to ENGL method performance requirements ⁽³⁾ and to the validation results on the individual events.

2. Step 1 (dossier reception and acceptance)

Bayer CropScience Division (now 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 cotton 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 three methods applied to T304-40 x GHB119 x COT102 cotton genomic DNA. Means are the average of eighteen replicates obtained through six runs performed with ABI 7900HT real-time PCR equipment. Percentages are expressed as GM DNA / total DNA x 100.

Note: Numerical values presented in the following tables were rounded keeping two digits for values ≤ 1, one digit for values between 1 and 10 and no digit for values ≥ 10, unless otherwise stated. The calculations in the MS Excel files however were done over not rounded data. This approach might create small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSD_r %) provided by the applicant for the T304-40, GHB119 and COT102 methods applied to GM stack T304-40 x GHB119 x COT102 cotton.

T304-40 *				
Sample GM %	Expected value (GMO %)			
	0.06	0.08	0.9	4.5
Mean	0.054	0.073	0.79	4.19
RSD _r (%)	15.1	19.5	10.9	7.1
Bias (%)	-10.1	-8.2	-12	-6.9
GHB119 *				
Sample GM %	Expected value (GMO %)			
	0.06	0.08	0.9	4.5
Mean	0.052	0.067	0.74	4.10
RSD _r (%)	23.8	15.8	7.5	8.2
Bias (%)	-12.9	-15.9	-18.2	-8.8
COT102 *				
Sample GM %	Expected value (GMO %)			
	0.08	0.9	2	4.5
Mean	0.067	0.76	1.7	4.6
RSD _r (%)	15.9	16.6	10.6	17.0
Bias (%)	-16.8	-15.1	-12.9	1.3

* 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 methods and the control samples were addressed to the applicant. The EURL GMFF verified the data and the complementary information received and accepted the received clarifications as satisfactory.

The dossier was therefore moved to step 3.

4. Step 3 (EURL GMFF experimental testing)

In step 3 the EURL GMFF implemented the three methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack T304-40 x GHB119 x COT102 cotton.

4.1 Materials

The following control samples were provided by the applicant:

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

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

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

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

* percentage expressed in copy number ratio.

The applicant introduced deviations to the protocols, which are described in §4.4.1. The EURL GMFF laboratory implemented the protocols already published for the individual 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 applicability of the quantitative real-time PCR methods developed for T304-40, GHB119 and COT102 *'will depend 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 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 some 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)'*.

The EURL GMFF recommends that laboratories using this validated method for testing complex or difficult matrices always verify that the extracted genomic DNA is of sufficient quality.

The protocol for the DNA extraction method is available at http://gmo-crl.jrc.ec.europa.eu/summaries/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 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 for T304-40 and GHB119 and *SAH7*, *Sinapis Arabidopsis Homolog 7* gene for COT102. 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 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 T304-40 x GHB119 x COT102 cotton using the single detection methods previously validated for the respective single GM events T304-40, GHB119 and COT102.

For detection of GM cotton events T304-40, GHB119 and COT102, DNA fragments of 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 (6-carboxytetramethylrhodamine) as a quencher dye at their 3'-end for all three events.

For quantification of GM cotton events T304-40 and GHB119, a taxon-specific reference system amplifies a 73-bp fragment of *alcohol dehydrogenase C (AdhC)*, a cotton endogenous gene (GenBank AF036569 and AF403330), using two *AdhC* gene-specific primers and a gene-specific probe labelled with VIC and TAMRA. For quantification of GM cotton event COT102, a taxon-specific reference system amplifies a fragment of *Sinapis Arabidopsis Homolog 7 (SAH7)*, a cotton endogenous gene (GenBank FN610856.1), using two *SAH7* gene-specific primers and a gene-specific probe labelled with VIC and TAMRA. The *SAH7* gene is present not only in the A-subgenome, but also in the D-subgenome of *Gossypium hirsutum*. The A-subgenome copy differs from the D-subgenome copy by several single or double nucleotide substitutions and small deletions/insertions. The primers and probe of the cotton-specific reference PCR system match perfectly without any single mismatch to both subgenome gene copies. However, due to sequence differences within the amplified region, the size of the amplicons resulting from the A- and D-subgenomes differs slightly, being respectively 115 bp and 123 bp of length.

For the relative quantification of GM cotton events T304-40, GHB119 and COT102 standard curves are generated both for the T304-40, GHB119 and COT102 and for the *AdhC* or the *SAH7* specific system by plotting Cq values of the calibration standards against the logarithm of the DNA amount and by fitting a linear regression into these data. Thereafter, the Cq values of the unknown samples are measured and, by means of the regression formula, the relative amount of 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

The following deviations from the original validated methods were introduced by the applicant:
- the labelling of the probes were modified with respect to the validated methods for: T304-40

(BHQ1 instead of TAMRA), GHB119 (BHQ1 instead of TAMRA), and *AdhC* (JOE instead of VIC and BHQ1 instead of TAMRA);

- T304-40 and GHB119 methods were run with a different number of PCR cycles (40 instead of 45);

- the dynamic range verified for COT102 method was from 0.08 % to 4.5 % GM content instead of the validated range from 0.078 % to 5 % GM content (in copy number ratio).

The EURL GMFF tested the methods as originally validated.

4.5 Results

Tables 3-5 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 T304-40, GHB119 and COT102. Slope values were rounded to two digits.

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

Run	T304-40			<i>AdhC</i>		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.37	98	1.00	-3.35	99	1.00
2	-3.35	99	1.00	-3.34	99	1.00
3	-3.42	96	1.00	-3.29	101	1.00
4	-3.30	101	1.00	-3.32	100	1.00
5	-3.31	101	1.00	-3.32	100	1.00
6	-3.34	99	1.00	-3.32	100	1.00
7	-3.37	98	1.00	-3.29	101	1.00
8	-3.30	101	1.00	-3.31	100	1.00
Mean	-3.34	99	1.00	-3.32	100	1.00

Table 4. Values of standard curve slope, PCR efficiency and R² coefficient for the GHB119 method on GM stack T304-40 x GHB119 x COT102 cotton.

Run	GHB119			<i>AdhC</i>		
	Slope	PCR Efficiency (%)	R ² coefficient	Slope	PCR Efficiency (%)	R ² coefficient
1	-3.34	99	1.00	-3.28	102	1.00
2	-3.34	99	1.00	-3.30	101	1.00
3	-3.37	98	1.00	-3.33	100	1.00
4	-3.38	98	1.00	-3.30	101	1.00
5	-3.39	97	1.00	-3.26	102	1.00
6	-3.29	101	1.00	-3.27	102	1.00
7	-3.31	100	1.00	-3.26	103	1.00
8	-3.28	102	1.00	-3.29	101	1.00
Mean	-3.34	99	1.00	-3.29	101	1.00

Table 5. Values of standard curve slope, PCR efficiency and R² coefficient for the COT102 method on GM stack T304-40 x GHB119 x COT102 cotton.

Run	COT102			<i>SAH7</i>		
	Slope	PCR Efficiency (%)	R ² coefficient	Slope	PCR Efficiency (%)	R ² coefficient
1	-3.34	99	1.00	-3.45	95	1.00
2	-3.35	99	1.00	-3.38	98	1.00
3	-3.26	103	1.00	-3.41	96	1.00
4	-3.35	99	1.00	-3.37	98	1.00
5	-3.29	101	1.00	-3.41	97	1.00
6	-3.33	100	1.00	-3.42	96	1.00
7	-3.25	103	1.00	-3.41	96	1.00
8	-3.36	98	1.00	-3.35	99	1.00
Mean	-3.32	100	1.00	-3.40	97	1.00

The mean PCR efficiencies of the GM and species-specific systems were above 90 % (99 % and 100 % for the T304-40 and the *AdhC* systems, 99 % and 101 % for the GHB119 and the *AdhC* systems, and 100 % and 97 % for the COT102 and the *SAH7* systems, respectively). The mean R² coefficient of the methods was 1.00 for all systems in all cases. The data presented in Tables 3-5 confirm the appropriate performance characteristics of the three methods when tested on GM stack T304-40 x GHB119 x COT102 cotton in terms of PCR efficiency and R² 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 three methods applied

to samples of DNA extracted from GM stack T304-40 x GHB119 x COT102 cotton see Tables 6-8.

Table 6. 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 T304-40 x GHB119 x COT102 cotton.

T304-40					
Unknown sample GM %	Expected value (GMO %)				
	4.5	2.0	0.90	0.40	0.10
Mean	4.5	2.0	0.89	0.39	0.10
SD	0.09	0.07	0.03	0.02	0.01
RSD _r (%)	2.0	3.4	3.4	6.0	6.5
Bias (%)	-0.15	2.0	-1.3	-1.4	-1.4

Table 7. 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 T304-40 x GHB119 x COT102 cotton.

GHB119					
Unknown sample GM %	Expected value (GMO %)				
	4.5	2.0	0.90	0.40	0.10
Mean	4.5	2.0	0.87	0.37	0.09
SD	0.11	0.06	0.04	0.02	0.01
RSD _r (%)	2.3	3.1	5.0	4.6	8.0
Bias (%)	0.68	-1.6	-3.8	-7.5	-10

Table 8. 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 T304-40 x GHB119 x COT102 cotton.

COT102					
Unknown sample GM %	Expected value (GMO %)				
	5.0	2.0	0.90	0.50	0.10
Mean	4.9	2.0	0.92	0.47	0.09
SD	0.14	0.16	0.04	0.03	0.01
RSD _r (%)	2.9	8.0	3.9	5.6	8.8
Bias (%)	-1.5	1.8	2.6	-5.8	-5.3

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 6-8, the values range from -1.4 % to 2.0 % for T304-40, from -10

% to 0.68 % for GHB119 and from -5.8 % to 2.6 % for COT102. Therefore, the three methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack T304-40 x GHB119 x COT102 cotton.

Tables 6-8 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 2.0 % and 6.5 % for T304-40, between 2.3 % and 8.0 % for GHB119 and between 2.9 % and 8.8 % for COT102, the three methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack T304-40 x GHB119 x COT102 cotton.

5. Conclusions

The performance of the three event-specific methods for the detection and quantification of cotton single line events T304-40, GHB119 and COT102, when applied to genomic DNA extracted from GM stack 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 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 T304-40 x GHB119 x COT102 cotton supposed that sufficient genomic DNA of appropriate quality is available.

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