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Report on the Verification of the Performance of GHB614, LLCotton25 and MON 15985 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack GHB614 x LLCotton25 x MON 15985

European Union Reference Laboratory for Genetically Modified Food and Feed

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EURL European Union Reference Laboratory for GM Food & Feed

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European Union Reference Laboratory for GM Food and Feed

17 June 2016

Executive Summary

An application was submitted by Bayer CropScience GmbH to request the authorisation of genetically modified stack (GM stack) GHB614 x LLCotton25 x MON 15985 cotton (glyphosate and glufosinate ammonium-tolerant and insect-resistant) 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 LLCotton25 x MON 15985 cotton is BCS-GHØØ2-5 x ACS-GHØØ1-3 x MON-15985-7.

The GM stack GHB614 x LLCotton25 x MON 15985 cotton has been obtained by conventional crossing between three genetically modified cotton events: GHB614, LLCotton25 and MON 15985, 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, LLCotton25 and MON 15985 (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 LLCotton25 x MON 15985 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 LLCotton25 x MON 15985 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 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, 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/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 events and if these events have been stacked by conventional crossing. These criteria are met for the GM stack GHB614 x LLCotton25 x MON 15985 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 (EC) No 641/2004 (Annex I).

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

2. Step 1 (dossier reception and acceptance)

Bayer CropScience GmbH submitted the detection methods, the data demonstrating their adequate performance, and the corresponding control samples consisting of DNA extracted from GM stack cotton GHB614 x LLCotton25 x MON 15985 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 %) for the three methods on the stack DNA calculated by the EURL based on the individual quantification values submitted by the applicant. Means are the average of eighteen replicates, obtained through three runs for each event, 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 %) for the GHB614, LLCotton25 and MON 15985 methods applied to GM stack GHB614 x LLCotton25 x MON 15985.

GHB614			
Sample GM%*	**Expected value (GMO %)**		
	0.08	**0.90**	**4.5**
Mean	0.070	0.76	4.31
RSD_r (%)	15.31	8.63	10.43
Bias (%)	14.29	15.25	7.00
LLCotton25			
Sample GM%*	**Expected value (GMO %)**		
	0.08	**0.90**	**4.5**
Mean	0.070	0.85	4.55
RSD_r (%)	9.54	5.39	8.97
Bias (%)	15.60	6.68	7.33
MON 15985			
Sample GM%*	**Expected value (GMO %)**		
	0.08	**0.90**	**4.5**
Mean	0.070	0.82	4.30
RSD_r (%)	11.27	8.07	6.47
Bias (%)	18.24	9.26	4.43

* 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 were reliable and seemed to confirm that the methods meet the ENGL performance criteria [(3)].

One request of complementary information was addressed to Bayer CropScience GmbH. This was related to the *in-house* verification of the methods, the DNA sequences and the amount of control sample provided. The EURL GMFF verified the data, the complementary information and the additional sample received and accepted the clarifications received 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 DNA extracted from GM stack GHB614 x LLCotton25 x MON 15985.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from leaves of plants harbouring the GHB614 x LLCotton25 x MON 15985 stacked events homozygously, as positive control sample.
- genomic DNA extracted from leaves of non-GM (conventional) cotton plants, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack GHB614 x LLCotton25 x MON 15985 and genomic DNA extracted from non-GM cotton in a constant amount of total cotton DNA. Table 2 shows the five GM concentrations used in the verification of the GHB614, LLCotton25 and MON 15985 methods when applying them to genomic DNA extracted from the GM stack GHB614 x LLCotton25 x MON 15985 cotton. These are the same concentrations used in the validation of these methods for the parental single GM lines.

Table 2. Percentage of GHB614, LLCotton25 and MON 15985 events in GHB614 x LLCotton25 x MON 15985 verification samples.

GHB614 GM%* (GM DNA / Total cotton DNA x 100)	**LLCotton25 GM%*** (GM DNA / Total cotton DNA x 100)	**MON 15985 GM%**** (GM DNA / total cotton DNA x 100)
4.5	3.3	6.0
2.0	2.0	2.5
0.9	0.9	0.9
0.4	0.4	0.4
0.09	0.15	0.10

* percentage expressed in copy number ratio

** percentage expressed in mass ratio

The protocols validated for the individual GM events GHB614, LLCotton25 and MON 15985 (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx), were followed in the *in-house* verification with the deviation reported in § 4.4.1.

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; the *AdhC* reference system was used for the relative quantification of events GHB614 and LLCotton25 while for MON 15985 the *acp1* reference system was used in accordance with the corresponding previously validated methods. 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, LLCotton25 and MON 15985), 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 LLCotton25 x MON 15985 using the single detection methods previously validated for the respective single GM events GHB614, LLCotton25 and MON 15985.

For detection of GM cotton events GHB614, LLCotton25 and MON 15985, DNA fragments of 120-bp, 79-bp and 82-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 the relative quantification of GM cotton events GHB614, LLCotton25 and MON 15985, two taxon-specific reference systems were used. For MON 15985, a 76-bp fragment of a cotton endogenous gene *acp1* (*acyl carrier protein* Genbank U48777) was used as the reference system, using two *acp1* gene-specific primers and a acp1 gene-specific probe labelled with 6-FAM and TAMRA. For GHB614 and LLCotton25, a 73-bp fragment of the cotton endogenous gene *AdhC* (*alcohol dehydrogenase C,* GenBank AF036569) was used as the reference system, using two *AdhC* gene-specific primers and a *AdhC* gene-specific probe labelled at its 5'-end with the reporter VIC (for relative quantification of GHB614 event) or 6-FAM (for relative quantification of LLcotton25 event) and TAMRA as the quencher at its 3'-end.

For relative quantification of GM cotton event GHB614 and MON 15985 in a test sample, standard curves are generated both for the GHB614 and for the cotton-specific reference systems (*AdhC* and *acp1*, 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 GHB614 and MON 15985 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.

For relative quantification of GM cotton event LLCotton25 DNA in a test sample, 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 LLCotton25 event DNA). The ΔCq values of the unknown samples are measured and, by means of the regression formula, the relative amount of LLCotton25 event is estimated.

4.1.1 Deviations from the validated methods

Updated bioinformatics analyses indicated that the acp1 target is present in two copies per cotton haploid genome while the methods for quantification of MON 15985 was originally validated as ratio of GM DNA copy numbers to target taxon-specific DNA copy number, assuming that the latter was only present in one copy per genome. Therefore, in the current report all quantification data are reported as mass fractions of GM DNA in the cotton stacked event GHB614 x LLCotton25 x MON 15985 (§ 4.1) in comparison to the mass fraction of the total cotton DNA in the sample.

Note: the under-estimation of the copy number for the reference gene is only relevant if the measurement is done in copy numbers and then a wrong conversion factor used to estimate the mass fraction. However, results should be expressed in the same unit of measurement of the calibrator. In most cases CRMs, certified in mass fractions of GM-material, are used as calibrators; consequently the copy number of the reference gene does not have an influence on the expression of results of quantification.

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 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 eight runs, for GM cotton events GHB614, LLCotton25 and MON 15985. Slope 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 LLCotton25 x MON 15985 cotton.

Run	GHB614			*AdhC*		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.38	98	1.00	-3.27	102	1.00
2	-3.32	100	1.00	-3.29	101	1.00
3	-3.25	103	1.00	-3.31	101	1.00
4	-3.24	104	1.00	-3.26	103	1.00
5	-3.29	101	1.00	-3.28	102	1.00
6	-3.42	96	1.00	-3.29	101	1.00
7	-3.28	102	1.00	-3.29	102	1.00
8	-3.30	101	1.00	-3.28	102	1.00
Mean	-3.31	**100**	**1.00**	**-3.28**	**102**	**1.00**

Table 4. Values of standard curve slope, PCR efficiency and R^2 coefficient for the LLCotton25 method on GM stack GHB614 x LLCotton25 x MON 15985 cotton.

Run	LLCotton25		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.31	101	1.00
2	-3.22	104	1.00
3	-3.24	104	1.00
4	-3.26	103	1.00
5	-3.33	100	1.00
6	-3.24	104	1.00
7	-3.20	105	1.00
8	-3.23	104	0.99
Mean	**-3.25**	**103**	**1.00**

Table 5. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 15985 method on GM stack GHB614 x LLCotton25 x MON 15985 cotton.

Run	MON 15985			*Acp1*		
	Slope	**PCR Efficiency (%)**	**R^2 coefficient**	**Slope**	**PCR Efficiency (%)**	**R^2 coefficient**
1	-3.38	98	1.00	-3.44	95	1.00
2	-3.47	94	1.00	-3.41	96	1.00
3	-3.33	100	1.00	-3.41	97	1.00
4	-3.37	98	1.00	-3.42	96	1.00
5	-3.38	98	1.00	-3.42	96	1.00
6	-3.34	99	1.00	-3.40	97	1.00
7	-3.33	100	1.00	-3.44	95	1.00
8	-3.36	98	1.00	-3.40	97	1.00
Mean	**-3.37**	**98**	**1.00**	**-3.42**	**96**	**1.00**

The mean PCR efficiencies of the GM and taxon-specific systems were 100 % for GHB614 and 102 % for the *AdhC* system respectively; for event LLCotton25 the mean PCR efficiency of the ΔCq slope was 103 %, while for MON 15985 it was 98 % and 96 % for *acp1*. The mean R^2 coefficient of the methods was 1.00 for all systems. 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 LLCotton25 x MON 15985 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 three methods applied to samples of DNA extracted from GM stack GHB614 x LLCotton25 x MON 15985 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 LLCotton25 x MON 15985 cotton.

GHB614					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.40**	**0.90**	**2.0**	**4.5**
Mean	0.07	0.39	0.84	2.0	4.5
SD	0.01	0.02	0.03	0.05	0.09
RSD_r (%)	13	5.8	3.8	2.5	2.1
Bias (%)	-19	-2.3	-7.0	-1.0	-1.1

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the LLCotton25 method applied to genomic DNA extracted from GM stack GHB614 x LLCotton25 x MON 15985 cotton.

LLCotton25					
Unknown sample GM%	**Expected value (GMO%)**				
	0.15	**0.40**	**0.90**	**2.0**	**3.3**
Mean	0.14	0.45	0.95	2.3	3.4
SD	0.01	0.03	0.08	0.07	0.15
RSD_r (%)	8.1	5.7	8.2	3.2	4.3
Bias (%)	-6.9	14	5.0	17	3.4

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 18985 method applied to genomic DNA extracted from GM stack GHB614 x LLCotton25 x MON 15985 cotton.

MON 15985					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**2.5**	**6.0**
Mean	0.10	0.38	0.96	2.4	6.2
SD	0.01	0.02	0.03	0.06	0.16
RSD_r (%)	4.9	4.3	3.0	2.3	2.6
Bias (%)	3.2	-4.5	6.3	-3.5	4.0

The trueness of a 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 ±25 % across the entire dynamic range. As shown in Tables 6, 7 and 8, the values range from -19 % to -1.1 % for GHB614, from -6.9 % to 17 % for LLCotton25 and from -4.5 % to 6.3 % for MON 15985. Therefore, the three methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack GHB614 x LLCotton25 x MON 15985 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 acceptance criteria and method performance requirements, the EURL GMFF requires RSD_r values to be below 25 %. As the values range between 2.1 % and 13 % for GHB614, between 3.2 % and 8.1 % for LLCotton25 and between 2.3 % and 4.9 % for MON 15985, the three methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack GHB614 x LLCotton25 x MON 15985 cotton.

6. Conclusions

The performance of the three event-specific methods for the detection and quantification of cotton events GHB614, LLCotton25 and MON 15985, when applied to genomic DNA extracted from GM stack GHB614 x LLCotton25 x MON 15985, 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 events GHB614, LLCotton25 and MON 15985, can be equally applied for the detection and quantification of the respective events combined in GM stack GHB614 x LLCotton25 x MON 15985 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 503/2003 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. 13 October 2008. http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf.

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