EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Cotton GHB119 Using Real-time PCR

Validation Report

11 October 2012

Corrected version 1 - 21/11/2012 (see page 2)

European Union Reference Laboratory for GM Food and Feed
Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Executive Summary

In line with its mandate[1] the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting and quantifying cotton event GHB119 (unique identifier BCS-GHØØ5-8). The validation study was conducted according to the EU-RL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and internationally accepted guidelines.

In accordance with current EU legislation[1], Bayer CropScience has provided the detection method and the samples (genomic DNA from cotton seeds harbouring the GHB119 event as positive control DNA, genomic DNA from conventional cotton seeds as negative control DNA). The EU-RL GMFF prepared the validation samples (calibration samples and blind samples at test GM percentage [DNA/DNA]), organised an international collaborative study, and analysed the results.

The study confirms that the method meets the method performance requirements as established by the EU-RL GMFF and the ENGL detailing the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004[1] and it fulfils the analytical requirements of Regulation (EU) No 619/2011[2].
This report is published at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

[1] Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed" and Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003".

[2] Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired.

Quality assurance

The EU-RL GMFF is ISO 9001:2008 certified (certificate number: CH-32232) and 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.

Corrected version 1 - 21/11/2012

Correction from the previous version:

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The paragraph under Figure 1 has been replaced by the following:
Overall, most mean relative deviations from the true values were within the ENGL acceptance criterion of maximum 25%. Indeed, at GM-level 0.1% eleven laboratories were within the limits, 12 laboratories at the 0.4%, 11 at the 0.9% GM-level, 12 at the 2.0% and 12 at the 4.5%. At GM-level 0.1%, a trend for overestimation of the GM-target can be noticed. Overall relative deviations from the true value were within the ± 25%, except one laboratory overestimating the 0.1% GM-content by about 37% and one laboratory underestimating the 0.9% GM-level by about 27%.

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit (MBG)
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, 21027 Ispra (VA) – Italy
Functional mailbox: eurl-gmff@jrc.ec.europa.eu

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1. Summary

In line with Regulation (EC) No 1829/2003, Bayer CropScience provided the EU-RL GMFF with a copy of the official application for authorisation of an event specific method for the detection and quantification of cotton event GHB119 (unique identifier BCS-GHØØ5-8) together with genomic DNA as positive control samples (February 2011).

In response to an earlier submission of the method, the EU-RL GMFF started its step-wise validation procedure (step 1: dossier reception) already in advance to the official dossier, before EFSA declared the dossier as complete and valid (November 2011).

The scientific dossier assessment (step 2) focused on the reported method performance characteristics assessed against the ENGL method acceptance criteria[3] (see http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf for a summary of method acceptance criteria and method performance requirements). It was positively concluded already in March 2011.

In step 3 of the validation procedure (Experimental testing), the EU-RL GMFF verified the purity of the control samples provided and conducted the in-house testing of samples and methods. The positive and negative control DNA - submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Reg.(EC) No 1829/2003 – were found of good quality.

The method characteristics were verified by quantifying five blind GM levels within the range 0.1%-4.5% on a genome copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision were within the limits established by the ENGL. In addition, and in line with the requirements of Reg. (EU) No 619/2011, the EU-RL GMFF also verified *i)* the zygosity ratio of the positive control sample submitted by investigating the GM- to reference- target ratio by means of digital PCR, in order to determine the conversion factor between copy numbers and mass fractions; and *ii)* the method's precision (relative repeatability standard deviation, RSDr %) at the 0.1% related to mass fraction of GM-material on fifteen replicates. Step 3 was finished in June 2012 with the conclusion to enter into a collaborative trial (step 4).

The collaborative trial (step 4) was organised and took place in June/July 2012. It demonstrated that the method is well suited to analyse DNA, appropriately extracted from food or feed, with regard to identifying the presence of GM event GHB119 and is therefore applicable for this purpose.

[3] EURL/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm)

2. Step 1 (dossier acceptance) and step 2 (dossier scientific assessment)

The early submitted dossier was found to be complete (step 1) and the verification against the formal dossier did not reveal any differences.

In step 2, the documentation and data provided by the applicant were evaluated by the EU-RL GMFF for compliance with the ENGL method acceptance criteria for the parameters of the calibration curves (slope, R^2 coefficient).

Table 1. Values of slope and R^2 obtained by the applicant

	GHB119		*AdhC*	
	Slope	**R^2**	**Slope**	**R^2**
Run 1	-3.4	1.00	-3.4	1.00
Run 2	-3.5	1.00	-3.4	1.00
Run 3	-3.4	0.99	-3.4	1.00

Table 1 indicates that the slope and the R^2 coefficient of the standard curves for the GM-system (GHB119) and the cotton–specific reference system (*AdhC*) (slope between -3.4 to -3.5 and R^2 coefficient $\geq$ 0.99) were within the ENGL acceptance criteria.

Table 2. Mean %, precision and trueness measured at three GM-levels by the applicant

Test Sample Expected GMO %			
	0.08	**0.9**	**4.5**
Mean %	0.088	0.96	5.01
Precision (RSDr %)	14	8.0	9.8
Trueness(bias %)	10	17	11

Table 2 reports precision and trueness for the three GM-levels tested by the applicant. Eighteen values for each GM-level were provided. Both parameters were within the ENGL acceptance criteria (trueness $\pm$ 25%, RSDr $\leq$ 25% across the entire dynamic range).

3. Materials and methods

3.1 DNA extraction

A "CTAB/Genomic-tip 20" method for DNA extraction suitable for the isolation of genomic DNA from ground cotton seeds and grains, submitted in support of the validation of the method for detection of event LLCotton25, was evaluated and tested by the EU-RL GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on testing were published in 2007 at http://gmo-crl.jrc.ec.europa.eu/summaries/LLCotton25_DNAExtr_report.pdf.
In agreement with the ENGL position, which endorses the modularity principle (see also Annex I to Reg. (EC) No 641/2004), the EU-RL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for analysis of event GHB119 cotton, given the similarity of the cotton matrix.

3.2 Method protocol for the PCR analysis

The PCR analysis method that was provided by the applicant (see the corresponding Validated Method at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) and subsequently validated by the EU-RL GMFF is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event GHB119 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton *AdhC* (alcoholdehydrogenase C) specific assay, and the GM target assay (GHB119) are performed in separate wells.

For the specific detection of cotton event GHB119, a 90-bp fragment of the region spanning the 3' insert-to-plant junction in cotton event GHB119 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein), as a reporter at its 5′ end, and TAMRA (carboxytetramethylrhodamine) at its 3' end as quencher dye.

For the relative quantification of GM event 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 a *AdhC* gene-specific probe labelled with VIC as reporter dye at its 5' end, and TAMRA as quencher at its 3' end.

Standard curves are generated for both the GHB119 and the *AdhC* systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves.

For relative quantification of event GHB119 DNA in a test sample, the GHB119 copy number is divided by the copy number of the cotton reference gene (*AdhC*) and multiplied by 100 to obtain the percentage value (GM% = GHB119/*AdhC* x 100).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (nanograms) by the published average 1C value for the cotton genome (2.33 pg) (Arumuganathan & Earle, 1991) (1). The copy number values used in the quantification, the GMO contents of the calibration samples and total DNA quantity used in PCR are listed in Table 3.

Table 3. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	300	100	20	5	1
Target taxon *AdhC* copies	128755	42918	8584	2146	429
GHB119 Cotton GM copies	12876	4292	858	215	43

3.3 EU-RL GMFF experimental testing (step 3)

3.3.1 Determination of the zygosity ratio in the positive control sample

Standard and test samples were prepared, using the genomic DNA provided by the applicant, at the EU-RL GMFF in terms of GM DNA copy numbers in relation to target taxon specific DNA copy numbers calculated as haploid genomes.

Annex II of Reg. (EU) No 619/2011 requires that "when results are primarily expressed as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EU-RL GMFF." In order to satisfy this requirement, the EU-RL GMFF conducted an experimental assessment of the zygosity (GM-target to reference target ratio) in the positive control sample that was submitted by the applicant for the preparation of standard and test items at various GM-concentrations. To this end, the copy number of the GHB119 and of the *AdhC* targets in the positive control sample submitted by the applicant were determined by digital PCR (dPCR), performed on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Four micrograms of genomic DNA were digested at 37 ºC overnight with 60 units of restriction enzyme DraI that do not cleave within the annealing sites for the primers of the GHB119 or *AdhC* amplification systems. DraI restriction sites are located outside the respective targeted sequences. Further to digestion, the DNA was precipitated with ammonium acetate 2.5 M final and two

volumes of absolute ethanol. The outcome of enzymatic digestion was controlled by running approximately 250 ng of digested DNA and 200 nanograms of undigested DNA in comparison with DNA molecular marker in 1% agarose-gel electrophoresis.

Digested DNA was used as template in digital PCR experiments. Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix with UNG (Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probe at the reaction concentrations indicated in the corresponding Validated Method (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm), 1 µL of DNA at a concentration of 1.5 ng/µL, suitable to avoid panel saturation after analysis (optimal between 200<positive partitions<500).

Loading of the digital chip was performed according to the manufacturer's instructions by using the IFC controller (Fluidigm). A volume of 9 µL of reaction mix was loaded into each well of which only approximately 4.6 µL were distributed into the 765 chambers (or partitions) constituting one panel. The analysis was repeated in three consecutive days; five replicates in five panels were run each day for both the GM- and reference assay, with a total number of thirteen data sets for both targets (two data were removed as considered outliers). No template controls were included. Amplification conditions were as reported in the Validated Method. Data analysis and copy number calculation was performed using the BioMark digital PCR Analysis software, the range of Ct retention was from 15 to 40.

Calculations of mean and variances where carried out according to the procedure outlined for random variables in the Annex 4 of the ENGL guidance document 'Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods[4].

3.3.2 In-house verification of the method performance against ENGL method acceptance criteria

The method performance characteristics were verified (EU-RL GMFF step 3) by quantifying on a copy number basis five blinded test samples with known GM levels, within the range 0.1%-4.5%. The experiments were performed on a Roche Light Cycler real-time platform under repeatability conditions. Test samples with GM-levels 4.5%, 2.0%, 0.9%, 0.4%, 0.1% were tested in two real-time PCR runs with two replicates for each GM-level on each plate (total of four replicates per GM-level). The test sample with GM-level 0.1% was tested in 15 replicates in one PCR real-time run. Average values of the slope and of the R^2 coefficient of the standard curves and method trueness and precision of quantification over the dynamic range were evaluated for compliance against the ENGL method acceptance criteria.

[4] Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. European Network of GMO laboratories (ENGL), 2011.
http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf

In order to assess the method compliance to Reg. (EU) No 619/2011, the EU-RL GMFF also determined the zygosity of the GM-insert in the positive control sample and estimated, based on 15 replicates, the method precision (RSDr) at 0.1% GM level in mass fraction.

3.4 International collaborative study (step 4)

The international collaborative study (EU-RL GMFF step 4) involved twelve laboratories, all being "National reference laboratories, assisting the CRL for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 1981/2006. The study was carried out in accordance with the following internationally accepted guidelines:

1. The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) (2)
2. ISO 5725 (1994) (3)

The objective of the international collaborative study was to verify in experienced laboratories the trueness and reproducibility of the PCR analytical method described under 3.2, above, that was provided by the applicant.

3.4.1 List of participating laboratories

The participants in the GHB119 validation study were randomly selected from the 23 NRLs that offered to participate.

Clear guidance was given to the selected laboratories to strictly follow the standard operational procedures that was provided for the execution of the protocol (the protocol is detailed in "the report of the Validated Method" that is available at, http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the validation of the detection method for cotton GHB119

Laboratory	Country
Bavarian Health and Food Safety Authority	DE
Austrian Agency for Health and Food Safety (AGES)	AT
National Veterinary Research Institute in Pulawy, Department of Feed Hygiene	PL
Tallinn University of Technology - Department of Gene Technology	EE
Agricultural Institute of Slovenia	SL
National Food Administration	SE
National Health Laboratory, Food Control Department	LU
National Food and Veterinary Risk Assessment Institute	LT
BioGEVES	FR
Plant Health Laboratory	FR
National Centre for Food, Spanish Food Safety Agency and Nutrition	ES
State Institute of Chemical and Veterinarian Analysis	DE

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: six laboratories used the ABI 7500, five used the ABI 7900, one used the Stratagene Mx3005p.

This variability of equipment, with its known potential influence on the PCR results, reflects the real life situation in the control laboratories and provides additional assurance that the method is robust and useable under real conditions.

3.4.3 Materials used in the international collaborative study

For the validation of the quantitative event-specific method, the following control samples were provided to the EU-RL GMFF by the applicant:

i) genomic DNA extracted by the applicant from homozygous cotton seeds harbouring the event GHB119, and

ii) genomic DNA extracted by the applicant from conventional cotton seeds genetically similar to those harbouring the GHB119 event.

These positive and negative control samples were used by the EU-RL GMFF to prepare standard and test samples (of unknown GM-content), containing mixtures of GHB119 cotton and non-GM cotton, as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes.

The calibration sample S1 was prepared by mixing the appropriate amount of GHB119 DNA with control non-GM cotton DNA to obtain a 10% GM GHB119 (12876 GM copies).
Calibration samples S2 to S4 were prepared by serial dilutions of the 10% standard. S2 contains 4292 cotton GM copies (3-fold dilution), S3 contains 858 GM copies (five-fold dilution), S4 contains 215 GM copies (four-fold dilution) and S5 contains 43 GM copies (five-fold dilution).

The 12 NRLs participating in the validation study received the following materials:

- ✓ Five calibration samples with known concentrations of GM-events (175 µL of DNA solution each) labelled from S1 to S5 (Table 3).
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution each at 40ng/uL) labelled from U1 to U20, representing 5 GM levels (Table 5).

Table 5. GHB119 GM contents in genome copy number and DNA mass

GHB119 GM% GM copy number/cotton genome copy number x 100
0.1
0.4
0.9
2.0
4.5

- ✓ Reaction reagents:
  - TaqMan® Universal PCR Master Mix (2x), two vials: 10 mL
  - distilled sterile water, one vial: 5 mL

- ✓ Primers and probes (1 tube each) as follows:
  AdhC taxon-specific assay

  - KVM157 (10 µM): 160 µL
  - KVM158 (10 µM): 160 µL
  - TM012 (VIC) (10 µM): 160 µL

GHB119 assay provided by the applicant

- SHA021 (10 µM): 320 µL
- NEL109 (10 µM): 320 µL
- TM082 (10 µM):160 µL

3.4.4 Design of the collaborative study

Twenty test samples (labelled from U1 to U20), representing five GM levels, each in 4 replicates, were included in the validation study (Table 5). On each PCR plate the samples were analysed for the GHB119 specific system and for the *AdhC* taxon-specific system. In total, two plates were run per participating laboratory. Participants determined the GM% according to the instructions provided and using the excel sheet supplied.

Each participating laboratory received the above described materials and prepared the master-mixes for the GHB119 and *AdhC* assay in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per recommended plate lay-out. The amplification reactions followed the cycling program provided. Raw data were recorded on an excel sheet designed, validated and distributed to participating laboratories by the EU-RL GMFF. The excel sheets with the original data were provided to the EU-RL GMFF and back-up copies on CDs were subsequently delivered via mail. All the data received are stored by the EU-RL GMFF on a dedicated and protected server.

The EU-RL GMFF then analysed the data against parameters and limits set by the ENGL: trueness, precision, amplification efficiency and linearity.

3.4.5 Deviations reported from the protocol

Eleven laboratories reported no deviations from the method protocol.
One laboratory eliminated, due to atypical curves, one replicate of the reference system for GM-level 0.9% and one replicate of the reference system for GM-level 0.4%. However, the EU-RL GMFF did not regard this deviation rendering the other data provided by the laboratory invalid.

4. Results

4.1 EU-RL GMFF experimental testing

4.1.1 Determination of the zygosity ratio in the positive control sample

The results of the tests to determine the zygosity ratio in the positive control sample are shown in Table 6.

Table 6. Summary of dPCR analysis conducted on the GHB119 and *AdhC* targets in the positive control sample.

Mean ratio (GHB119/*AdhC*)	1.01
Standard deviation	0.083
RSD_r (%)	8.2
Standard error of the mean	0.023
Upper 95% CI of the mean	1.06
Lower 95% CI of the mean	0.96

In conclusions, the 95% confidence interval (CI) spans around 1 and therefore the mean ratio is not significantly different from an expected ratio of 1, assuming a GM homozygous and a single-copy reference target, for an alpha = 0.05.

Hence, given the above experimental data on the positive control sample provided by the applicant, the GM% expressed in GM- DNA copy numbers in relation to target taxon DNA copy numbers calculated in terms of haploid genomes equals the GM% expressed in DNA mass fraction:

GM % in DNA copy number ratio = GM % in mass fraction

4.1.2 In-house verification of method performance against ENGL method acceptance criteria

Test samples with GM-levels 4.5%, 2.0%, 0.9% and 0.4% were tested in the EU-RL GMFF laboratories in two real-time PCR runs (run A and B), with two replicates for each GM-level on each plate (total of four replicates per GM-level). The test sample with GM-level 0.1% was tested in 15 replicates in one run (run C). Results are shown in Table 7 and 8.

Table 7. Standard curve parameters

	GHB119-system			*AdhC* reference system		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.25	103	1.00	-3.32	100	1.00
Run B	-3.27	102	1.00	-3.32	100	1.00
Run C	-3.27	102	0.99	-3.33	99	0.99

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98.
Table 7 documents that the slope of the standard curve and the R^2 coefficient were within the limits established by the ENGL.

Table 8. Outcome of the in-house verification, with regards to the quantification of the five test samples

GM-levels %	Measured GM %	Bias %	Precision (RSDr %)
0.1	0.09	-10	14
0.4	0.34	-16	7.0
0.9	0.76	-15	6.0
2.0	2.03	1.7	5.9
4.5	4.4	-2.0	7.6

According to the ENGL method acceptance criteria, the method trueness (measured as bias %) should be within ±25% of the accepted reference values over the dynamic range. The method's precision as RSDr % should be ≤25% over the dynamic range. Table 8 documents that trueness (estimated through bias %) and precision of quantification (estimated through relative standard deviation of repeatability, RSDr) were within the limits established by the ENGL.

4.2 Results of the international collaborative study

4.2.1 PCR efficiency and linearity

Standard curve slopes, from which the PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$, and R^2 values (expressing the linearity of the regression), reported by participating laboratories for the GHB119 and the *AdhC* assays are reported in Table 9.

Table 9. Values of slope, PCR efficiency and R^2 obtained during the validation study

		GHB119			***AdhC***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.30	101	1.00	-3.39	97	1.00
	B	-3.34	99	1.00	-3.38	98	1.00
2	A	-3.48	94	1.00	-3.58	90	1.00
	B	-3.37	98	1.00	-3.55	91	1.00
3	A	-3.50	93	1.00	-3.56	91	1.00
	B	-3.52	92	1.00	-3.56	91	1.00
4	A	-3.36	99	0.97	-3.23	104	0.99
	B	-3.25	103	0.99	-3.26	103	0.98
5	A	-3.52	92	1.00	-3.47	94	1.00
	B	-3.57	91	1.00	-3.50	93	1.00
6	A	-3.51	93	1.00	-3.49	93	0.99
	B	-3.47	94	0.99	-3.46	94	1.00
7	A	-3.47	94	1.00	-3.38	98	1.00
	B	-3.35	99	1.00	-3.38	97	1.00
8	A	-3.38	98	1.00	-2.94	119	0.99
	B	-3.42	96	1.00	-2.96	118	0.99
9	A	-3.48	94	1.00	-3.45	95	1.00
	B	-3.54	92	1.00	-3.28	102	0.99
10	A	-3.39	97	1.00	-3.44	95	1.00
	B	-3.36	99	1.00	-3.39	97	1.00
11	A	-3.44	95	1.00	-3.51	93	1.00
	B	-3.50	93	1.00	-3.40	97	1.00
12	A	-3.53	92	1.00	-3.60	90	1.00
	B	-3.46	95	1.00	-3.59	90	1.00
	Mean	**-3.44**	**96**	**1.00**	**-3.41**	**97**	**1.00**

Table 9 indicates that the efficiency of amplification for the GHB119 system ranges from 91 to 103% and the linearity from 0.97 to 1.00, the amplification efficiency for the cotton-specific reference system ranges from 90% to 119% and the linearity from 0.98 to 1.00. The mean PCR efficiency was 96% for the GHB119 assay and 97% for the *AdhC* assay, with both values within the ENGL acceptance criteria. The average R^2 of the methods was 1.00 both for GHB119 and *AdhC* assays.

The results confirm the appropriate performance characteristics of the method tested in terms of efficiency and linearity.

4.2.2 GMO quantification

Table 10 reports the values of the four replicates for each GM level as provided by all laboratories.

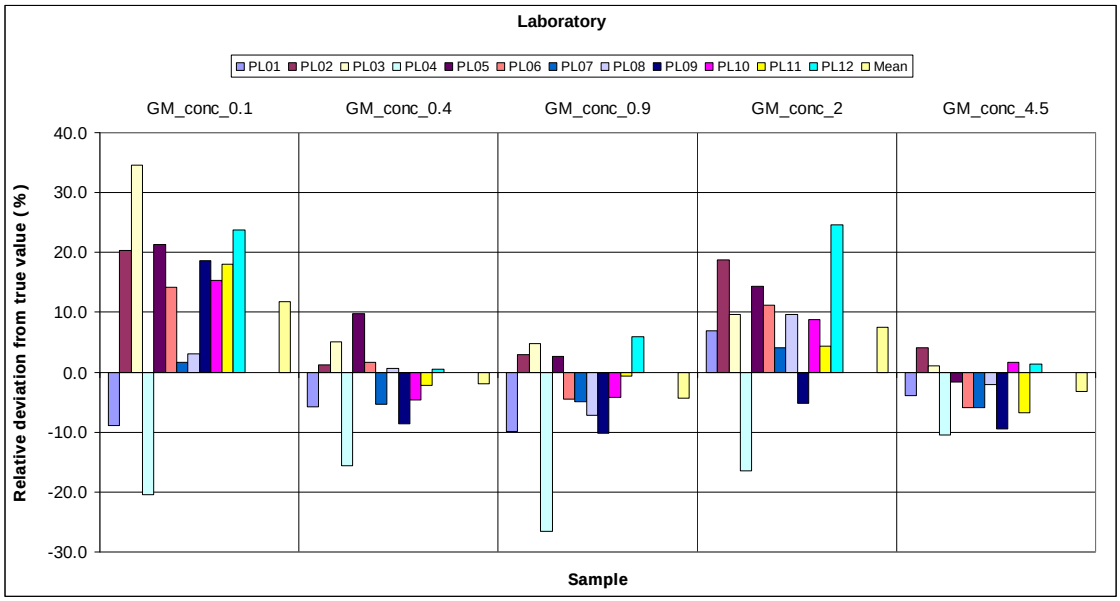
Table 10. GM% values determined by laboratories for test samples

	GMO content GMO% = GMO copy number/cotton genome copy number x 100 and GM DNA mass/cotton DNA mass x 100																			
LAB	0.1				0.4				0.9				2.0				4.5			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.09	0.08	0.09	0.10	0.47	0.29	0.36	0.38	0.76	0.79	0.84	0.85	2.19	2.15	2.11	2.10	4.36	3.92	4.36	4.65
2	0.12	0.14	0.13	0.10	0.38	0.45	0.40	0.39	0.85	0.95	1.03	0.87	2.27	2.51	2.44	2.29	4.32	5.02	4.49	4.91
3	0.12	0.14	0.15	0.12	0.44	0.42	0.41	0.41	0.97	1.01	0.93	0.87	2.20	2.37	2.05	2.15	4.25	4.69	4.71	4.54
4	0.07	0.05	0.09	0.10	0.41	0.23	0.36	0.35	0.49	0.57	0.83	0.76	0.99	1.58	2.16	1.95	4.35	4.23	4.03	3.52
5	0.10	0.13	0.14	0.11	0.47	0.44	0.42	0.42	0.89	0.99	0.88	0.93	2.36	2.43	2.15	2.20	4.42	4.66	4.31	4.32
6	0.12	0.13	0.09	0.11	0.42	0.36	0.38	0.46	0.81	0.93	0.85	0.85	2.06	2.29	2.17	2.36	4.13	3.94	4.28	4.57
7	0.10	0.09	0.09	0.13	0.36	0.34	0.41	0.40	0.80	0.80	0.87	0.94	2.02	2.13	2.14	2.03	4.27	4.15	4.38	4.12
8	0.10	0.10	0.10	0.11	0.38	0.41	0.42	0.40	0.81	0.94	0.84	0.76	2.17	2.16	2.28	2.16	4.53	4.54	4.49	4.07
9	0.11	0.13	0.11	0.12	0.32	0.38	0.38	0.39	0.83	0.75	0.85	0.80	1.65	1.71	2.07	2.15	3.84	3.76	4.28	4.43
10	0.12	0.12	0.10	0.12	0.37	0.36	0.38	0.41	0.84	0.86	0.84	0.92	2.22	2.28	2.19	2.02	4.69	4.41	4.40	4.78
11	0.11	0.11	0.12	0.14	0.39	0.39	0.38	0.40	0.94	0.93	0.87	0.85	2.09	2.11	2.11	2.04	4.18	4.16	4.14	4.30
12	0.13	0.11	0.13	0.13	0.39	0.36	0.49	0.36	0.92	0.85	0.90	1.15	2.93	2.35	2.32	2.38	4.59	4.49	4.66	4.51

Data of all the replicates were retained for the statistical analysis and for tests of outliers (Cochrane and Grubbs) whose results are reported in Table 11.

In Figure 1 the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the deviation of the GM level measured by the respective laboratory in % of the true GM level; the light yellow bar on the right represents the mean relative deviation over all 12 participating laboratories for each true GM level.

Figure 1. Relative deviation (%) from the true value of GHB119 for all laboratories



Overall, most mean relative deviations from the true values were within the ENGL acceptance criterion of maximum 25%. Indeed, at GM-level 0.1% eleven laboratories were within the limits, 12 laboratories at the 0.4%, 11 at the 0.9% GM-level, 12 at the 2.0% and 12 at the 4.5%. At GM-level 0.1%, a trend for overestimation of the GM-target can be noticed. Overall relative deviations from the true value were within the ± 25%, except one laboratory overestimating the 0.1% GM-content by about 37% and one laboratory underestimating the 0.9% GM-level by about 27%.

All the data were retained for the statistical analysis reported in Table 11.

The mean bias generated by all laboratories is +12% at the 0.1% GM-level and within ±10% at all the other GM-levels, therefore the method's bias is well within the accepted limits at all GM levels.

5. Method performance requirements

Among the performance requirements established by the ENGL and adopted by the EU-RL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), repeatability and reproducibility are to be assessed through an international collaborative trial. Table 11 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the collaborative trial.

According to the ENGL method performance requirements, the relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 35% at the target

concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 11, the method satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R is 18% at the 0.1% GM level, thus within the acceptance criterion.

Table 11. GHB119, summary of validation results expressed as GM- DNA copy numbers in relation to target taxon specific DNA copy numbers

	Test Sample Expected GMO %				
	0.1	**0.4**	**0.9**	**2.0**	**4.5**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	0	1	0
Reason for exclusion	-	-	-	C	-
Mean value	**0.11**	**0.39**	**0.86**	**2.2**	**4.4**
Relative repeatability standard deviation, RSD_r (%)	13	11	9.2	6.7	5.6
Repeatability standard deviation	0.02	0.0	0.08	0.15	0.24
Relative reproducibility standard deviation, RSD_R (%)	18	12	12	9.2	6.8
Reproducibility standard deviation	0.02	0.05	0.11	0.20	0.3
Bias (absolute value)	0.01	- 0.01	-0.04	0.2	- 0.14
Bias (%)	12	- 1.9	- 4.3	9.7	-3.2

C= Cochran's test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2 (3).
Bias is estimated according to ISO 5725 data analysis protocol.

Table 11 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for a collaborative study, the EU-RL GMFF requires that the RSD_r value is below 25%, as indicated by the ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). As it can be observed from the values reported, the method showed a repeatability standard deviation below 25% at all GM levels, with the highest value of RSD_r (%) of 13% at 0.1% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. In this case, the method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 12% at the 0.1% GM level.

6. Compliance of the method of detection of event GHB119 with the requirements of Regulation (EU) No 619/2011

To verify the compliance of the method under validation with the requirements of Regulation (EU) No 619/2011, the following steps were carried out and their outcome is summarised in Table 12:

- at step 2 of the validation process (scientific assessment of the dossier), the EU-RL GMFF acknowledged the applicants data indicating that the RSDr at the level of 0.08% in terms of GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers was about 14% on 18 replicates (Table 2), hence below 25%. The EU-RL GMFF accepted therefore the applicant's data on method performance.

- at step 3 of the validation process (in-house testing of the method), the EU-RL GMFF determined the RSDr at the level of 0.1% expressed as mass fraction of GM-material on the basis of fifteen replicates carried out under repeatability conditions. The RSDr was 14% (Table 8), hence of the same level as derived from the applicant data and below 25%.

- further to the conclusion of step 4 of the validation process (ring trial), the EU-RL GMFF analysed the data generated by the 12 participating laboratories for determining the method performance parameters. It found that the RSDr of the method at the level of 0.1% related to mass fraction of GM-material was 13 %, therefore below the limit of 25% established by the ENGL.

Table 12. Precision of the method for quantitative detection of GHB119

Source	RSDr %	GM %
Applicant' method optimisation*	14 %	0.08 %
EU-RL GMFF tests	14 %	0.1 %
Collaborative study	13 %	0.1%

* GM- DNA copy numbers in relation to target taxon specific DNA copy numbers

Based on the results of in-house verification and of the collaborative study, it is concluded that the method RSDr is equal or less than 25% at the level of 0.1% related to mass fraction of GM-material, hence the method for quantitative detection of event GHB119 cotton meets the requirement laid down in Regulation (EU) No 619/2011.

7. Conclusion

The method provided by the applicant and described in detail under 3.2 (protocol is also available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) has been validated in accordance to the EU-RL GMFF validation scheme; respecting all requirements of the relevant EU legislation and international standards for method validation.

The dossier was found complete (step 1) and the scientific dossier analysis (step 2) concluded that the method appeared to meet the ENGL minimum performance criteria for entering into validation.
The subsequent in-house verification of the method (step 3) by the EU-RL GMFF confirmed this conclusion.
The international collaborative study (step 4) yielded data that also indicated that the method meets all acceptance criteria and method performance requirements recommended by the ENGL (as detailed at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) for a valid quantitative PCR method for detection and quantification of GM-events.

In conclusion, the validation study confirmed that the method is applicable to the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004 and (EU) No 619/2011. The EU-RL further concludes that the method, if carried out in accordance with the validated method protocol (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm), is applicable to appropriately extracted cotton DNA.

8. References

1. Arumuganathan K. and Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9, 208-218.
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3. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.