

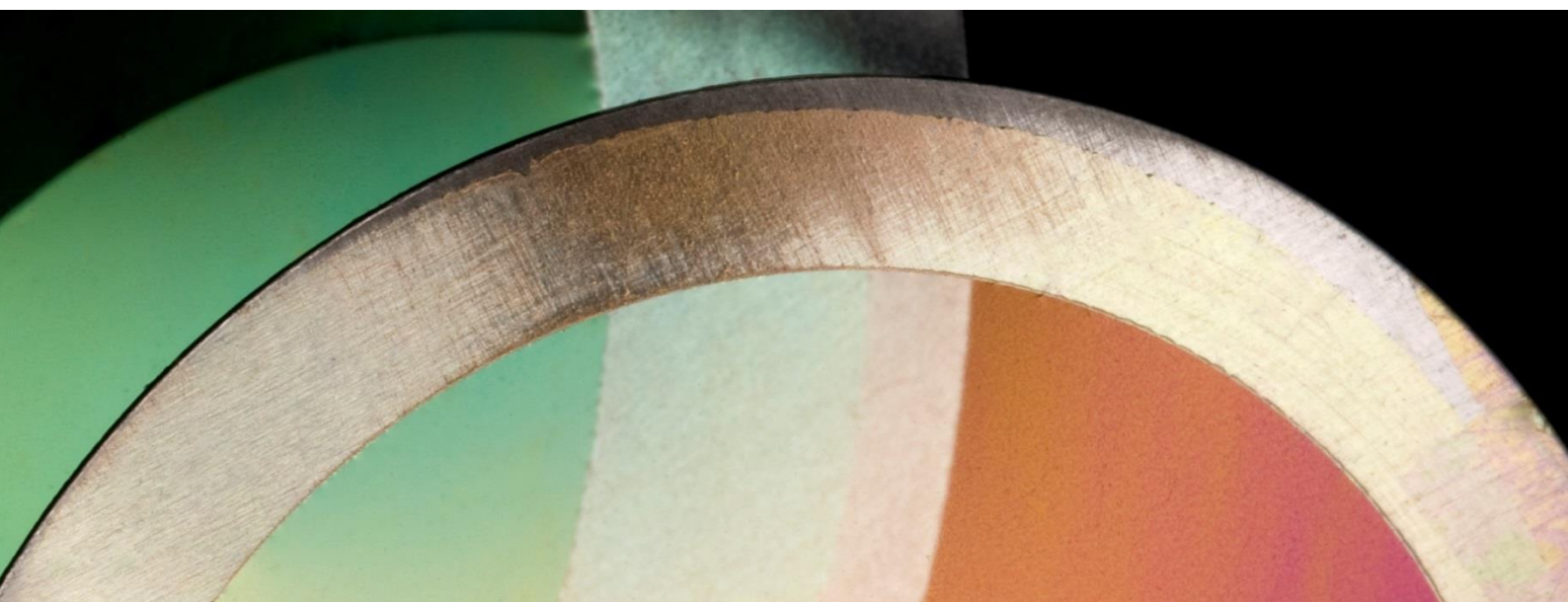
JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Event-specific Method for the Quantification of Cotton GHB811 Using Real-time PCR

Validation Report

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European Union Reference Laboratory for
Genetically Modified Food and Feed

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EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Health, Consumers & Reference Materials
Food and Feed Compliance



Event-specific Method for the Quantification of Cotton GHB811 Using Real-time PCR

Validation Report

25 June 2020

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate¹, the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), validated an event-specific real-time polymerase chain reaction (qPCR) method for detecting and quantifying cotton event GHB811 (unique identifier BCS-GHB811-4). The validation study was conducted according to the EURL GMFF validation procedure [<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>] and the relevant internationally accepted guidelines²⁻⁶.

In accordance with current EU legislation⁷, BASF Agricultural Solutions Seed US LLC provided the detection method and the positive and negative control samples (genomic DNA from leaves of GHB811 cotton as positive control DNA, and genomic DNA from leaves of conventional cotton as negative control DNA). The EURL GMFF verified the method performance data provided by the applicant, where necessary experimentally, prepared the validation samples (calibration samples and blind samples at different GM percentage (copies GM/total cotton haploid genome copies), organised an international collaborative study and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL, in line with the provisions of Annex III-3.C.2 to Regulation (EU) No 503/2013⁷, and it fulfils the analytical requirements of Regulation (EU) No 619/2011⁸. This validation 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

In line with Regulation (EC) No 1829/2003¹, BASF Agricultural Solutions Seed US LLC provided the EURL GMFF with an event-specific method for detection and quantification of cotton event GHB811 (unique identifier BCS-GHB811-4) together with genomic DNA as positive and negative control samples.

The dossier was found complete (step 1 of the EURL GMFF validation procedure) and the scientific dossier assessment (step 2) concluded that the reported method performance characteristics, assessed against the ENGL method acceptance criteria⁹, allowed moving the method forward to step 3 of the procedure (experimental testing), where the EURL GMFF verified the purity of the control samples provided and conducted an in-house testing of samples and method.

The positive and negative control DNA, submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Regulation (EC) No 1829/2003, were found of good quality.

Step 3 was completed with the conclusion that the method could be submitted to the collaborative study (step 4). This study confirmed that the method is well suited for quantifying genomic DNA of GM cotton GHB811, appropriately extracted from food or feed, down to a GM content level of 0.1 % m/m.

The preparation of the report (step 5) was aligned with the timeline communicated by EFSA for its risk assessment.

2. Dossier reception and acceptance (Step 1)

BASF Agricultural Solutions Seed US LLC submitted the detection methods, data demonstrating their adequate performance when applied to genomic DNA extracted from the GM event, the corresponding control samples of DNA extracted from the GM event cotton GHB811 and from non-GM cotton, and a sample of food and feed consisting in a mixture of 1 % of homozygous GHB811 grains.

The dossier was found to be complete and thus was moved to step 2.

3. Scientific assessment and bioinformatics analysis (Step 2)

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL and with regard to their documentation and reliability.

Two requests of complementary information regarding validity and limitation of the DNA extraction method module, limit of quantification, instruments used were addressed to the applicant. The EURL GMFF verified the data and the complementary information received and accepted the received clarifications as satisfactory.

3.1. Specificity assessment conducted by the applicant

The specificity of the event-specific assay was verified by the applicant and confirmed by the EURL GMFF by means of bioinformatics analysis, based on the sequence data provided by the applicant.

The specificity of the event-specific assay was assessed by the applicant in duplicate real-time PCR reactions, according to the method described in Annex 1, using "approximately 100 copies of target DNA and 2500 copies of non-target DNA from:

- GHB811 cotton event as positive control sample for the GHB811 cotton system reaction;
- Non-genetically modified *Gossypium hirsutum* as positive control sample for the reference system reaction;
- Relevant events from the GHB811 cotton event production pipe-line, authorized BASF GM events that are currently being commercialized, that have been commercialized or being phased out i.e. rice LLRICE62; corn T25; oilseed rape (OSR) MS1, MS8, MS11, RF1, RF2, RF3, Topas19/2, T45, OXY-235; soybean A2704-12, A5547-127 and cotton LLCOTTON25, T304-40, GHB614, GHB119, GHB814, GHB507;
- GM events not originating from BASF for which official Reference Materials are available (DNA and/or seed powders), i.e. corn BT176, BT11, MON810, GA21, NK603, MON863, TC1507, event 3272, MIR604, MIR162, DAS-59122-7, event 98140, MON88017, MON89034, MON87460, MON87427, DAS-40278-9; oilseed rape (OSR) RT73, MON88302, event 73496; cotton MON1445, MON531, MON 15985, MON88913, 281-24-236x3006-210-23, COT102 and soybean GTS 40-3-2, MON89788, MON87701, MON87708, DP-356043-5, DP-305423-1, CV127-9, MON87769, MON87705, DAS-68416-4, DAS-81419-2, DAS-44406-6, FG72;
- Non-genetically modified soybean, rice, oilseeds and corn."

According to the method developer, the GHB811 assay did not react with any sample except the positive control.

In addition, the applicant performed an in-silico specificity analysis by using the amplicon sequence as a query for BLASTN 2.2.28+ algorithm search against public sequence of National Center for Biotechnology Information (NCBI) and Patent_Genbank databases. No sequence showed the alignment of both forward and reverse primers or with the full length of the probe, besides relevant similarity hits to GHB811 patents.

For identification and quantification of *G. hirsutum*, the reference system reaction targeting the *G. hirsutum* endogenous D-subgenome alcohol dehydrogenase C gene (Adh C) gene is used.

The specificity of the *G. hirsutum* reference system reaction was determined in a Real-Time PCR experiment using genomic DNA samples of different crops and different *G. hirsutum* varieties.

200 ng genomic DNA extracted from conventional oilseeds, rice, cotton, soybean and corn was applied in duplicate.

Apart from the *G. hirsutum* positive control reaction, the method did not react with any other crop DNA sample.

3.2. Specificity assessment conducted by the EURL GMFF

The detection method spans the 5' insert-to-plant junction in cotton GHB811. The forward primer PRIM0638 binds to the cotton genomic border adjacent to the insertion 5' end of the insert. The reverse primer PRIM1870 binding site was found in the T-DNA region. The TM2207 probe binds to the "5' flanking genomic sequence".

The amplicon size is expected to be 144 bp, consistent to what reported by the applicant. The sequence of the amplicon was analysed by BLAST (NCBI) against local copies of the "nt" and "patents" databases, and no significant similarity was found with any other published sequence. In addition, the primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System (CCSIS) of the JRC, as well as the whole genomes of more than 80 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays*) using the e-PCR prediction tool (NCBI), and no potential amplicon was identified.

A perfect match of the amplicon and of the primers was identified with the sequence deposited for the GHB811.

3.3. Verification of the ENGL acceptance parameters

The applicant prepared the calibration curve from a DNA solution (S1) of 10 % cotton event GHB811 genomic DNA (expressed as copy number ratio) which was serially diluted to obtain solutions S2, S3, S4 and S5. The parameters (slope, R^2 coefficient) of six runs of the calibration curve are reported as provided by the applicant (Table 1).

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

GHB811		<i>Adh C</i>	
Slope	R^2	Slope	R^2
-3.1	0.9949	-3.2	0.9972
-3.2	0.9918	-3.2	0.9958
-3.1	0.9953	-3.2	0.9942
-3.5	0.9989	-3.3	0.9990
-3.5	0.9986	-3.3	0.9995
-3.5	0.9974	-3.3	0.9995

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 1 indicates that the slope and R^2 coefficient of the standard curves for the GM-system (GHB811) and the cotton-specific D-subgenome alcohol dehydrogenase C gene (*Adh C*) system, as established by the applicant, were within the ENGL acceptance criteria.

Precision and trueness of the method were established by the applicant and 18 values for each of four GM levels (expressed as haploid genome copy fraction of GM-material) were provided. Table 2

reports precision and trueness values for the four GM-levels as provided by the applicant. Both parameters were within the ENGL acceptance criteria (trueness ± 25 %, $RSD_r \leq 25$ % across the entire dynamic range).

Table 2. Mean %, precision and trueness values provided by the applicant.

Expected GM %	Test results			
	0.06%	0.08%	0.9%	4.5%
Measured mean GM %	0.054	0.065	0.80	5.21
Precision (RSD_r %)	21.06	18.11	14.29	11.05
Trueness (bias %)	-9.85	-19.03	-11.44	15.74

Numbers are not rounded but are presented as reported by the applicant

In accordance to the data in Table 2, the limit of quantification (LOQ %) was found to be at least 0.06 %. Therefore, the LOQ is in line with the ENGL acceptance criteria (below or equal 0.09 % or 50 copies).

The limit of detection (LOD) of the real time PCR methods was assessed by the applicant in 60 PCR replicates at 10, 5 and 1 copies per reaction of the GHB811 cotton and of cotton. The LOD was found to be below 10 haploid genome copies for GHB811 and for the reference system. The applicant tested the LOD of the combined PCR modules in six-fold and three real time PCR runs (18 replicates) and it was found to be at least 0.023 % of GHB811 in 200 ng of cotton DNA per reaction. The LOD is in line with the ENGL acceptance criteria (below 0.045 % or 25 copies with a level of confidence of 95%).

The robustness of the method was assessed in 8 combinations of the following variations: exact/+5%/-5% enzyme mix concentration, exact/-10% primer concentration, exact/+10%/-10% probe concentration, exact/+1 μ L/-1 μ L reaction volume, +/-1 °C in annealing temperature. The RSD_r and trueness calculated for a combination of changes on sample at LOQ level (0.08%) did not exceed 30 %, thus meeting the ENGL requirement for robustness.

Precision and trueness of the method were tested in a transferability study: 18 values for each of the five GM levels (expressed as haploid genome copy fraction of GM-material) were provided. Table 2B reports precision and trueness values for the four GM-levels as provided by laboratory different from the method developer. Both parameters were within the ENGL acceptance criteria (trueness ≤ 25 %, $RSD_r \leq 25$ % across the entire dynamic range).

Table 2B. Mean %, precision and trueness values obtained in the transferability study.

Expected GM %	Test results				
	0.08%	0.4%	0.9%	2.0%	4.5%
Measured mean GM %	0.07	0.34	0.79	1.98	4.91
Precision (RSD _r %)	13.04	10.48	10.25	8.24	10.33
Trueness (bias %)	-15.48	-16.03	-11.95	-1.18	9.04

3.4. DNA extraction

Genomic DNA was isolated from ground cotton seeds, using a DNA extraction procedure "CTAB/Genomic-tip 20" method previously submitted by Dow AgroSciences and validated by the EURL GMFF in the context of submission of 3006-210-23/281-24-236 Cotton (CRLVL-14/05XP) and LLCOTTON25 (CRLVL13/04XP). The protocol for DNA extraction and a report on testing are published at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

According to the experimental data submitted by the applicant, the validated method provided DNA of suitable quantity and quality for PCR based applications when applied to ground seeds from the cotton event GHB811.

Therefore, the validated method CRLVL-14/05XP has been successfully applied in the context of the application for authorisation of GHB811 cotton.

Consequently, the EURL GMFF did not verify the DNA extraction method proposed by the applicant.

Annex III to Reg. (EU) No 503/2013⁷ 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 "*the submitted method for DNA extraction is suitable for the isolation of genomic DNA from a wide variety of matrices (e.g. cotton seed, grain and other cotton tissues). The suitability of isolated DNA as an analyte for PCR based detection of GMOs 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 difficult processed matrices may require adaptation. In fact, food processes can influence the quality and intactness of the DNA contained in the final processed products¹⁰⁻¹³. 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^{14,15}. Therefore, DNA extraction from certain of these processed matrices may require additional rounds of purification in order to achieve the quality standards needed for quantitative real-time PCR^{14,15}.*

In conclusion, the DNA extraction module has been successfully validated on cotton seeds and grains, which is the most representative matrix of food and feed cotton samples in the context of the current application for authorisation".

In agreement with the ENGL position, endorsing the modularity principle (see also Annex III to Reg. (EU) No 503/2013)⁷, and given the similarity in the matrix, the EURL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for cotton event GHB811.

Whenever DNA is extracted from more complex and processed matrices, a thorough control of the quality of the DNA is recommended in order to ensure that it has the required quality for subsequent PCR analysis.

4. Materials and methods

4.1. Samples

The following positive and negative control samples were provided by the applicant to the EURL GMFF:

- genomic DNA extracted by the applicant from homozygous cotton leaves harbouring the GHB811 event, and
- genomic DNA extracted by the applicant from conventional cotton leaves genetically similar to those harbouring the GHB811 event.

4.2. Method for the PCR analysis

The PCR method provided by the applicant is an event-specific, quantitative, real-time TaqMan[®] PCR procedure for the determination of the relative content of GM event GHB811 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton specific assay targeting the endogenous gene *D-subgenome alcohol dehydrogenase C (Adh C)*, and the GM target assay for GHB811 are run in separate wells. The validated method is published by the EURL GMFF at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx> and can be found in Annex 1 to this report.

For the detection of GM event GHB811, a 144 bp fragment of the region spanning the 5' insert-to-plant junction in cotton GHB811 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and MGBNFQ (Minor Groove Binder Non-Fluorescent Quencher) as quencher dye at its 3' end.

For the relative quantification of GM event GHB811, a cotton taxon-specific system amplifies a 73 bp fragment of an cotton *D-subgenome alcohol dehydrogenase C (Adh C)* endogenous gene, using *Adh C* gene-specific primers and a *Adh C* gene-specific probe labelled with JOE[™] (5'-Dichloro-Dimethoxy-Fluorescein) as reporter dye at its 5' end and BHQ1 (Black Hole Quencher 1) as quencher dye at its 3' end.

Standard curves are generated for both the GHB811 and the *Adh C* systems by plotting the C_q 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 the relative quantification of event GHB811 DNA in a test sample, the GHB811 copy number is divided by the copy number of the cotton haploid genome and multiplied by 100 to obtain the percentage value ($GM\% = [GHB811 / \text{cotton haploid genome}] \times 100$).

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)¹⁶. The copy number values used in the quantification, the GMO contents of the calibration samples, and the total DNA quantity used in the PCR reactions are listed in Table 3.

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 with 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 3. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of cotton DNA in the reaction (ng)	300	100	20	5.0	1.0
Target taxon haploid genome copies	128755	42918	8584	2146	429
Target GHB811 copies	12876	4292	858	215	43

4.3. EURL GMFF experimental testing

4.3.1. Determination of the zygosity ratio in the positive control sample

Annex II of Regulation (EU) No 619/2011⁸ 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 EURL GMFF.” This requires knowledge of the zygosity of the event. In order to satisfy this requirement, the EURL GMFF assessed the zygosity (GM-target to reference target ratio) in the positive control sample submitted by the applicant.

The copy number of the GHB811 and of the *Adh C* targets in the positive control sample were determined by digital PCR (dPCR) performed on the BioMark HD System using the 37K digital arrays (Fluidigm).

Reaction mixes were prepared in order to test the zygosity in five replicates to a final volume of 9 μL and contained 1X TaqMan® Universal PCR Master Mix (Applied Biosystems, Cat. number 4318157), 1X GE sample loading reagent (Fluidigm PN 85000746), primers and probes at concentrations indicated in the corresponding validated method (PRIM0638 and PRIM1870 primers at 400 nM each, TM2207 probe at 200 nM; KVM157 and KVM158 at 200 nM each, TM1304 probe at 200 nM), and 1.8 μL of DNA at a concentration of 1.5 ng/ μL ; the DNA concentration was chosen in order to avoid panel saturation (optimal between 200<positive partitions<700).

Loading of the digital chip was performed according to the manufacturer's instructions by using the IFC controller (Fluidigm). Approximately 4 µL of the reaction mixes were loaded into each well and distributed into the 765 partitions constituting one panel. The experiment was repeated three times for a total number of eighteen data sets for the GM target and eighteen for the reference target. 'No template controls' were included. Amplification conditions were as reported in Annex 1 or in the Validated Method document at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>. Data analysis and copy number calculations were performed using the BioMark digital PCR Analysis software. The range of Cq retention was from 15 to 35.

Calculations of means and variances were 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 interlaboratory validated methods - Version 2'¹⁷.

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

The method performance characteristics were verified by quantifying on a copy number basis five blind test samples distributed over a range of GM levels (0.10 % - 4.5 %). The experiments were performed on an ABI 7500, an ABI 7900HT and a Roche LC480 real-time platforms under repeatability conditions and following the protocol provided by the applicant. Test samples with GM levels 0.50 %, 0.90 %, 2.0 % and 4.5 % 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 0.10 % was tested in 15 replicates in an additional run for each platform. Average values of the slope and of the R² coefficient of the standard curves and method trueness and precision over the dynamic range were evaluated against the ENGL method acceptance criteria. On Roche LC480 platform, the method was run at 45 cycles as described in the validated method published at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx> and in Annex 1 below, and analysed with the second derivative maximum method.

4.4. International collaborative study (step 4)

The international collaborative trial involved twelve randomly selected laboratories, all being "national reference laboratories, assisting the EURL GMFF for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 120/2014¹⁸ who had expressed their interest in participation. The study was carried out in accordance with the following internationally accepted guidelines:

- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995)²
- ISO 5725 "Accuracy (trueness and precision) of measurement methods and results", Part 1 and Part 2 (ISO, 1994); ISO 5725-1:1994/Cor 1 (ISO 1998) and ISO 5725-2:1994/Cor 1 (ISO, 2002)³⁻⁶

The objective of the international collaborative study was to verify in experienced laboratories the trueness and precision of the PCR analytical method provided by the applicant and verified in-house by the EURL GMFF.

4.4.1. List of participating laboratories

The twelve laboratories participating in GHB811 international collaborative study were randomly selected from 23 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories for strictly following the validation protocol that was provided to them. The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the validation of the detection method for cotton event GHB811

Laboratory	Country
Walloon Agricultural Research Centre - Department Valorization des productions	BE
National Center of Public Health and Analyses (NCPHA)	BG
Federal Institute for Risk Assessment	DE
Saxon State Company of Environmental and Agriculture	DE
Laboratory for the Detection of GMO in Food - Bad Langensalza	DE
Departamento de OGM/Técnicas Biomoleculares Laboratorio Arbitral Agroalimentario (LAA)	ES
Service commun des laboratoires du ministere de l'economie et des finances Etablissement de Strasbourg	FR
CREA-DC Sede di Tavazzano	IT
Wageningen Food Safety Research (WFSR)	NL
National Veterinary Research Institute, Department of Hygiene of Animal Feedingstuff	PL
National Food Agency	SE
National Institute of Biology	SI

4.4.2. Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: four laboratories used ABI 7500, three used ABI 7900HT, one used ABI QuantStudio 5, one used ABI QuantStudio 6flex, one used ABI 7300, one used Roche LC 480 and one used Bio-Rad CFX96.

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

4.4.3. Materials used in the international collaborative study

For the validation of the quantitative event-specific method, test samples were provided by the EURL GMFF to the participating laboratories.

The test samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant (see 4.2 for details) in accordance to Regulation (EC) No 1829/2003¹, Art 2.11^a.

The control samples were used by the EURL GMFF to prepare standards (of known GMO content) and test samples (of undisclosed GM content = blind samples) by mixing GHB811 cotton DNA and non-GM cotton DNA.

The calibration sample S1 was prepared by mixing the appropriate amount of GHB811 DNA with control non-GM cotton DNA to obtain a 10 % (in copy number ratio related to haploid genome copies) GM sample. Calibration samples S2-S5 were prepared by serial dilutions from the S1 sample.

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

- Five calibration samples with known concentrations of GM-event (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 40 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 5)

Table 5. GHB811 blinded samples GM % contents

GHB811 GM % GM copy number/cotton haploid genome copy number x 100
4.5
2.0
0.90
0.50
0.10

Reaction reagents:

- TaqMan[®] Universal PCR Master Mix (2x), one vial: 8 mL
- distilled sterile water, one vial: 4 mL

Primers and probes (1 tube each) as follows:

Adh C taxon-specific assay

- KVM157 (10 µM): 160 µL
- KVM158 (10 µM): 160 µL
- TM1304 (10 µM): 160 µL

^a Control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample).

GHB811 assay

- PRIM0638 (10 µM): 320 µL
- PRIM1870 (10 µM): 320 µL
- TM2207 (10 µM): 160 µL

4.4.4. Design of the collaborative study

Participating laboratories received a detailed validation protocol that included the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the GHB811 event-specific system and for the *Adh C* taxon-specific system. In total, two plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for the cotton event GHB811 and the *Adh C* assay in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per pre-determined plate layout.

The amplification reaction followed the cycling program specified in the protocol. Participants determined the GM % in the test samples according to the instructions and also reported the raw data to the EURL GMFF on an Excel sheet that was designed, validated and distributed by the EURL GMFF. All data are stored by the EURL GMFF on a dedicated and protected server.

The EURL GMFF analysed the data against the parameters and the limits set by the ENGL, i.e. trueness, precision, amplification efficiency and linearity.

4.4.5. Deviations reported from the protocol

Six laboratories reported no deviations from the validation protocol. Two laboratories reported a plate set up different from the one described in the validation protocol. Two laboratories reported the use of HEX or VIC as reporter for the reference system instead of JOE. One laboratory performed the denaturation time for 25 seconds instead of 15 seconds. One laboratory repeated one of the two runs due to a pipetting error.

5. Results

5.1. EURL GMFF experimental testing (Step 3)

5.1.1. Zygoty ratio in the positive control sample

The results of the digital PCR analysis conducted by the EURL GMFF on the GHB811 and *Adh C* targets to determine the zygoty ratio in the positive control samples are reported in Table 6.

Table 6. Zygoty ratio of the GHB811 and *AdhC* targets in the positive control sample.

Mean ratio (GHB811/ <i>Adh C</i>)	0.99
Standard deviation	0.12
RSDr (%)	11.61
Standard error of the mean	0.03
Upper 95 % CI of the mean	1.05
Lower 95 % CI of the mean	0.93

The mean ratio (GHB811/*Adh C*) was 0.99. The 95 % confidence interval (CI) spans around 1.0, the expected ratio for a cotton control sample, homozygous for the GM-locus and assuming single-copy endogenous gene target. Therefore, the measured mean ratio is not significantly different from the expected ratio, for an $\alpha = 0.05$.

Hence, the relative (%) GM content of the samples expressed in haploid genome copy numbers corresponds to the GM content expressed in mass fraction. For example, the 0.10 GM % in DNA copy number ratio corresponds to a 0.10 GM % in mass fraction.

Note: the zygoty ratio herein reported is valid for the positive control sample in the context of the present validation study. It is used to assess the method performance at 0.1% GM level, expressed as mass fraction of GM material, in relation to the provisions of Reg. (EU) No 619/2011.

When analytical results of official laboratories are primarily expressed as ratio of GM DNA copy numbers, they shall be translated into mass fraction results by means of the specific conversion factor published in the document "Conversion factors (CF) for certified references materials (CRM)" (<https://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>).

5.1.2. In-house verification of method performance against ENGL method acceptance criteria

Test samples with GM levels from 0.5 % to 4.5 % 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 sample at 0.10 % GM-level was tested for its precision in quantification in 15 replicates in separate runs.

Tests were conducted on ABI 7500, ABI 7900HT and Roche LC480.

The standard curve parameters and the results of efficiency, linearity, trueness and precision obtained in the three real-time PCR runs with the test samples are shown in Tables 7A, 7B, 8, 9 and 10.

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 7A and 7B document that the slopes of the standard curves and the R^2 coefficients were within the limits established by the ENGL. The EURL GMFF in-house results confirm the data provided by the applicant.

Table 7A. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, ABI 7900HT and Roche LC480 to quantify GM-levels in the range 0.5 % to 4.5 % in four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	GHB811			Adh C		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.45	95	1.00	-3.36	98	1.00
Run B	-3.52	93	1.00	-3.36	98	1.00
Run C	-3.41	97	1.00	-3.42	96	1.00
Run D	-3.49	93	1.00	-3.39	97	1.00
Run E	-3.52	92	1.00	-3.43	96	1.00
Run F	-3.48	94	1.00	-3.42	96	1.00

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

Runs A-B were carried out on ABI 7500; Runs C-D were carried out on ABI 7900HT; Runs E and F were carried out on Roche LC480.

Table 7B. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, ABI 7900HT, and Roche LC480 to quantify the GM-level 0.10 % in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	GHB811			Adh C		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run G	-3.54	92	1.00	-3.32	100	1.00
Run H	-3.48	94	1.00	-3.36	98	1.00
Run I	-3.44	95	1.00	-3.40	97	1.00

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

Run G was carried out on ABI 7500; Run H was carried out on ABI 7900HT; Run I was carried out on Roche LC480.

According to the ENGL method acceptance criteria the method trueness (measured as bias in % of the target GM level) should be within ± 25 % of the accepted reference value over the entire dynamic range and the precision, expressed as RSD_r % (relative standard deviation of repeatability), should be ≤ 25 %, also over the entire dynamic range.

Tables 8, 9 and 10 show that trueness and precision of quantification were within the limits established by the ENGL for the PCR machines used.

Table 8. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI 7500. GM % in copy/copy haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD _r %)
4.5	4.5	0.41	1.0
2.0	2.2	7.6	2.9
0.90	0.87	-3.0	1.6
0.50	0.51	2.2	4.4
0.10	0.10	2.0	19.4

Table 9. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI 7900HT. GM % in copy/copy haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD _r %)
4.5	4.8	6.2	2.3
2.0	2.1	6.7	4.7
0.90	0.97	7.6	4.5
0.50	0.51	1.8	6.3
0.10	0.10	1.7	12.3

Table 10. Values of trueness and precision as established by the EURL GMFF in its in-house verification using a Roche LC480. GM % in copy/copy haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD _r %)
4.5	4.8	7.0	3.4
2.0	1.9	-2.5	2.6
0.90	0.91	0.96	4.5
0.50	0.49	-3.0	5.5
0.10	0.10	-0.27	9.9

5.2. International collaborative study (Step 4)

5.2.1. PCR efficiency and linearity

The PCR efficiency (%) and R² values (expressing the linearity of the regression) for the standard curve, reported by participating laboratories are displayed in Table 11. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

$$\text{Efficiency (\%)} = \left(10^{\frac{-1}{\text{slope}}} - 1 \right) \times 100$$

Table 11 indicates that the efficiency of amplification for the GHB811 system ranges from 80 % to 111 % and the linearity from 0.92 to 1.00; the amplification efficiency for the cotton-specific system ranges from 93 % to 105 % and the linearity from 0.98 to 1.00. The mean PCR efficiency was 93 % for GHB811 assay and 97 % for the *Adh C*. The average R^2 of the methods was 0.99 and 1.00 for the GHB811 and *Adh C* assays, respectively. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

Table 11. Values of slope, PCR efficiency and R^2 obtained during the international collaborative trial. Slope and R^2 coefficient values were rounded to two digits.

Lab	Plate	GHB811			<i>Adh C</i>		
		Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.45	95	1.00	-3.34	99	1.00
	B	-3.36	98	1.00	-3.36	98	1.00
2	A	-3.53	92	1.00	-3.42	96	1.00
	B	-3.59	90	1.00	-3.35	99	1.00
3	A	-3.56	91	1.00	-3.38	97	1.00
	B	-3.50	93	1.00	-3.40	97	1.00
4	A	-3.46	95	0.92	-3.20	105	0.98
	B	-3.64	88	1.00	-3.38	98	0.99
5	A	-3.46	95	1.00	-3.44	95	1.00
	B	-3.41	96	1.00	-3.40	97	1.00
6	A	-3.47	94	1.00	-3.44	95	1.00
	B	-3.45	95	1.00	-3.38	98	1.00
7	A	-3.39	97	1.00	-3.36	98	1.00
	B	-3.58	90	1.00	-3.49	93	1.00
8	A	-3.60	90	1.00	-3.36	98	1.00
	B	-3.59	90	1.00	-3.33	100	1.00
9	A	-3.63	89	1.00	-3.43	96	1.00
	B	-3.61	89	1.00	-3.36	98	1.00
10	A	-3.93	80	1.00	-3.50	93	1.00
	B	-3.08	111	0.96	-3.43	96	1.00
11	A	-3.54	91	1.00	-3.35	99	1.00
	B	-3.47	94	1.00	-3.40	97	1.00
12	A	-3.57	91	1.00	-3.36	99	1.00
	B	-3.49	93	1.00	-3.40	97	1.00
Mean		-3.51	93	0.99	-3.39	97	1.00

These results confirm the appropriate performance characteristics of the methods tested in terms of efficiency and linearity.

5.2.2. GMO quantification

Table 12 reports the values of quantification for the four replicates of each GM level as reported by each of the twelve participating laboratories.

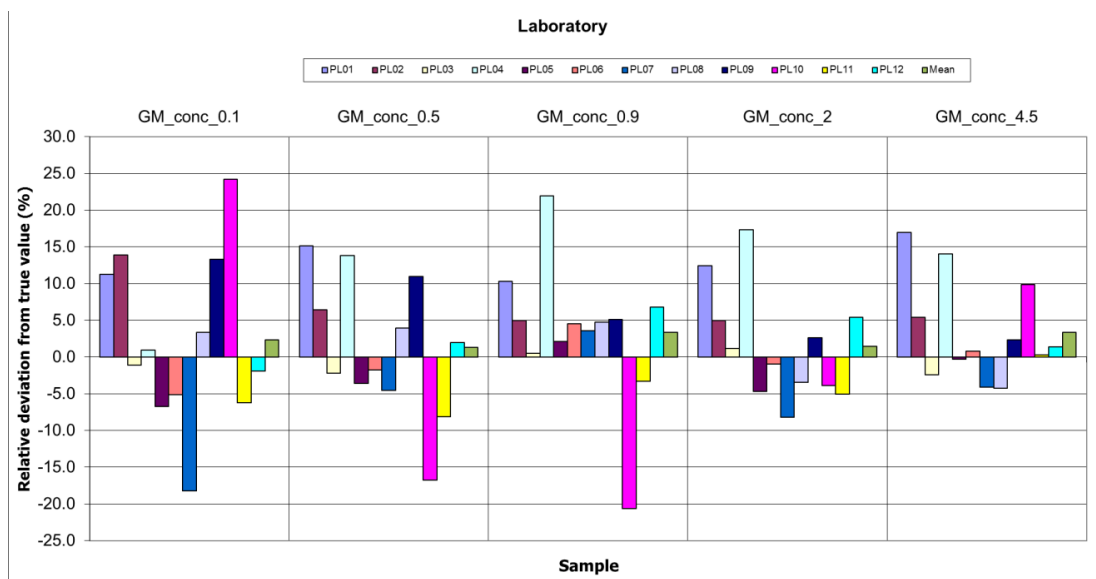
Table 12. GM % values determined by laboratories for test samples

LAB	GMO content (%) *																			
	0.10				0.50				0.90				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	0.12	0.13	0.09	0.10	0.57	0.59	0.55	0.59	0.92	0.96	1.0	1.1	2.2	2.3	2.1	2.4	5.2	5.2	5.1	5.5
2	0.11	0.12	0.11	0.12	0.51	0.52	0.55	0.55	0.99	0.92	0.96	0.91	2.1	2.1	2.0	2.2	4.8	4.7	4.8	4.6
3	0.10	0.11	0.09	0.10	0.51	0.45	0.48	0.52	0.94	0.91	0.88	0.89	2.0	1.9	2.0	2.2	4.5	4.7	4.4	4.0
4	0.09	0.10	0.11	0.11	0.52	0.55	0.60	0.61	1.0	1.1	1.1	1.2	2.2	2.5	2.2	2.5	5.2	5.5	4.9	5.0
5	0.10	0.09	0.08	0.10	0.49	0.46	0.54	0.43	0.96	0.91	0.91	0.90	2.0	1.8	2.0	1.9	4.6	4.6	4.4	4.3
6	0.09	0.09	0.10	0.11	0.48	0.50	0.52	0.46	0.89	0.99	0.95	0.93	2.0	2.0	2.0	2.0	4.5	4.5	4.5	4.6
7	0.09	0.07	0.10	0.08	0.39	0.39	0.63	0.49	0.83	0.82	1.0	1.1	1.7	1.7	1.9	2.1	4.7	4.5	4.0	4.1
8	0.10	0.11	0.10	0.11	0.53	0.48	0.56	0.51	0.91	0.90	0.99	0.98	1.9	1.9	1.9	2.0	4.5	4.6	4.2	4.0
9	0.13	0.12	0.11	0.10	0.55	0.58	0.55	0.53	0.93	0.91	0.97	0.98	2.1	2.1	2.0	2.0	4.9	4.7	4.4	4.4
10	0.12	0.15	0.15	0.08	0.49	0.51	0.24	0.42	1.2	0.99	0.37	0.32	1.8	1.9	1.5	2.4	4.5	4.4	5.6	5.3
11	0.11	0.09	0.09	0.09	0.45	0.45	0.52	0.42	0.91	0.85	0.88	0.84	1.8	1.8	2.0	2.0	4.5	4.5	4.5	4.5
12	0.10	0.09	0.10	0.10	0.52	0.47	0.47	0.59	0.92	1.0	0.98	0.93	2.0	1.9	2.1	2.4	3.9	4.4	5.1	4.9

* $\text{GMO \%} = (\text{GMO copy number} / \text{cotton haploid genome copy number}) \times 100$

A graphical representation of the data reported in Table 12 is provided in Figure 1 that shows the relative deviation from the true value for each GM level tested for the participating laboratory. The coloured bars represent the deviation of the GM level measured in % of the true GM level; the green bar on the right represents the mean relative deviation over all data before eliminating outliers for each GM level.

Figure 1. Relative deviation (%) from the true value of GM level*



PL = participating laboratory.

Overall, laboratories' mean relative deviations from the true values were within a maximum of ± 25 %.

5.2.3. Method performance requirements

Among the performance requirements established by ENGL and adopted by the EURL GMFF (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>), repeatability and reproducibility are to be assessed through an international collaborative trial. Table 13 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study (see Table 4 for a list of the participant laboratories).

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 13, the method satisfies this requirement at all GM levels tested. Indeed, the highest value of RSD_R % is 14 % at the 0.50 % GM level, thus within the acceptance criterion.

Table 13. Summary of validation results for the GHB811 method, expressed as GM copy numbers in relation to target-taxon haploid genome copy numbers.

	Expected GMO %				
	0.10	0.50	0.90	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	1	0	2	0	0
Reason for exclusion	C*	-	C*	-	-
Mean value	0.10	0.51	0.95	2.0	4.7
Relative repeatability standard deviation, RSD_r (%)	9.3	12	4.6	8.0	6.6
Repeatability standard deviation	0.01	0.06	0.04	0.2	0.3
Relative reproducibility standard deviation, RSD_R (%)	13	14	7.5	10	8.8
Reproducibility standard deviation	0.01	0.07	0.07	0.2	0.4
Bias** (absolute value)	0.00	0.01	0.05	0.03	0.2
Bias (%)	0.32	1.3	5.8	1.5	3.3

* C = Cochran's test; identification and removal of outliers through Cochran and Grubb tests, according to ISO 5725-2.

** Bias is estimated according to ISO 5725 data analysis protocol.

Table 13 also reports the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for collaborative study, the EURL GMFF and ENGL require that the RSD_r value indicated by the applicant and confirmed by the EURL GMFF through in-house experiments, is below 25 % (see ENGL document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing"⁹ <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>). As it can be observed from the values reported, the relative repeatability standard deviation is below 25 % at all GM levels, with the highest value of 12 % at the 0.50 % 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. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 5.8 % at the 0.90 % GM level.

5.3. Compliance of the method for detection and quantification of event GHB811 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 619/2011⁸, the following steps were carried out and their outcome is summarised in Table 14:

- at step 2 of the validation process (scientific assessment of the dossier), the EURL GMFF acknowledged that the RSD_r value at the 0.085 % level shown by the applicant's dossier (expressed as fraction of copies of GM-material) was 18.11 %, based on 18 replicates (Table 2) and 13.04 % at 0.08 % level in the transferability study (Table 2B), hence below the maximum value of 25 % required by the ENGL. The EURL GMFF therefore concluded that it could accept the applicant's data on method performance;
- at step 3 of the validation process (experimental testing of samples and methods), the EURL GMFF determined the RSD_r % value at the level of 0.1 % in mass fraction of GM-DNA (corresponding to 0.10 % expressed in terms of copy number ratio to haploid genome copy numbers). The experiments were carried out under repeatability conditions on fifteen replicates. The RSD_r resulted to range between 9.9 % and 19.4 % (Table 8, 9 and 10) depending on the qPCR platform applied, hence also below 25 %;
- the collaborative study (step 4 of the validation process) established that over the twelve participating laboratories at the level of 0.1 % related to mass fraction of GM-DNA the RSD_r of the method was 9.3 %, therefore also below 25 % and well in line with the previous data.

The outcome of the different steps is summarised Table 14.

Table 14. Precision of the event-specific method for quantitative detection of GHB811 at or around 0.1 % level related to mass fractions of GM material.

Source	RSD _r %	GM %
Applicant's method optimisation	18.11 %	0.08 %
	13.04 %	0.08 %
EURL GMFF tests	9.9 – 19.4 %	0.1 %
Collaborative study	9.3 %	0.1 %

Based on the results of the EURL GMFF in-house verification and of the international collaborative study, it is concluded that the method RSD_r % is lower than 25 % at the level of 0.1 % related to mass fraction of GM material, hence the method meets the requirement laid down in Regulation (EU) No 619/2011⁸.

6. Conclusion

The method provided by the applicant has been validated in accordance to the EURL GMFF validation process, respecting all requirements of the relevant EU legislation and international standards for method validation.

This validation study confirmed that the method is applicable to the control samples provided by the applicant (see paragraph 4.4.3), in accordance with the requirements of Annex I-3.C.2 to Commission Regulation (EU) No 503/2013⁷ and (EU) No 619/2011⁸ and meets all method performance requirements established by the ENGL and the EURL GMFF. The method is therefore valid to be used for regulatory purposes, including the quantification of low level presence of 0.1 % (m/m) of the GM event. It can be assumed that it is applicable to any appropriately extracted cotton genomic DNA.

In any case the user of the method is advised to verify the quality of the extracted genomic DNA in order to ensure that it is suitable for the subsequent PCR analysis. This is particularly relevant for more complex matrices of samples from food and feed products.

The validated method is described in detail as "Validated Method" at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx> and in Annex 1.

7. References

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Annex 1: Event-specific Method for the Quantification of soybean GHB811 by Real- time PCR

Validated Method

Method development:

BASF Agricultural Solutions Seed US LLC

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan[®] PCR (polymerase chain reaction) procedure for the determination of the relative content of cotton event GHB811 DNA to total cotton DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assays. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are also recommended to ensure suitability of the extracted DNA.

For the detection of GM event GHB811, a 144 bp fragment of the region spanning the 5' insert-to-plant junction in cotton GHB811 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and MGBNFQ (Minor Groove Binder Non-Fluorescent Quencher) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event GHB811, an cotton taxon-specific system amplifies a 73 bp fragment of a cotton D-subgenome alcohol dehydrogenase C (*Adh C*) endogenous gene (Accession number, GeneBank: AF036569), using *Adh C* gene-specific primers and a *Adh C* gene-specific probe labelled with JOE[™] as reporter dye at its 5' end and BHQ-1 (Black Hole Quencher[®] 1) as non-fluorescent quencher dye at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Cq" value. For quantification of the amount of GHB811 DNA in a test sample, Cq values for the GHB811 and the *Adh C* systems are determined for the sample. Standard curves are then used to estimate the relative amount of GHB811 DNA to total cotton DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from genetically modified and conventional cotton leaves, seeds and grain. Precision and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF). The study was undertaken with twelve participating laboratories in June-July 2019.

A detailed validation report can be found at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.023 % (related to copy fraction of GM material) in 200 ng of total suitable cotton DNA. The LOD_{abs} of the real time PCR modules were found to be below 10 haploid genome copies for GHB811 and for the reference system. The LOD was not assessed in the collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.06 % (related to copy fraction of GM material) in 200 ng of total suitable cotton DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1 % (mass fraction of GM-material).

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' insert-to-plant junction in cotton GHB811 and is therefore event-specific for the event GHB811. This was confirmed by the applicant's specificity studies and by in silico analysis performed by the applicants and the EURL GMFF.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. "forward flow direction" during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.
- PCR reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment should be sterilised prior to use and any residue of DNA should have been removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed regularly
- Laboratory benches and equipment should be cleaned periodically, with 10% sodium hypochlorite solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps, unless specified otherwise, should be carried out at room temperature.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of cotton event GHB811

3.2.1 General

The real-time PCR set-up for the taxon (*Adh C*) and the GMO (event GHB811) target sequences are carried out in separate vials. Multiplex qPCR (using differential fluorescent labels for the probes) has not been tested or validated by the EURL GMFF.

The method is developed for a total volume of 25 µL per reaction mixture for the GM (event GHB811) and the taxon (*Adh C*) assay with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curves have to be established on at least five samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % cotton GHB811 DNA in a total of 300 ng of cotton DNA (corresponding to 128755 cotton haploid genome copies with one haploid genome assumed to correspond to 2.33 pg of cotton genomic DNA) ⁽¹⁾. Standards S2 to S5 are to be prepared by serial dilutions according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of cotton DNA in reaction (ng)*	300	100	20	5	1
Cotton haploid genome copies	128755	42918	8584	2146	429
GHB811 copies	12876	4292	858	215	43

* Total nanograms are rounded to the integral value

A calibration curve is to be produced by plotting the C_q values against the logarithm of the target copy number for the calibration points. This can be done by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the software.

The copy number measured for each unknown sample DNA is obtained by interpolation from the standard curves.

3.2.3 Real-time PCR set-up

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.
2. In two tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mixes for the GHB811 cotton specific system (Table 2) and the *Adh C* reference gene system (Table 3). Please note that additional volume is included in the total to cover pipetting variability due to the viscosity of the solution.

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the GHB811 assay.

Component	Final concentration	µL/reaction
TaqMan [®] Universal PCR Master Mix (2x)	1x	12.5
PRIM0638 (10 µM)	400 nM	1.00
PRIM1870 (10 µM)	400 nM	1.00
TM2207* (10 µM)	200 nM	0.50
Nuclease free water	-	5.0
DNA	-	5.0
Total reaction volume:		25 µL

*TaqMan[®] probe labelled with 6-FAM at its 5'-end and MGBNFQ at its 3'-end

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the cotton *Adh C* assay.

Component	Final concentration	µL/reaction
TaqMan [®] Universal PCR Master Mix (2x)	1x	12.5
KVM157 (10 µM)	200 nM	0.50
KVM158 (10 µM)	200 nM	0.50
TM1304* (10 µM)	200 nM	0.50
Nuclease free water	-	6
DNA	-	5
Total reaction volume:		25 µL

*TaqMan[®] probe is labelled with JOE at its 5'-end and BHQ1 at its 3'-end

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for the cotton GHB811 and one for the *Adh C* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).

5. Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (70 μ L for the GHB811 cotton system and 70 μ L for the *Adh C* system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (17.5 μ L DNA). The volume for the additional 0.5 repetition will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
6. Spin down the tubes. Aliquot 25 μ L for GHB811 system and for the *Adh C* reference system in each well.
7. Place an optical cover on the reaction plate and briefly centrifuge the plate.
8. Place the reaction plate in the real-time PCR apparatus (possibly apply a compression pad, depending on the model), according to the manufacturer's instructions and your Standard Operating Procedures and start the run.
9. Select FAM as reporter dye for the GHB811 and JOE for the *Adh C* reference system. Define MGBNFQ or non-fluorescent as quencher dye for GHB811 specific system and BHQ1 or non-fluorescent for *Adh C* reference system. Select ROX as the passive reference dye. Enter the correct reaction volume (25 μ L).
10. Run the PCR with the cycling program described in Table 4. Users who plan to use the second derivative maximum analysis method (an option e.g. on Roche LC480 instruments) are advised to program 45 cycles instead of 40, in order to be able to quantify down to Cq 40.

Table 4. Cycling program for GHB811/*Adh C* assays.

Step	Stage	T (°C)	Time (s)	Acquisition	Cycles	
1	UNG*	50	120	No	1	
2	Initial denaturation	95	600	No	1	
3	Amplification	Denaturation	95	15	No	40**
		Annealing & Extension	60	60	Yes	

*UNG: Uracil-N-glycosylase

** see comment above for users of second derivative maximum analysis method

3.3 Data analysis

After the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold following the automatic or the manual mode. In the manual mode display the amplification curves of the event specific assay in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the "update" button to ensure changes affect C_q values (only needed for some analysis software). Switch to the linear view mode by clicking on the Y axis of the amplification plot and check that the threshold previously set falls within the exponential phase of the curves.
- b) Set the baseline following the automatic or the manual mode. In the manual mode: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest C_q = 25, set the baseline crossing at C_q = 25 – 3 = 22).
- c) Save the settings.
- d) Repeat the procedure described in a), b) and c) on the amplification plots of the taxon specific system.
- e) Save the settings and export all the data for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the C_q values for each reaction.

The standard curves are generated both for the *Adh C* and the GHB811 specific assays by plotting the C_q values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the DNA copy number in the unknown samples.

To obtain the percentage value of event GHB811 DNA in the unknown sample, the GHB811 copy number is divided by the copy number of the cotton endogenous gene *Adh C* and multiplied by 100 (GM% = GHB811/*Adh C* x 100).

4. Equipment and Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition) and appropriate analysis software
- 96-well reaction plates
- Optical caps/adhesion covers

- Microcentrifuge
- Micropipettes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex
- Racks for reaction tubes, also cooled
- 0.5, 1.5 mL and 5 or 15 mL DNase free reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix. Applied Biosystems Part No 4304437.

4.3 Primers and Probes

Table 5. Primers and probes for the GHB811 and *Adh C* methods

	GHB811	DNA Sequence (5' to 3')	Length (nt)
GHB811			
Forward primer	PRIM0638	5'-CgA ATA gTT CCA TCA ATT TTA TCA TTT ATg-3'	30
Reverse primer	PRIM1870	5'-CgC TTT AAC gTC CCT CAg ATT T-3'	22
Probe	TM2207	FAM-5'-AAG CCT TgA AAC AgA ACA-3'-MGBNFQ	18
<i>Adh C</i>			
Forward primer	KVM157	5'-CAC ATg ACT Tag CCC ATC TTT gC-3'	23
Reverse primer	KVM158	5'-CCC ACC CTT TTT Tgg TTT AgC-3'	21
Probe	TM1304	JOE-5'- TgC Agg TTT Tgg TgC CAC TgT gAA Tg-3'-BHQ1	26

FAM: 6-carboxyfluorescein; MGBNFQ: minor groove binder non fluorescent quencher; JOE: 4,5-dichloro-dimethoxy-fluorescein; BHQ1: black hole quencher.

5. References

1. Arumuganathan, K. & Earle, E. D. Nuclear DNA content of some important plant species. Plant Mol. Biol. Report. 9 (1991).

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