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

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11 March 2014

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Bayer CropScience AG to request the authorisation of genetically modified GM stack GHB614 x LLCotton25 cotton (tolerant to glufosinate ammonium and glyphosate-containing herbicides) 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) N° 1829/2003 GM Food and Feed. The unique identifier assigned to GM stack GHB614 x LLCotton25 cotton is BCS-GHØØ2-5xACS-GHØØ1-3.

The GM stack GHB614 x LLCotton25 cotton has been obtained by conventional crossing of two genetically modified cotton events: GHB614 and LLCotton25, without any new genetic modification.

The EU-RL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events GHB614 and LLCotton25 (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 EU-RL GMFF has carried out only an in-house verification of the performance of each validated method when applied to DNA extracted from GM stack GHB614 x LLCotton25 cotton.

The herewith reported *in-house* verification study lead to the conclusion that the individual methods meet the ENGL performance criteria also when applied to DNA extracted from the GM stack GHB614 x LLCotton25 cotton.

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

Quality assurance

The EU-RL 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 EU-RL GMFF quality system.

The EU-RL 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 EU-RL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection (IHCP) provided by CERMET.

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Content

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 (GM 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 EU-RL 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 EU-RL 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 EU-RL GMFF carries out an *in-house* verification of the performance of each event-specific methods if this method has previously been validated by the EU-RL 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 LLCotton25 cotton.

Upon reception of methods, samples and related data (step 1), the EU-RL 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 on the individual events.

2. Step 1 (dossier reception and acceptance)

Bayer CropScience AG submitted the detection methods, data demonstrating their adequate performance, and the corresponding control samples DNA extracted from the GM stack GHB614 x LLCotton25 cotton 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 two methods on the stack DNA. Means are the average of

eighteen replicates obtained through three runs. Percentages are expressed as GM DNA / total DNA x 100.

Table 1. Estimates of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) for the GHB614 and LLCotton25 methods applied to GM stack GHB614 x LLCotton25 cotton.

GHB614			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.90**	**4.50**
Mean	0.077	0.90	4.2
RSD_r (%)	13	14	9.3
Bias (%)	-3.4	0.42	-7.2
LLCotton25			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.90**	**4.50**
Mean	0.087	0.84	4.7
RSD_r (%)	14	12	11
Bias (%)	8.4	-6.8	5.4

The EU-RL GMFF verified the data and concluded that they were reliable and seemed to confirm that the methods meet the ENGL performance criteria [(3)].

Three requests for complementary information regarding the DNA sequences of the GM events GHB614 and LLCotton25 were addressed to the applicant. Further to the request, the applicant provided the full sequence of the inserts. Additionally, clarifications were requested on the relation between GHB614 and LLCotton25 events and existing patents that were identified by the EU-RL GMFF with bioinformatics analysis, on the length of the GHB614 target amplicon and on the cotton plant origin of the 3' region of GHB614 target amplicon. The EU-RL GMFF accepted the received clarifications as satisfactory.

The dossier was therefore moved to step 3.

4. Step 3 (EU-RL GMFF experimental testing)

In step 3 the EU-RL GMFF implemented the two methods in its own laboratory and performed a verification of their performance when applied to DNA extracted from GM stack GHB614 x LLCotton25 cotton.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from leaves of GM stack GHB614 x LLCotton25 cotton
- genomic DNA extracted from leaves of non GM cotton plants.

The EU-RL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack GHB614 x LLCotton25 cotton and genomic DNA extracted from non GM cotton in a constant amount of background total cotton DNA. The same 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 and LLCotton25 methods when applying them to genomic DNA extracted from the GM stack GHB614 x LLCotton25 cotton. These are the same concentrations used in the validation of these methods for the parental single line GMOs.

Table 2. Percentage of GHB614 and LLCotton25 in GHB614 x LLCotton25 in the verification samples.

GHB614 GM% (GM DNA / Non-GM DNA x 100)	**LLCotton25 GM%** (GM DNA / Non-GM DNA x 100)
0.09	0.15
0.40	0.40
0.90	0.90
2.00	2.00
4.50	3.30

The protocols (reagents, concentrations, primer/probe sequences) described by the applicant were implemented precisely in the EU-RL GMFF laboratory. The *in-house* verification followed the protocols already published as validated methods for the individual GHB614 and LLCotton25 single events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx), with the small modifications described in section 4.5, below.

4.2 DNA extraction

A method for DNA extraction from cotton seeds was previously evaluated by the EU-RL GMFF with regard to its performance characteristics and was considered valid i.e. fit for 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/GHB-DNAExtr_report.pdf.

Consequently, the EU-RL 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 *AdhC* (*alcohol dehydrogenase C gene*). 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 and LLCotton25), 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 EU-RL GMFF carried out parallel tests on DNA extracted from GM stack GHB614 x LLCotton25 cotton using the methods previously validated for the respective single line GM cotton events GHB614 and LLCotton25, respectively.

For the detection of GM cotton events GHB614 and LLCotton25, DNA fragments of 119-bp and 79-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) is used as reporter dye at its 5′-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3′-end.

For the quantification of GM cotton events GHB614 and LLCotton25 DNA, a taxon-specific reference system amplifies a 73-bp fragment of *AdhC* (*alcohol dehydrogenase C*) cotton endogenous gene (GeneBank: AF036569 and AF403330), using two *AdhC* gene-specific primers and an *AdhC* gene-specific probe labelled with VIC and TAMRA.

For the relative quantification of GM event GHB614 in a test sample, standard-curves are generated both for the GHB614 event and for the *AdhC* specific systems by plotting the Ct values of the calibration standards against the logarithm of the DNA copy numbers of GHB614 event, and fitting a linear regression into these data. For the relative quantification of GM event LLCotton25 in a test sample, the normalised ΔCt values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCt values against the logarithm of the amount of LLCotton25 event DNA). Thereafter, the normalised Ct or ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of GHB614 and LLCotton25 events respectively, 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

For the LLCotton25 event, the analytical method used by the applicant in its *in-house* method verification was a Ct-based method; however, the EU-RL GMFF validated method is ΔCt based. Therefore the verification studies for LLCotton25 method applied to stack cotton GHB614 x LLCotton25 carried out by the EU-RL GMFF have been performed using the original validated method (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

4.6 Results

Tables 3 and 4 present the values of the slopes of the different standard curves generated by the EU-RL 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 cotton events GHB614 and LLCotton25, respectively.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the GHB614 method on GM stack GHB614 x LLCotton25 cotton.

Run	GHB614			*AdhC*		
	Slope	**PCR Efficiency (%)**	**Linearity (R^2)**	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**
1	-3.31	101	0.99	-3.36	99	1.00
2	-3.25	103	1.00	-3.42	96	1.00
3	-3.27	102	1.00	-3.46	94	1.00
4	-3.46	95	1.00	-3.43	96	1.00
5	-3.40	97	1.00	-3.41	96	1.00
6	-3.38	98	1.00	-3.44	95	1.00
7	-3.35	99	1.00	-3.37	98	1.00
8	-3.39	97	1.00	-3.31	101	1.00
Mean	-3.35	99	1.00	-3.40	97	1.00

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the LLCotton25 method on GM stack GHB614 x LLCotton25.

Run	LLCotton25		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.34	99	1.00
2	-3.43	96	1.00
3	-3.36	99	1.00
4	-3.41	97	1.00
5	-3.31	100	1.00
6	-3.42	96	1.00
7	-3.22	105	1.00
8	-3.21	105	1.00
Mean	-3.34	99	1.00

The mean PCR efficiencies of the calibration curves for each of the two event-specific methods were above 90% (99% for GHB614 and 99% for LLCotton25, respectively). The linearity of both methods (R^2) was 1.00. The data presented in Tables 3 and 4 confirm the appropriate performance characteristics of the two methods when tested on GM stack GHB614 x LLCotton25 cotton in terms of PCR efficiency and linearity.

The EU-RL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as RSDr %, relative repeatability standard deviation) of the two methods applied to samples of DNA extracted from GM stack GHB614 x LLCotton25 cotton, see tables 5 and 6.

Table 5. 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 cotton.

GHB614					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.40**	**0.90**	**2.0**	**4.50**
Mean	0.08	0.40	0.93	2.05	4.59
SD	0.02	0.03	0.06	0.14	0.31
RSD_r (%)	22	7.5	6.5	6.6	6.8
Bias (%)	-9.3	-0.8	3.3	2.3	2.0

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the LLCotton25 method applied to genomic DNA extracted from GM stack GHB614 x LLCotton25 cotton.

LLCotton25					
Unknown sample GM%	**Expected value (GMO%)**				
	0.15	**0.40**	**0.90**	**2.00**	**3.30**
Mean	0.16	0.41	0.88	2.12	3.42
SD	0.02	0.04	0.12	0.26	0.28
RSD_r (%)	11	10	13	12	8.1
Bias (%)	4.4	3.7	-2.3	5.8	3.8

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 ± 25% across the entire dynamic range. As shown in Tables 5 and 6, the values range from -9.3% to 3.3% for GHB614 and from -2.3% to 5.8% for LLCotton25. Therefore, the two methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack GHB614 x LLCotton25 cotton.

Tables 5 and 6 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 EU-RL GMFF requires RSD_r values to be below 25%. As the values range between 6.5% and 22% for GHB614 and between 8.1% and 13% for LLCotton25, the two methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack GHB614 x LLCotton25 cotton.

5. Comparison of method performance when applied to genomic DNA extracted from GM stack GHB614 x LLCotton25 cotton and to DNA extracted from the single-line GM events

An indicative comparison of the performance (bias, RSDr %) of the two methods applied to GM stack GHB614 x LLCotton25 cotton 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 GHB614 detection method applied to genomic DNA extracted from GM stack GHB614 x LLCotton25 cotton and to genomic DNA extracted from the single line event GHB614.

Trueness and repeatability of GHB614 quantification on GHB614 x LLCotton25			**Trueness and repeatability of GHB614 quantification on single event GHB614***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.09	-9.3	22	**0.09**	15	9.4
0.40	-0.8	7.5	**0.40**	14	15
0.90	3.3	6.5	**0.90**	7.5	6.8
2.00	2.3	6.6	**2.00**	8.8	3.3
4.50	2.0	6.8	**4.50**	2.0	4.1

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

Table 8. Qualitative comparison of the performance of the LLCotton25 detection method applied to genomic DNA extracted from GM stack GHB614 x LLCotton25 cotton and to genomic DNA extracted from the single line events LLCotton25.

Trueness and repeatability of LLCotton25 quantification on GHB614 x LLCotton25			**Trueness and repeatability of LLCotton25 quantification on single event LLCotton25***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.15	4.4	11	**0.15**	12	23
0.40	3.7	10	**0.40**	17	28
0.90	-2.3	13	**0.90**	20	18
2.00	5.8	12	**2.00**	11	18
3.30	3.8	8.1	**3.30**	8.1	24

*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 cotton events GHB614 and LLCotton25, when applied to DNA extracted from GM stack GHB614 x LLCotton25 cotton, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant.

The method verification has demonstrated that the PCR efficiency, linearity, trueness and repeatability of the methods were within the limits established by the ENGL.

In conclusion, the verification study confirmed that the two methods are capable to detect, identify and quantify each of the GM events when applied to genomic DNA of suitable quality, extracted from GM stack GHB614 x LLCotton25 cotton.

Therefore these methods, developed and validated to detect and quantify the single events in the single event parental GMOs, can be equally applied for the detection and quantification of the respective events combined in GM stack GHB614 x LLCotton25 cotton.

7. References

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Abstract

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