

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Event-specific Method for the Quantification of Cotton COT102 Using Real-time PCR Validation Report

Savini C., Sacco M. G.,
Mazzara M., Emons H.

European Union Reference Laboratory for
Genetically Modified Food and Feed

2020



This publication is a Validated Methods, Reference Methods and Measurements report by the Joint Research Centre (JRC), the European Commission's science and knowledge service. It aims to provide evidence-based scientific support to the European policymaking process. The scientific output expressed does not imply a policy position of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication.

Contact information

Name: EURL GMFF

Email: JRC-EURL-GMFF@ec.europa.eu

JRC Science Hub

<https://ec.europa.eu/jrc>

JRC 119913

Ispra: European Commission, 2020

© European Union, 2020

Reuse is authorised provided the source is acknowledged. The reuse policy of European Commission documents is regulated by Decision 2011/833/EU (OJ L 330, 14.12.2011, p. 39).

For any use or reproduction of photos or other material that is not under the EU copyright, permission must be sought directly from the copyright holders.

How to cite this report: European Union Reference Laboratory for GM Food and Feed, Joint Research Centre. "Event-specific Method for the Quantification of Cotton COT102 Using Real-time PCR", 2020. <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

All images © European Union 2020

Event-specific Method for the Quantification of Cotton COT102 Using Real-time PCR

Validation Report

4 February 2020

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate^a 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 COT102 (unique identifier SYN-IR102-7). 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 guidelines⁽¹⁻⁵⁾.

In accordance with current EU legislation^b, Syngenta Crop Protection NV/SA provided the detection method and the positive and negative control samples (genomic DNA from seeds of COT102 cotton as positive control DNA, and genomic DNA from seeds 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 503/2013, and it fulfils the analytical requirements of Regulation (EU) No 619/2011^c. This validation report is published at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

^a Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed".

^b Regulation (EC) No 503/2013 of 3 April 2013 "on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006".

^c 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.

Content

1. INTRODUCTION.....	5
2. DOSSIER RECEPTION AND ACCEPTANCE (STEP 1).....	5
3. SCIENTIFIC ASSESSMENT AND BIOINFORMATICS ANALYSIS (STEP 2)	6
3.1. SPECIFICITY ASSESSMENT CONDUCTED BY THE APPLICANT	6
3.2. SPECIFICITY ASSESSMENT CONDUCTED BY THE EURL GMFF	6
3.3. VERIFICATION OF THE ENGL ACCEPTANCE PARAMETERS	7
3.4. DNA EXTRACTION	9
4. MATERIALS AND METHODS.....	10
4.1 SAMPLES	10
4.2. METHOD PROTOCOL FOR THE PCR ANALYSIS	10
4.3. EURL GMFF EXPERIMENTAL TESTING (STEP 3)	12
4.3.1. <i>Determination of the zigosity ratio in the positive control sample.....</i>	<i>12</i>
4.3.2. <i>In-house verification of the method performance against ENGL method acceptance criteria</i>	<i>12</i>
4.4. INTERNATIONAL COLLABORATIVE STUDY (STEP 4)	13
4.4.1. <i>List of participating laboratories.....</i>	<i>13</i>
4.4.2. <i>Real-time PCR equipment used in the study</i>	<i>14</i>
4.4.3. <i>Materials used in the international collaborative study.....</i>	<i>15</i>
4.4.4. <i>Design of the collaborative study.....</i>	<i>16</i>
4.4.5. <i>Deviations reported from the protocol.....</i>	<i>16</i>
5. RESULTS	17
5.1. EURL GMFF EXPERIMENTAL TESTING	17
5.1.1. <i>Zygosity factor in the positive control sample</i>	<i>17</i>
5.1.2 <i>In-house verification of method performance against ENGL method acceptance criteria 18</i>	<i>18</i>
5.2. RESULTS OF THE INTERNATIONAL COLLABORATIVE STUDY	21
5.2.1. <i>PCR efficiency and linearity</i>	<i>21</i>
5.2.2. <i>GMO quantification</i>	<i>23</i>
5.2.3. <i>Method performance requirements.....</i>	<i>24</i>
6. COMPLIANCE OF THE METHOD FOR DETECTION AND QUANTIFICATION OF EVENT COT102 WITH THE REQUIREMENTS OF REGULATION (EU) NO 619/2011.....	26
7. CONCLUSIONS.....	27
8. REFERENCES.....	28

**ANNEX 1: EVENT-SPECIFIC METHOD FOR THE QUANTIFICATION OF COTTON
COT102 BY REAL-TIME PCR 29**

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: Belac 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) by DNA extraction, DNA identification and real Time PCR in food and feed)] 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.

Address of contact laboratory:

European Commission
Directorate General Joint Research Centre
Directorate F – Health, Consumers and Reference Materials
European Union Reference Laboratory for GM Food and Feed
Food & Feed Compliance (F.5)
Via E. Fermi, 2749. TP201
I-21027 Ispra (VA), Italy

Functional mailbox: JRC-EURL-GMFF@ec.europa.eu

1. Introduction

In line with Regulation (EC) No 1829/2003, Syngenta Crop Protection NV/SA provided the EURL GMFF with an event-specific method for detection and quantification of cotton event COT102 (unique identifier SYN-IR1Ø2-7) 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 criteria ⁽⁶⁾, 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 COT102, 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)

Syngenta Crop Protection NV/SA submitted the identification and quantification method, data demonstrating its adequate performance when applied to genomic DNA extracted from GM event cotton COT102 and from non-GM cotton.

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

3. Scientific assessment and bioinformatics analysis (step 2)

Documentation and data supplied by the applicant were evaluated by the EURL GMFF for compliance with the ENGL method acceptance criteria (step 2) ⁽⁶⁾.

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.

3.1. Specificity assessment conducted by the applicant

The specificity of the event-specific assay was assessed by the applicant in triplicate real-time PCR reactions, according to the method described (Annex 1, Tables 2 and 4), using 215 copies/reaction of COT102 genomic DNA and at least 2500 copies/reaction of non-target genomic DNA extracted from: maize Bt176, Bt11, MON810, GA21, NK603, 1507, 3272, MIR604, 59122, 98140, DAS-40278-9, T25, MON88017, MON87460, MON89034, MIR162, 5307, MON87427, VCO-01981-5, MZIR098, MZHG0JG; cotton 281-24-236 x 3006-210-23, GHB119, T304-40, GHB614, LLCotton25, MON1445, MON531, MON15985, MON88931; potato EH92-527-1, AM04-1020, AV43-6-G7, PH05-026-0048; oilseed rape 73496, Ms1, Rf1, Rf2, Topas19/2, T45, Ms8, Rf3, RT73, MON88302; LLRice62; soybean GTS 40-3-2, 356043, 305423, DAS-68416-4, DAS-44406-6, DAS-81419-2, FG72, A2704-12, A5547-127, MON87701, MON87769, MON89788, CV127, MON87708, MON87705, SYHT0H2, SYHT04R; sugar beet H7-1. The event-specific assay was also tested against the respective conventional counterparts. According to the method developer the COT102 assay did not react with any sample except the positive control.

A previously validated cotton-specific PCR method (http://gmo-crl.jrc.ec.europa.eu/summaries/281-24-36_cotton_Protocol.pdf), which amplifies a 115 bp and a 123 bp fragment of *SAH7*, a putative cotton gene (*Sinapis Arabidopsis Homolog 7*) present in the A- and D- sub genomes of cotton (*Gossypium hirsutum*), was used as a reference method ⁽⁷⁾.

The specificity of the taxon-specific assay was assessed by the applicant in triplicate real-time PCR reactions, according to the method described (Annex 1, Tables 3 and 4), using 100 ng genomic DNA extracted from: conventional cotton, soybean, mung bean, rapeseed, rice, wheat, maize, potato, sugar beet, tobacco, barley, oat, rye, spelt and alfalfa. According to the method developer the *SAH7* assay did not react with any sample except the positive control represented by conventional cotton DNA.

3.2. Specificity assessment conducted by the EURL GMFF

The detection method spans the 3' insert-to-plant junction in cotton COT102. The forward primer "COT102_3_89F" binds to the insert. The reverse primer "COT102_3_181R" binding site was found in the 3' cotton (*G. hirsutum*) genomic border adjacent to the insertion. The probe

"COT102_3_115T" binds to the edge of the insert and spans with a few nucleotides over the junction at the 3' cotton genomic region.

The amplicon size is expected to be 101 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 (except for those of related patents). 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 100 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.

3.3. Verification of the ENGL acceptance parameters

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL ⁽⁶⁾ and with regard to their documentation and reliability.

Table 1 shows the parameters of the calibration curves (slope, R² coefficient) determined by the applicant in eight runs used to quantify test samples at different GM levels.

Table 1. Summary of the slope and R² values obtained by the applicant

	COT102		SAH7	
Runs	Slope	R²	Slope	R²
1	-3.41	1.00	-3.47	1.00
2	-3.50	1.00	-3.46	1.00
3	-3.50	1.00	-3.41	1.00
4	-3.35	1.00	-3.48	1.00
5	-3.41	0.99	-3.56	1.00
6	-3.57	1.00	-3.47	1.00
7	-3.55	1.00	-3.48	1.00
8	-3.46	1.00	-3.48	1.00
Mean	-3.47	1.00	-3.48	1.00

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 R² coefficient shall be ≥ 0.98 .

Table 1 indicates that the slope and R^2 coefficient of the standard curves for the GM-system (COT102) and the cotton-specific (*SAH7*) system, as established by the applicant, were within the ENGL acceptance criteria.

Precision (expressed as RSDr %) and trueness (expressed as bias %) of the method were established by the applicant. Means are the average of sixteen replicates for each of five GM levels (calculated in terms of GM-DNA copy numbers/total cotton haploid genome copies) and obtained through real-time PCR runs performed with Applied Biosystems® 7500 Fast Real-Time PCR system (standard 7500 run mode).

Table 2 shows values of trueness (expressed as bias %) and precision (expressed as RSDr %) of the method as estimated by the applicant. Both parameters were within the ENGL acceptance criteria (trueness ± 25 %, RSDr ≤ 25 % across the entire dynamic range).

Table 2. Mean %, precision and trueness values provided by the applicant estimated for single measurements

Expected GM %	Test results				
	0.078	0.1	0.9	2.0	5.0
Measured mean GM %	0.072	0.092	0.801	1.80	4.59
Precision (RSDr %)	15.6	14.5	5.5	8.3	8.0
Trueness (bias %)	-7.7	-8.0	-11.0	-10.0	-8.2

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

The method met the ENGL acceptance criteria for trueness and precision also at the lowest GM level, i.e. 0.078 %. The applicant showed that a sample at 50 copies of COT102 was quantified with acceptable precision and trueness. Hence, based on the applicant's information the relative LOQ for COT102 is at least 0.078 % and the absolute LOQ is at least 50 copies. According to the applicant's information, the absolute LOQ for the SAH7 reference system is at least 50 cotton genome copies (ENGL acceptance criteria for LOQ: below or equal to 0.09 % or 50 copies).

The absolute limit of detection (LOD_{abs}) of the SAH7 reference real-time PCR method was assessed by the applicant in 60 PCR replicates at 20 cotton genome copies^d. All the replicates reacted with the SAH7 taxon-specific reference system. Therefore, the data showed that LOD_{abs} of the SAH7 system is at least 20 cotton genome copies per reaction.

The relative Limit of Detection (LOD_{rel}) of the combined method was assessed by the applicant in 60 PCR replicates at 0.035 % and 0.015 % GM level. All samples reacted with the COT102 specific system. Hence, the data showed that the LOD_{rel} for COT102 system is at least 0.015 % (related to copies GM/total haploid genome copies) in 150 ng of total cotton DNA per reaction; this

^d Cotton genome copy numbers based on a haploid genome weight of 2.33 pg (applicant's source)

corresponds to 10 GM copies^d. The absolute and the relative LOD are 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 eight combinations of the following variations to the method: (unchanged/-10 % master mix concentration; unchanged/-30 % primer concentration; unchanged/-30 % probe concentration; +1/-1 µL master mix volume; +/-1 °C in annealing temperature; ABI® 7900HT/Bio-Rad CFX96 Touch™ real time PCR equipment). The RSD_r and the trueness calculated for each combination of variations on a sample at the LOQ level (0.078 %) did not exceed 30 %, thus meeting the ENGL acceptance criteria.

Trueness of the method was also tested in a transferability study: mean of triplicate values for each of the five GM levels (expressed as copies GM/total haploid genome copies) were provided by two laboratories. Further to removal of outlying Cq values from one sample at the LOQ level, the trueness (expressed as bias %) was within the ENGL acceptance criteria (≤ 25 %) across the dynamic range for each laboratory.

3.4. DNA extraction

Genomic DNA used in the applicant in-house testing was isolated from ground cotton seeds, using a protocol for DNA extraction previously submitted with the quantitative event-specific detection methods for cotton event 3006-210-23 x 281-24-236 (http://gmo-crl.jrc.ec.europa.eu/summaries/281-3006%20Cotton_DNAExtr.pdf), which was already validated by the EURL GMFF. Consequently, the EURL GMFF did not verify the DNA extraction method proposed by the applicant.

Annex III to Reg. (EU) No 503/2013 ⁽⁸⁾ requires the applicant to discuss the validity and limitations of the detection method in the various types of foods and feeds (matrices) that are expected to be placed on the market. To this regard, the applicant stated that: *"The event-specific real-time PCR detection method developed by Syngenta for COT102 will work on DNA extracted from various matrices if good quality, intact DNA of a sufficient quantity can be extracted from the materials. Most GM detection methods have been validated with DNA samples extracted from seeds. When compared to other matrices (e.g. leaves), seeds are considered a recalcitrant matrix from which high quality DNA is relatively difficult to obtain. Syngenta's event-specific real-time PCR COT102 detection method has been validated and accurately quantitates DNA that is extracted from such a recalcitrant seed matrix, yet this DNA is of sufficient quality and quantity for this method to perform as intended. This strongly suggests that Syngenta's COT102 detection method would work with other matrices as well.*

In addition, DNA extraction methods that have been developed and validated for difficult matrices like seeds are generally sufficient for extracting DNA from various common food/feed materials. It should be noted that many types of seed processing (dry heating, wet heating, grinding, extrusion, etc.) will denature DNA resulting in degradation, reduction, or even elimination of DNA from a given type of processed material (cottonseed oil, cottonseed meal, etc.) ⁽⁹⁾. Also, different

manufacturing processes for the same product can lead to various degree of degradation of DNA. For example, DDGS (Distillers Dried Grains with Solubles) from different plants have variable amounts of DNA present after processing. Finally, to ensure high quality results, all DNA samples used for event-specific real-time PCR should be monitored for intactness and concentration prior to running any analysis."

In agreement with the ENGL position, endorsing the modularity principle (see also Annex III to Reg. (EU) No 503/2013), 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 COT102.

Whenever DNA is extracted from more complex or 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 in accordance to Regulation (EC) No 1829/2003 Art 2.11^e:

- i) genomic DNA extracted by the applicant from cotton seeds homozygous for the COT102 event, and
- ii) genomic DNA extracted by the applicant from conventional cotton seeds genetically similar to those harbouring the COT102 event.

4.2. Method protocol 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 COT102 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton specific assay targeting the endogenous gene (*SAH7*), and the GM target assay for COT102 are performed in separate wells. The validated method is available at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

For the detection of GM event COT102, a 101 bp fragment of the region spanning the 3' insert-to-plant junction in cotton COT102 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with

^e 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).

FAM™ (6-carboxyfluorescein) as reporter dye at its 5' end and TAMRA™ (carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of GM event COT102, a cotton taxon-specific system amplifies two fragments of a cotton (*SAH7*) endogenous gene of respectively 115 bp and 123 bp (§ 2), using *SAH7* gene-specific primers and a *SAH7* gene-specific probe labelled with VIC® as reporter dye at its 5' end and TAMRA™ as quencher dye at its 3' end.

Standard curves are generated for both the COT102 and the *SAH7* systems by plotting the Cq 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 COT102 DNA in a test sample, the COT102 copy number is divided by the copy number of the cotton haploid genome and multiplied by 100 to obtain the percentage value (GM % = COT102/ cotton haploid genome 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) ⁽¹⁰⁾. 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 over not rounded data. This approach might create small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.

Table 3. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4
Total amount of cotton DNA in the reaction (ng) *	150	19	4.7	0.94
Target taxon haploid genome copies	64378	8047	2012	402
Target COT102 copies	6438	805	201	40

4.3. EURL GMFF experimental testing (step 3)

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

The EURL GMFF experimentally verified the zygosity ratio (GM-target to reference target ratio) in the positive control sample 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.

The copy number of the COT102 and of the *SAH7* targets in the positive control sample were determined by digital PCR (dPCR) performed on the BioMark HD System using the 12.765 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 (COT102_3_89F and COT102_3_181R primers at 600 nM each, COT102_3_115T probe at 150 nM; primers SAH7-uni-f1 at 350 nM and SAH7-uni-r1 at 250 nM each, SAH7-uni-S1 probe at 175 nM), and 1 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 4.6 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 fifteen data sets for the GM target and fifteen 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 inter-laboratory validated methods'^f.

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 (GM-DNA copy numbers/total cotton haploid genome copies) five test samples distributed over a range of GM levels (0.10 % - 5.0 %). The experiments were performed on an ABI 7500, ABI 7900HT and

^f Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods. European Network of GMO Laboratories (ENGL), 2011.
<http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf>

a Roche LC480 real-time platform under repeatability conditions and followed the protocol provided by the applicant. Test samples with GM levels 0.50 %, 0.90 %, 2.0 % and 5.0 % 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 R^2 coefficient of the standard curves and method trueness and precision over the dynamic range were evaluated against the ENGL method acceptance criteria.

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 120/2014⁹ 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) ⁽¹⁾
- 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) ⁽²⁻⁵⁾

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 COT102 international collaborative study were randomly selected from 24 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.

⁹ Commission Implementing Regulation (EU) No 120/2014 of 7 February 2014 amending Regulation (EC) No 1981/2006 on detailed rules for the implementation of Article 32 of Regulation (EC) No 1829/2003 of the European Parliament and the Council as regards the Community reference laboratory for genetically modified organisms. OJ L 39, 8.2.2014, p. 46–52

Table 4. Laboratories participating in the validation of the detection method for cotton event COT102

Laboratory	Country
BioGEVES - Groupement d'Intérêt Public	FR
Institute of Food Safety, Animal Health and Environment "BIOR"	LV
Italian National Institute of Health (ISS) Food Safety, Nutrition and Veterinary Public Health	IT
LUFA Speyer	DE
National Centre for Food, Spanish Agency for Consumer Affairs, Food Safety and Nutrition	ES
National Food and Veterinary Risk Assessment Institute Molecular Biology and GMO Department	LT
National Health Laboratory	LU
National Institute of Biology	SI
Sciensano, Service Platform Biotechnology and Bioinformatics (PBB)	BE
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE
The Netherlands Food and Consumer Product Safety Authority	NL
Veterinary Public Health Institute for Lazio and Toscana Regions; National Reference Centre for GMO Analysis	IT

4.4.2. Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: three laboratories used ABI 7500, the following platforms were used by only one laboratory: ABI 7300, ABI 7700, ABI 7900HT, BioRad CFX, Quantstudio 5, Quantstudio 7flex, Roche LC 480, StepOnePlus and ViiA 7.

This variability of equipment, with its known potential influence on PCR results, reflects the real-life situation in the control laboratories and provides additional assurance that the method is robust and usable under real 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 4.1 for details). Their concentration was not disclosed to the laboratories participating to the ring-trial (blind samples).

The EURL GMFF made use of the control samples to prepare standards (of known GMO content) and test samples (of undisclosed GM content = blind samples) by mixing COT102 cotton DNA and non-GM cotton DNA to the aim of validating the quantitative event-specific method.

The calibration sample S1 was prepared by mixing the appropriate amount of COT102 DNA with control non-GM cotton DNA to obtain a 10 % (in copy number ratio related to haploid genome copies) GM sample. Calibration sample S2 was prepared by 8-fold dilution from the S1 sample; the S3 sample was obtained by 4-fold dilution from the S2 sample and sample S4 by a 5-fold dilution from the S3 sample.

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

- ✓ Four calibration samples with known concentrations of GM-event (175 µL of DNA solution each) labelled from S1 to S4 (Table 3).
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution, each at 30 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 5)

Table 5. COT102 blinded samples GM % contents

COT102 GM % (GM copy number/cotton haploid genome copy number) x 100
5.0
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:
 - SAH7* taxon-specific assay
 - SAH7-uni-f1 primer (10 µM): 280 µL
 - SAH7-uni-r1 primer (10 µM): 200 µL
 - SAH7-uni-s1 probe (10 µM): 140 µL
 - COT102 assay
 - COT102_3_89F primer (10 µM): 480 µL
 - COT102_3_181R primer (10 µM): 480 µL
 - COT102_3_115T probe (10 µM): 120 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 COT102 event-specific system and for the *SAH7* taxon-specific system. In total, two plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for the cotton event COT102 and the *SAH7* 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

Nine laboratories reported no deviations from the validation protocol. One laboratory selected JOE, instead of VIC, as reporter dye of the reference gene assay. One laboratory reported a missing amplification of one out of three replicates of a test sample (U16) amplified with the reference-specific amplification system; the average of the two remaining replicates was used for determining the value of the *SAH7* genome copies for that sample. One laboratory eliminated one of three replicates amplified with the reference-specific assay (sample U10), because it showed an outlying Cq value.

5. Results

5.1. EURL GMFF experimental testing

5.1.1. Zygosity factor in the positive control sample

In this validation study the relative quantification of event COT102 is performed in relation to the taxon-specific reference system *SAH7*. According to the information available and to bioinformatics analyses two amplicons are generated by the *SAH7* method on each cotton haploid genome (two *SAH7* copies per cotton haploid genome, composed of the A- and D- sub-genomes, § 3) ⁽⁷⁾. The unit of measurement in the present validation is expressed as GM-DNA copy numbers in relation to haploid cotton genome copies (Annex to Reg. (EU) No 619/2011). According to the applicant, the positive control sample provided is homozygous for the COT102 event (i.e. it contains one COT102 event copy per cotton haploid genome). Hence, the ratio between the GM-DNA copy numbers and the cotton haploid genome copies should be equal to 1. In order to verify the validity of this assumption, a zygosity test was carried out on the positive control sample submitted, as described in 4.3.1. Since two copies of *SAH7* are hosted per cotton haploid genome, it is expected that the ratio between COT102 event and *SAH7* reference gene copies corresponds to 0.5.

The results of the digital PCR analysis conducted by the EURL GMFF on the COT102 and *SAH7* targets to determine the zygosity factor in the positive control samples are shown in Table 6.

Table 6. Zygosity factor of the COT102 and *Sah7* targets in the positive control sample.

Mean ratio (COT102/ <i>SAH7</i>)	0.50
Standard deviation	0.06
RSD _r (%)	11
Standard error of the mean	0.01
Upper 95 % CI of the mean	0.53
Lower 95 % CI of the mean	0.47

The mean ratio of GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes was 0.5. The 95 % confidence interval (CI) spans around 0.50, i.e. the expected ratio for a cotton control sample homozygous for the GM-locus and assuming two copies of endogenous gene target.

Therefore, the measured mean ratio is not significantly different from the expected ratio, for an $\alpha = 0.05$. The zygosity of the positive control sample reported above provides the evidence for a homozygous status of the COT102 event in the positive control sample.

Standard curve and test samples have been prepared as ratio of GM copies to haploid genome copy numbers.

Hence, the sample at 0.1 % in haploid genome copy numbers prepared with the control samples used in the present validation study corresponds to a 0.1 % sample expressed in mass fraction: 0.1 GM % in DNA copy number ratio related to haploid genome copy numbers = 0.1 GM % in mass fraction.

Note:

The zygosity ratio herein reported is valid for the positive control sample DNA 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, according 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)", available at <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 0.50 % to 5.0 % 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 0.1 % GM-level was tested for its precision in quantification in 15 replicates in separate runs.

Tests were conducted on ABI 7500, and on ABI 7900 and Roche LC480 for assessing method's robustness.

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 5.0 % in four replicates each. Slope and R² coefficient values were rounded to two digits.

	COT102 system			SAH7 system		
	Slope	PCR efficiency*	R ²	Slope	PCR efficiency*	R ²
Run A	-3.34	99	1.00	-3.43	96	1.00
Run B	-3.48	94	1.00	-3.44	95	1.00
Run C	-3.31	100	1.00	-3.38	98	1.00
Run D	-3.26	103	1.00	-3.38	98	1.00
Run E	-3.23	104	1.00	-3.47	94	1.00
Run F	-3.41	96	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 with 40 cycles of amplification.

Table 7B. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, ABI 7900, and Roche LC480 to quantify the GM-level 0.1 % in 15 replicates. Slope and R² coefficient values were rounded to two digits.

	COT102 system			SAH7 system		
	Slope	PCR efficiency*	R ²	Slope	PCR efficiency*	R ²
Run G	-3.30	101	1.00	-3.45	95	1.00
Run H	-3.39	97	0.99	-3.45	95	1.00
Run I	-3.32	100	1.00	-3.46	95	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 with 40 cycles of amplification.

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 %)
5.0	4.9	-2.9	3.5
2.0	1.9	-6.1	2.4
0.90	0.85	-5.7	8.6
0.50	0.48	-4.9	8.9
0.10	0.08	-19	11

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 %)
5.0	4.4	-12	6.2
2.0	1.8	-9.7	6.3
0.90	0.77	-14	2.4
0.50	0.41	-18	15
0.10	0.08	-17	18

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 %)
5.0	4.9	-2.2	3.5
2.0	1.8	-8.7	4.4
0.90	0.83	-8.2	4.2
0.50	0.43	-15	8.0
0.10	0.09	-12	15

5.2. Results of the international collaborative study

5.2.1. PCR efficiency and linearity

The PCR efficiency (%) and R^2 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:

$$\text{Efficiency} = (10 * (-1/\text{slope})) - 1) \times 100$$

Table 11 indicates that the efficiency of amplification for the COT102 system ranges from 92 % to 118 % and the linearity from 0.99 to 1.00; the amplification efficiency for the cotton-specific system ranges from 91 % to 109 % and the linearity from 0.97 to 1.00. The mean PCR efficiency was 99 % for the COT102 assay and 95 % for *SAH7*. The average R^2 of the methods was 1.00 for the COT102 and 1.00 for the *SAH7* 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	COT102			SAH7		
		Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.29	101	0.99	-3.45	95	1.00
	B	-3.37	98	1.00	-3.45	95	1.00
2	A	-3.17	107	1.00	-3.51	93	1.00
	B	-3.17	107	1.00	-3.49	94	1.00
3	A	-3.21	105	1.00	-3.38	98	1.00
	B	-3.35	99	1.00	-3.36	98	1.00
4	A	-2.96	118	0.99	-3.51	93	0.97
	B	-3.04	113	0.99	-3.13	109	0.99
5	A	-3.47	94	1.00	-3.55	91	1.00
	B	-3.45	95	1.00	-3.53	92	0.99
6	A	-3.35	99	1.00	-3.36	98	0.99
	B	-3.45	95	1.00	-3.48	94	0.99
7	A	-3.25	103	1.00	-3.51	93	1.00
	B	-3.43	96	1.00	-3.45	95	1.00
8	A	-3.30	101	1.00	-3.32	100	0.99
	B	-3.39	97	1.00	-3.37	98	0.99
9	A	-3.39	97	0.99	-3.52	92	1.00
	B	-3.45	95	1.00	-3.51	93	1.00
10	A	-3.51	93	0.99	-3.31	101	1.00
	B	-3.50	93	0.99	-3.55	91	1.00
11	A	-3.44	95	1.00	-3.50	93	1.00
	B	-3.53	92	0.99	-3.50	93	1.00
12	A	-3.38	97	1.00	-3.41	96	1.00
	B	-3.46	94	1.00	-3.43	96	1.00
Mean		-3.35	99	1.00	-3.44	95	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

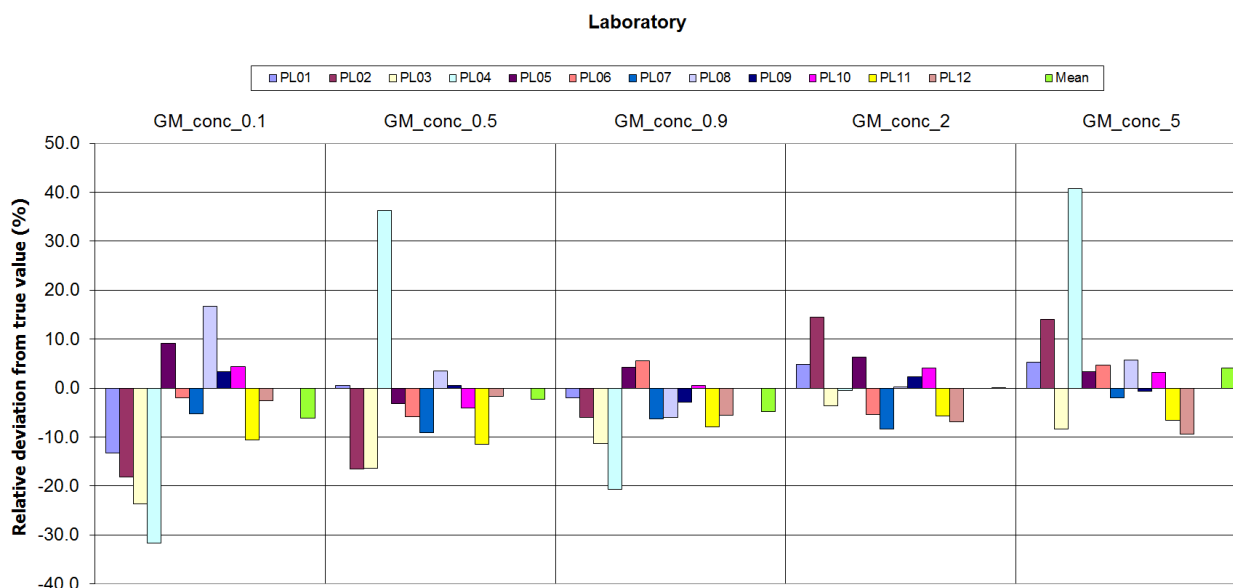
LAB	GMO content (%) *																			
	0.10				0.50				0.90				2.0				5.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.08	0.09	0.11	0.08	0.53	0.48	0.54	0.47	0.89	0.85	0.95	0.84	2.0	2.0	2.2	2.2	5.3	4.8	5.3	5.7
2	0.08	0.09	0.08	0.08	0.43	0.41	0.47	0.36	1.1	0.74	0.76	0.81	2.5	2.2	2.2	2.3	5.2	6.2	5.7	5.7
3	0.08	0.08	0.09	0.06	0.41	0.48	0.40	0.39	0.88	0.70	0.88	0.73	1.8	1.9	2.0	2.0	4.3	4.9	4.8	4.3
4	0.09	0.05	0.05	0.08	0.31	0.30	1.37	0.75	0.49	1.1	0.44	0.83	1.3	2.9	2.7	1.0	3.3	8.9	11.2	4.7
5	0.10	0.12	0.11	0.11	0.49	0.45	0.54	0.46	0.96	1.0	0.85	0.94	2.2	2.1	2.2	2.0	5.2	4.7	5.8	5.0
6	0.08	0.10	0.12	0.09	0.50	0.50	0.44	0.44	1.0	0.92	1.0	0.85	2.1	1.9	1.9	1.6	5.0	5.8	5.4	4.7
7	0.08	0.10	0.11	0.08	0.49	0.47	0.45	0.41	0.89	0.79	0.86	0.83	1.9	1.9	1.7	1.9	4.4	5.1	5.1	5.0
8	0.13	0.15	0.10	0.09	0.65	0.60	0.45	0.36	0.82	0.87	1.1	0.56	2.4	2.1	1.9	1.7	6.5	4.8	4.9	5.0
9	0.11	0.10	0.10	0.10	0.50	0.52	0.49	0.51	0.78	0.85	0.95	0.91	2.0	2.0	2.2	2.1	4.7	5.0	5.1	5.1
10	0.10	0.11	0.09	0.11	0.48	0.49	0.46	0.49	0.92	0.97	0.85	0.89	2.1	2.2	2.1	2.0	5.3	5.4	5.1	4.9
11	0.09	0.09	0.08	0.10	0.44	0.46	0.44	0.43	0.85	0.83	0.91	0.73	1.9	1.9	1.9	1.9	5.0	5.1	4.6	3.9
12	0.09	0.12	0.09	0.09	0.47	0.53	0.47	0.50	0.80	0.85	0.93	0.81	1.8	1.8	1.9	2.0	4.5	4.2	4.6	4.8

* GMO % = (GMO copy number/cotton haploid genome copy number) x 100

n.a. not available

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 *



* For PL8 at level 2.0 % a very small relative deviation from the true value was observed and therefore the corresponding bar does not show up in Figure 1. PL = participating laboratory.

Overall, most laboratories' mean relative deviations from the true values were within a maximum of ± 25 %. At GM-levels 0.90 % and 2.0 % twelve laboratories were within the limit; at GM-levels 0.10 %, 0.50 % and 5.0 % eleven laboratories were within the limit. One laboratory underestimated GM-level 0.10 % and overestimated GM-level 0.50 % and 5.0 % by more than 25 %. No clear trend for over- or underestimation was observed.

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 20 % at the 0.10 % GM level, thus within the acceptance criterion.

Table 13. Summary of validation results for the COT102 method, expressed as GM copy numbers in relation to target taxon haploid genome copy numbers.

	Test Sample Expected GMO %				
	0.10	0.50	0.90	2.0	5.0
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	2	2	2	1
Reason for exclusion	-	2C	2C	2C	1C
Mean value	0.09	0.47	0.87	2.0	5.0
Relative repeatability standard deviation, RSD _r (%)	15	7.1	9.2	5.6	8.6
Repeatability standard deviation	0.01	0.03	0.08	0.11	0.43
Relative reproducibility standard deviation, RSD _R (%)	20	9.2	9.7	8.8	10
Reproducibility standard deviation	0.02	0.04	0.08	0.18	0.52
Bias** (absolute value)	-0.01	-0.03	-0.03	0.00	0.04
Bias (%)	-6.1	-6.7	-3.1	0.21	0.85

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

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

Table 13 also documents 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" <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>). As it can be observed from the values reported, the repeatability standard deviation is below 25 % at all GM levels, with the highest value of 15 % at the 0.10 % 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. As shown in Table 13 the bias ranges from -6.7 % to 0.85 %. Therefore, the method satisfies this requirement across the dynamic range tested.

6. Compliance of the method for detection and quantification of event COT102 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 14:

- at step 2 of the validation process (scientific assessment of the dossier), the EURL GMFF acknowledged that the RSD_r value at the 0.1 % level shown by the applicant's dossier (expressed as GM-DNA copy numbers/total cotton haploid genome copies, equivalent to mass fractions of GM-DNA) was 14.5 %, based on 16 replicates (Table 2), 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-material (corresponding to 0.1 % expressed in terms of GM 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 11 % and 18 % (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-material the RSD_r of the method was 15 %, therefore also below 25 % and well in line with the previous data.

The outcome of the different steps is summarised in Table 14.

Table 14. Precision of the event-specific method for quantitative detection of COT102 at or around 0.1 % level related to mass fractions of GM material.

Source	RSD_r %	GM %
Applicant's method optimisation	14.5 %	0.1 %
EURL GMFF tests	11 - 18 %	0.1 %
Collaborative study	15 %	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 619/2011.

7. Conclusions

The method provided by the applicant was 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 4.1), in accordance with the requirements of Annex I-3.C.2 to Commission Regulation (EU) No 503/2013 and (EU) No 619/2011 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.

8. References

1. Horwitz W. Protocol for the design, conduct and interpretation of method- performance studies, *Pure and Appl. Chem.* 1995; 67: 331-343.
2. International Standard (ISO) 5725-1, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 1: General principles and definitions. International Organization for Standardization, Genève, Switzerland.
3. ISO 5725-1:1994/Cor 1:1998.
4. International Standard (ISO) 5725-2, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method. International Organization for Standardization, Genève, Switzerland.
5. ISO 5725-2:1994/Cor 1:2002.
6. European Network of GMO Laboratories (ENGL), 'Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing', 2015. http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf
7. EURL GMFF Event-specific methods for the quantitation of the hybrid cotton line 281-24-236/3006-210-23 using real-time PCR. Validation Report. Chapter 5, http://gmo-crl.jrc.ec.europa.eu/summaries/281-3006_val_report.pdf
8. Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on Applications for Authorisation of Genetically Modified Food and Feed in Accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006.
9. Guan Q, Wang X, Teng D, Yang Y. 2011. DNA Degradation of Genetically Modified Cottonseed Meal During Feed Processing. *Appl Biochem Biotechnol* 169: 368-379.
10. Plant DNA C-values Database, <http://data.kew.org/cvalues/>

Annex 1: Event-specific Method for the Quantification of Cotton COT102 by Real-time PCR

Validated Method

Method development:

Syngenta Crop Protection NV/SA

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 COT102 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 COT102, a 101 bp fragment of the region spanning the 3' insert-to-plant genome junction in cotton COT102 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 TAMRA™ (carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantitation of event COT102, a cotton-specific reference system amplifies part of the intron sequences of the gene for the putative SAH7 protein of *Gossypium hirsutum*. The *SAH7* gene is present not only in the A-sub-genome but also in the D-sub-genome of *G. hirsutum*. The A-sub-genome specific copy differs from the D-sub-genome specific copy by several single or double nucleotide substitutions and small deletions/insertions. Primers and probe of the cotton-specific reference PCR system match perfectly (no mismatch) to both sub-genomes gene copies. However, due to sequence differences within the amplified region, the size of the amplicons resulting from the A- and D- sub-genomes differ slightly, being respectively 115 bp and 123 bp long (CRLVL14/05VP, http://gmo-crl.jrc.ec.europa.eu/summaries/281-24-36_cotton_Protocol.pdf); (Accession number, GeneBank: X14555). Amplification of the target *SAH7* sequence is achieved using *SAH7* gene-specific primers and a *SAH7* gene-specific probe labelled with VIC® as reporter dye at its 5' end and TAMRA™ as quencher dye at its 3' end.

The 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 COT102 DNA in a test sample, Cq values for the COT102 and the *SAH7* systems are determined for the sample. Standard curves are then used to estimate the relative amount of COT102 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 seeds. 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 2018.

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 0.015 % (related to copy number ratio calculated in terms of haploid genomes) in 150 ng of total suitable cotton DNA. The relative 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 0.078 % (related to copy number ratio calculated in terms of haploid genomes) in 150 ng of total suitable cotton DNA, corresponding to 50 copies of COT102 event. 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 3' insert-to-plant genome junction in cotton COT102 and is therefore event-specific for the event COT102. This was confirmed in the validation study.

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 COT102

3.2.1 General

The real-time PCR set-up for the taxon (*SAH7*) and the GMO (event COT102) 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 COT102) and the taxon (*SAH7*) 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 four samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % cotton COT102 DNA in a total of 150 ng of cotton DNA (corresponding to 64378 cotton haploid genome copies with one haploid genome assumed to correspond to 2.33 pg of cotton genomic DNA)⁽¹⁾. Standards S2 to S4 are prepared by serial dilutions (dilution factor 8 for standard S2, dilution factor 4 for standard S3, dilution factor 5 for standard S4) according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4
Total amount of cotton DNA in reaction (ng)	150	18.8	4.7	0.94
cotton haploid genome copies	64378	8047	2012	402
COT102 copies	6438	805	201	40

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 COT102 cotton specific system (Table 2) and the *SAH7* 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 COT102 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
COT102_3_89F (10 µM)	600 nM	1.500
COT102_3_181R (10 µM)	600 nM	1.500
COT102_3_115T (10 µM)	150 nM	0.375
Nuclease free water	-	4.125
DNA	-	5
Total reaction volume:		25 µL

TaqMan® probe labelled with 6-FAM™ at its 5'-end and TAMRA at its 3'-end

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the cotton *SAH7* assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
SAH7-uni-f1 (10 µM)	350 nM	0.875
SAH7-uni-r1 (10 µM)]	250 nM	0.625
SAH7-uni-s1 (10 µM)	175 nM	0.438
Nuclease free water	-	5.562
DNA	-	5
Total reaction volume:		25 µL

TaqMan® probe is labelled with VIC® at its 5'-end and TAMRA at its 3'-end

- Mix well and centrifuge briefly.
- Prepare two 0.5 mL reaction tubes (one for the cotton COT102 and one for the *SAH7* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (70 µL for the COT102 cotton system and 70 µL for the *SAH7* system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (17.5 µL 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.
- Spin down the tubes. Aliquot 25 µL for COT102 system and for the *SAH7* reference system in each well.
- Place an optical cover on the reaction plate and briefly centrifuge the plate.
- 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.
- Select FAM™ as reporter dye for the COT102 and VIC® for the *SAH7* reference system. Define TAMRA as quencher dye for COT102 specific system and for *SAH7* reference system. Select ROX (if necessary according to the instructions of the real-time PCR manufacturer) as the passive reference dye. Enter the correct reaction volume (25 µL).
- Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for COT102/ *SAH7* 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	No	40*
		Annealing & Extension	60	Yes	

*UNG: Uracil-N-glycosylase

3.3 Data analysis

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

- 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.
- 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).
- Save the settings.
- Repeat the procedure described in a), b) and c) on the amplification plots of the taxon specific system.
- 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 *SAH7* and for the COT102 specific assays by plotting the Cq 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 COT102 DNA in the unknown sample, the COT102 copy number is divided by the copy number of the cotton endogenous gene *SAH7* and multiplied by 100 ($GM\% = COT102/SAH7 \times 100$).

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. ThermoFisher Scientific™ Catalogue Number 4318157

4.3 Primers and Probes

Table 5. Primers and probes for the COT102 and *SAH7* methods

	ID	DNA Sequence (5' to 3')	Length (nt)
COT102			
Forward primer	COT102_3_89F	TCT CCG CTC ATG ATC AGA TTG TC	23
Reverse primer	COT102_3_181R	CAG TAA CAG TAC AGT CGG TGT AGG G	25
Probe	COT102_3_115T	6-FAM™– TCC CGC CTT CAG TTT AAA CTA TCA GTG TTT AAT-TAMRA	33
<i>SAH7</i>			
Forward primer	SAH7-uni-f1	AGT TTG TAG GTT TTG ATG TTA CAT TGA G	28
Reverse primer	SAH7-uni-r1	GCA TCT TTG AAC CGC CTA CTG	21
Probe	SAH7-uni-s1	VIC®-AAA CAT AAA ATA ATG GGA ACA ACC ATG ACA TGT- TAMRA™	33

FAM™: 6-carboxyfluorescein; VIC®; TAMRA™: carboxytetramethylrhodamine.

5. References

1. Plant DNA C-values Database. Royal Botanic Gardens, Kew, <http://data.kew.org/cvalues/>

JRC Mission

As the science and knowledge service of the European Commission, the Joint Research Centre's mission is to support EU policies with independent evidence throughout the whole policy cycle.



EU Science Hub
ec.europa.eu/jrc



@EU_ScienceHub



EU Science Hub - Joint Research Centre



Joint Research Centre



EU Science Hub