



JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Event-specific Method for the Quantification of Cotton DAS-81910-7 Using Real-time PCR Validation Report

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Genetically Modified Food and Feed

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Event-specific Method for the Quantification of Cotton DAS-81910-7 Using Real-time PCR

Validation Report

17 October 2019

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 DAS-81910-7 (unique identifier DAS-81910-7). 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, Dow AgroSciences LLC provided the detection method and the positive and negative control samples (genomic DNA from homogenised seeds of DAS-81910-7 cotton as positive control DNA, and genomic DNA from homogenised 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.....	4
2. STEP 1 (DOSSIER ACCEPTANCE) AND STEP 2 (SCIENTIFIC DOSSIER ASSESSMENT AND BIOINFORMATICS ANALYSIS).....	4
3. STEP 3 (EXPERIMENTAL TESTING OF THE SAMPLES AND METHOD).....	7
3.1 DNA EXTRACTION.....	7
3.2 METHOD PROTOCOL FOR THE PCR ANALYSIS	7
3.3 EURL GMFF EXPERIMENTAL TESTING	9
3.3.1 <i>Determination of the zygosity ratio in the positive control sample</i>	<i>9</i>
3.3.2 <i>In-house verification of the method performance against ENGL method acceptance criteria</i>	<i>10</i>
3.4 INTERNATIONAL COLLABORATIVE STUDY (STEP 4)	10
3.4.1 <i>List of participating laboratories</i>	<i>11</i>
3.4.2 <i>Real-time PCR equipment used in the study.....</i>	<i>12</i>
3.4.3 <i>Materials used in the international collaborative study.....</i>	<i>12</i>
3.4.4 <i>Design of the collaborative study</i>	<i>13</i>
3.4.5 <i>Deviations reported from the protocol</i>	<i>14</i>
4. RESULTS	14
4.1 EURL GMFF EXPERIMENTAL TESTING	14
4.1.1 <i>Zygosity ratio in the positive control sample</i>	<i>14</i>
4.1.2 <i>In-house verification of method performance against ENGL method acceptance criteria</i>	<i>15</i>
4.2 RESULTS OF THE INTERNATIONAL COLLABORATIVE STUDY	17
4.2.1 <i>PCR efficiency and linearity.....</i>	<i>17</i>
4.2.2 <i>GMO quantification.....</i>	<i>18</i>
4.2.3 <i>Method performance requirements.....</i>	<i>20</i>
5. COMPLIANCE OF THE METHOD FOR DETECTION AND QUANTIFICATION OF EVENT DAS-81910-7 WITH THE REQUIREMENTS OF REGULATION (EU) NO 619/2011.....	22
6. CONCLUSION	23
7. REFERENCES.....	23
ANNEX 1: EVENT-SPECIFIC METHOD FOR THE QUANTIFICATION OF COTTON DAS-81910-7 BY REAL-TIME PCR	24

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: Belac 268 TEST (Flexible Scope for DNA extraction, DNA identification and real Time 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 1829/2003, Dow AgroSciences LLC provided the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) with an event-specific method for detection and quantification of cotton event DAS-81910-7 (unique identifier DAS-81910-7) 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 European Network of GMO Laboratories (ENGL) method acceptance criteria^d, 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 DAS-81910-7, 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 the European Food Safety Authority (EFSA) for its risk assessment.

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

Documentation and data supplied by the applicant were evaluated by the EURL GMFF for completeness (step 1) and compliance with the ENGL method acceptance criteria (step 2).

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

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

Specificity assessment by the applicant

The specificity of the event-specific assay was assessed by the applicant in triplicate real-time Polymerase Chain Reactions (PCR) according to the method described in Annex 1 (Tables 2, 3, 4 and 5) and using at least 2500 haploid genome copies/reaction of genomic DNA extracted from: oilseed rape Ms1, Ms8, Rf1, Rf2, Rf3, Topas19/2, T45, RT73, MON 88302, 73496; soybean A2704-12, A5547-127, FG72, GTS 40-3-2, MON 89788, MON 87701, MON 87708, 356043, 305423, CV127, MON 87769, MON 87705, DAS- 68416-4, DAS-81419-2, 44406; maize T25, Bt176, Bt11, MON 810, GA21, NK603, MON 863, 1507, 3272, MIR604, MIR162, 59122, 98140, MON 88017, MON 89034, 40278, 5307, MON 87427, VCO-01981-5; cotton LLCotton25, T304-40, GHB614, GHB119, MON 1445, MON 531, MON 15985, MON 88913, 281-24-236 x 3006-210-23, DAS-81807-3; rice LLRICE62; sugar beet H7-1; potato EH92-527-1, AM04-1020, AV43-6-G7, PH05-026-048 and conventional oilseed rape, soybean, maize, cotton, rice, wheat, potato, sugar beet. According to the method developer the DAS-81910-7 assay did not react with any sample except the positive control. In addition, an in-silico specificity test was performed against public sequence databases, such as NCBI and the patent database, with the DAS-81910-7 amplicon and no 100 % match was identified other than the intended target.

A modified version of 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 and a 123 base pair (bp) fragment of the *Sinapis Arabidopsis Homolog 7 (SAH7)* of *Gossypium hirsutum*, was used as a reference method. The specificity of the taxon-specific assay was assessed by the applicant in triplicate real-time PCR reactions, according to the method described in Annex 1 (Tables 2, 3, 4 and 5), using a minimum of 50 ng genomic DNA extracted from: conventional cotton, oilseed rape, soybean, potato, conventional maize, wheat (*Triticum aestivum*) and sugar beet. According to the method developer the *SAH7* assay did not react with any sample except the positive control.

Bioinformatics specificity assessment by the EURL GMFF

The detection method spans the 5' plant-to-insert junction in cotton DAS-81910-7. The forward primer "1706-f2" binds to cotton (*Gossypium hirsutum*) genomic border adjacent to the insertion. The reverse primer "1706-r3" and the probe "1706-p3" are located within the DAS-81910-7 transgenic insert.

The amplicon size is expected to be 120 bp, consistent to what reported by the applicant. The sequence of the amplicon was analysed by BLAST 2.3.0 (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 Joint Research Centre (JRC) of the Commission, 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).

A potential amplicon of 138 bp was identified using only the reverse primer "1706-r3" as forward and reverse primer on the GM Event Florigene Moonvista Carnation (FLO-40685-2), 3rd insertion site. Also the probe "1706-p3" matches perfectly on the potential amplicon.

The amplification of this potential target was excluded by the EURL GMFF by testing DNA from the GM Event Florigene Moonvista Carnation (FLO-40685-2).

Verification of the ENGL acceptance parameters

The applicant prepared the calibration curve from a DNA solution (S1) of 10 % cotton event DAS-81910-7 genomic DNA (expressed as copies GM/total haploid genome copies) which was serially diluted 1:5 to obtain solutions S2 and S3, and 1:8 to obtain solution S4. The parameters (slope and R² coefficient) of eight runs of the calibration curve are reported as provided by the applicant (Table 1).

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

	DAS-81910-7		<i>SAH7</i>	
	Slope	R²	Slope	R²
Run 1	-3.37	1.00	-3.48	1.00
Run 2	-3.39	1.00	-3.36	1.00
Run 3	-3.30	1.00	-3.41	1.00
Run 4	-3.30	1.00	-3.28	1.00
Run 5	-3.48	1.00	-3.25	1.00
Run 6	-3.45	1.00	-3.34	1.00
Run 7	-3.41	1.00	-3.34	1.00
Run 8	-3.28	1.00	-3.30	1.00
Mean	-3.37	1.00	-3.35	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 R² coefficient of the standard curves for the GM-system (DAS-81910-7) and the cotton-specific *Sinapis Arabidopsis Homolog 7* (*SAH7*) system, as established by the applicant, were within the ENGL acceptance criteria.

Also precision and trueness of the method were established by the applicant and 16 values for each of five GM levels (expressed as copies GM/total haploid genome copies) were provided. Table 2 reports precision and trueness values for the five GM-levels as provided by the applicant. Both parameters were within the ENGL acceptance criteria (trueness ± 25 %, RSD_r ≤ 25 % across the entire dynamic range).

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

	Test results				
Expected GM %	5.00	2.00	0.900	0.100	0.078
Measured mean GM %	4.82	1.95	0.893	0.102	0.073
Precision (RSD _r %)	11.9	4.4	7.4	14.0	16.3
Trueness (bias %)	-3.6	-2.5	-0.8	2.0	-6.4

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

3. Step 3 (experimental testing of the samples and method)

3.1 DNA extraction

Genomic DNA was isolated from ground cotton seeds, using a "CTAB/Genomic-tip 20" protocol previously submitted for detection of cotton stacked events 281-24-236 x 3006-210-23 (DAS-24236-5 x DAS-21Ø23-5) and already validated by the EURL GMFF. The protocol for DNA extraction and a report on testing can be consulted at http://gmo-crl.jrc.ec.europa.eu/summaries/281-3006%20Cotton_DNAExtr.pdf.

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 DAS-81910-7.

Whenever DNA is extracted from more complex and difficult 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.

3.2 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 DAS-81910-7 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton specific assay targeting the endogenous gene *Sinapis Arabidopsis Homolog 7 (SAH7)*, and the GM target assay for DAS-81910-7 are performed in separate wells. The validated method protocol 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 DAS-81910-7, a 120 bp fragment of the region spanning the 5' plant-to-insert junction in cotton DAS-81910-7 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 (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of GM event DAS-81910-7, a cotton taxon-specific system amplifies a fragment of the cotton *Sinapis Arabidopsis Homolog 7* (*SAH7*) endogenous gene, using *SAH7* gene-specific primers and a *SAH7* gene-specific probe labelled with FAM as reporter dye at its 5' end and TAMRA as quencher dye at its 3' end. The *SAH7* gene is present not only in the A-subgenome, but also in the D-subgenome of *Gossypium hirsutum*.

The A-subgenome specific copy differs from the D-subgenome specific copy for several single or double nucleotide substitutions and small deletions/insertions. The primers and probe of the cotton-specific reference PCR system match perfectly without any single mismatch to both subgenome gene copies. However, due to sequence differences within the amplified region, the size of the amplicons resulting from the A- and D-subgenomes differs slightly, being respectively 115 bp and 123 bp.

Standard curves are generated for both the DAS-81910-7 and the *SAH7* systems by plotting the threshold cycle (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 DAS-81910-7 DNA in a test sample, the DAS-81910-7 copy number is divided by the copy number of the cotton haploid genomes and multiplied by 100 to obtain the percentage value (GM % = DAS-81910-7/ cotton haploid genomes 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) ⁽⁶⁾. 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)	175	35	7	0.87
Target taxon haploid genome copies	75107	15021	3004	376
Target DAS-81910-7 copies	7511	1502	300	38

3.3 EURL GMFF experimental testing

3.3.1 Determination of the zygosity ratio in the positive control sample

Annex II of Regulation (EU) No 619/2011 requires that “when results are primarily expressed as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the 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 DAS-81910-7 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 (1706-f2 and 1706-r3 primers at 300 nM each, 1706-p3 probe at 150 nM; Sah7-uni-f1 primer at 350 nM, Sah7-uni-r1 primer at 250 nM, 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 each for the GM and 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 Annex 4 of the ENGL guidance document 'Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods'^e.

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

The method performance characteristics were verified by quantifying five blind test samples distributed over a range of GM levels (5.0 % - 0.10 %, copies GM/copies haploid genomes). The experiments were performed on an ABI 7500, an ABI 7900HT and a Roche LC480 real-time platform under repeatability conditions and followed the protocol provided by the applicant. Test samples with GM levels 5.0 %, 2.0 %, 0.90 % and 0.50 % 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.

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.

3.4 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 (EU) No 120/2014^f 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⁾

^e 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>

^f 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

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.

3.4.1 List of participating laboratories

The twelve laboratories participating in the DAS-81910-7 international collaborative study were randomly selected from 25 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 DAS-81910-7

Laboratory	Country
BioGEVES - Groupement d'Intérêt Public – Groupe d'Etude et de contrôle des Variétés et des Semences	FR
Federal Office of Consumer Protection and Food Safety - Berlin	DE
Hellenic Agricultural Organisation Demeter	GR
Institute of Food Safety, Animal Health and Environment "BIOR"	LV
LUFA Speyer	DE
National Centre for Food, Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN)	ES
National Health Laboratory	LU
National Institute of Biology	SI
Plant Health Laboratory	FR
Service commun des laboratoires du ministère de l'économie et des finances - Etablissement de Strasbourg	FR
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE
The Netherlands Food and Consumer Product Safety Authority	NL

3.4.2 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 or ABI7500 Fast, one used Roche LC 480, one used ABI ViiA7, two ABI Quantstudio 5, one ABI QuantStudio 7 Flex, one ABI 7300, one ABI 5700 and one used Biorad CFX96.

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.

3.4.3 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. They were derived from:

- i) genomic DNA extracted by the applicant from homozygous cotton homogenised seeds harbouring the DAS-81910-7 event, and
- ii) genomic DNA extracted by the applicant from conventional cotton homogenised seeds genetically similar to those harbouring the DAS-81910-7 event.

The control samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11⁹.

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 DAS-81910-7 cotton DNA and non-GM cotton DNA.

The calibration sample S1 was prepared by mixing the appropriate amount of DAS-81910-7 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-S3 were prepared by 5-fold serial dilutions from the S1 sample and sample S4 by an 8-fold serial 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)

⁹ 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).

Table 5. DAS-81910-7 blinded samples GM % contents

DAS-81910-7 GM %
GM copy number/cotton haploid genome copy number x 100
5.0
2.0
0.90
0.50
0.10

✓ Reaction reagents:

- JumpStart™ Taq ReadyMix™ (2x), one vial: 8.0 mL
- 100 mM MgCl₂, one vial: 0.58 mL
- ROX Reference Dye (1x), three vials: 0.50 mL each
- Nuclease-free water, one vial: 4.0 mL

✓ Primers and probes (1 tube each) as follows:

SAH7 taxon-specific assay

- Sah7-uni-f1 primer (10 µM): 300 µL
- Sah7-uni-r1 primer (10 µM): 210 µL
- Sah7-uni-s1 probe (10 µM): 150 µL

DAS-81910-7 assay

- 1706-f2 primer (10 µM): 250 µL
- 1706-r3 primer (10 µM): 250 µL
- 1706-p3 probe (10 µM): 130 µL

3.4.4 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 DAS-81910-7 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 DAS-81910-7 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.

3.4.5 Deviations reported from the protocol

Ten laboratories reported no deviations from the validation protocol. One laboratory reported an inverted position between two standard curve samples in one plate; one laboratory reported running the method at 45 cycles instead of 40 on an instrument for which the use of 45 cycles is suggested.

4. Results

4.1 EURL GMFF experimental testing

4.1.1 Zygosity ratio in the positive control sample

The results of the digital PCR analysis conducted by the EURL GMFF on the DAS-81910-7 and *SAH7* targets to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Zygosity ratio of the DAS-81910-7 and *SAH7* targets in the positive control sample.

Mean ratio (DAS-81910-7/ <i>SAH7</i>)	0.52
Standard deviation	0.03
RSD _r (%)	5.6
Standard error of the mean	0.01
Upper 95 % CI of the mean	0.53
Lower 95 % CI of the mean	0.50

The mean ratio (DAS-81910-7/*SAH7*) is 0.52. The 95 % confidence interval (CI) spans around 0.50, the expected ratio for a cotton control sample, homozygous for the GM-locus and assuming double-copy 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 provides the evidence for a homozygous status of the DAS-81910-7 event in *G. hirsutum*. The samples of the standard curve and test samples have been prepared as ratio of GM copies to haploid genome copy numbers.

Hence:

$$\begin{aligned}
 &0.1 \text{ GM \% in DNA copy number ratio related to haploid genome copy numbers} \\
 &= \\
 &0.1 \text{ GM \% in mass fraction}
 \end{aligned}$$

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

Test samples with GM levels from 5.0 % to 0.50 % 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 for robustness.

The standard curve parameters and the results of efficiency, linearity, trueness and precision obtained in the 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 5.0 % to 0.50 % in four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	DAS-81910-7 system			SAH7 system		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.32	100	1.00	-3.26	103	1.00
Run B	-3.33	100	1.00	-3.29	101	1.00
Run C	-3.32	100	1.00	-3.29	101	1.00
Run D	-3.27	102	1.00	-3.25	103	1.00
Run E	-3.30	101	1.00	-3.32	100	1.00
Run F	-3.29	101	1.00	-3.33	100	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 R² coefficient values were rounded to two digits.

	DAS-81910-7 system			SAH7 system		
	Slope	PCR efficiency*	R ²	Slope	PCR efficiency*	R ²
Run G	-3.30	101	1.00	-3.26	103	1.00
Run H	-3.34	99	1.00	-3.27	102	1.00
Run I	-3.33	100	1.00	-3.29	101	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 copies GM/copies haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	5.0	0.23	3.2
2.0	2.0	-2.2	1.9
0.90	0.93	3.1	4.1
0.50	0.48	-3.1	1.6
0.10	0.10	-1.4	18

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

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	4.9	-1.7	8
2.0	1.9	-4.2	11
0.90	0.86	-4.5	11
0.50	0.44	-11	10
0.10	0.10	-1.5	11

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

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	5.0	-0.66	2.2
2.0	2.0	-0.42	2.6
0.90	0.90	-0.17	1.8
0.50	0.50	0.35	7.9
0.10	0.11	5.4	15

4.2 Results of the international collaborative study

4.2.1 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 DAS-81910-7 system ranges from 86 % to 110 % and the linearity from 0.94 to 1.00; the amplification efficiency for the cotton-specific system ranges from 94 % to 114 % and the linearity from 0.98 to 1.00. The mean PCR efficiency was 101 % for DAS-81910-7 assay and 102 % for the *SAH7* one. The average R^2 of the methods was 0.99 and 1.00 for the DAS-81910-7 and *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	DAS-81910-7			SAH7		
		Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.70	86	0.98	-3.47	94	1.00
	B	-3.65	88	0.94	-3.25	103	0.98
2	A	-3.10	110	1.00	-3.19	106	1.00
	B	-3.21	105	1.00	-3.17	107	1.00
3	A	-3.24	103	1.00	-3.33	100	1.00
	B	-3.24	104	1.00	-3.33	100	1.00
4	A	-3.44	95	1.00	-3.42	96	0.99
	B	-3.37	98	1.00	-3.36	99	1.00
5	A	-3.45	95	1.00	-3.35	99	1.00
	B	-3.31	101	1.00	-3.35	99	1.00
6	A	-3.29	101	1.00	-3.29	101	1.00
	B	-3.27	102	0.99	-3.22	104	1.00
7	A	-3.36	99	1.00	-3.27	102	1.00
	B	-3.26	103	1.00	-3.32	100	1.00
8	A	-3.29	101	1.00	-3.32	100	1.00
	B	-3.20	105	1.00	-3.30	101	1.00
9	A	-3.24	104	1.00	-3.30	101	1.00
	B	-3.31	101	1.00	-3.24	104	1.00
10	A	-3.25	103	1.00	-3.25	103	1.00
	B	-3.46	95	0.99	-3.27	102	1.00
11	A	-3.15	108	1.00	-3.02	114	1.00
	B	-3.24	104	1.00	-3.05	112	1.00
12	A	-3.26	103	1.00	-3.22	104	1.00
	B	-3.31	101	1.00	-3.27	102	1.00
Mean		-3.32	101	0.99	-3.27	102	1.00

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

4.2.2 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

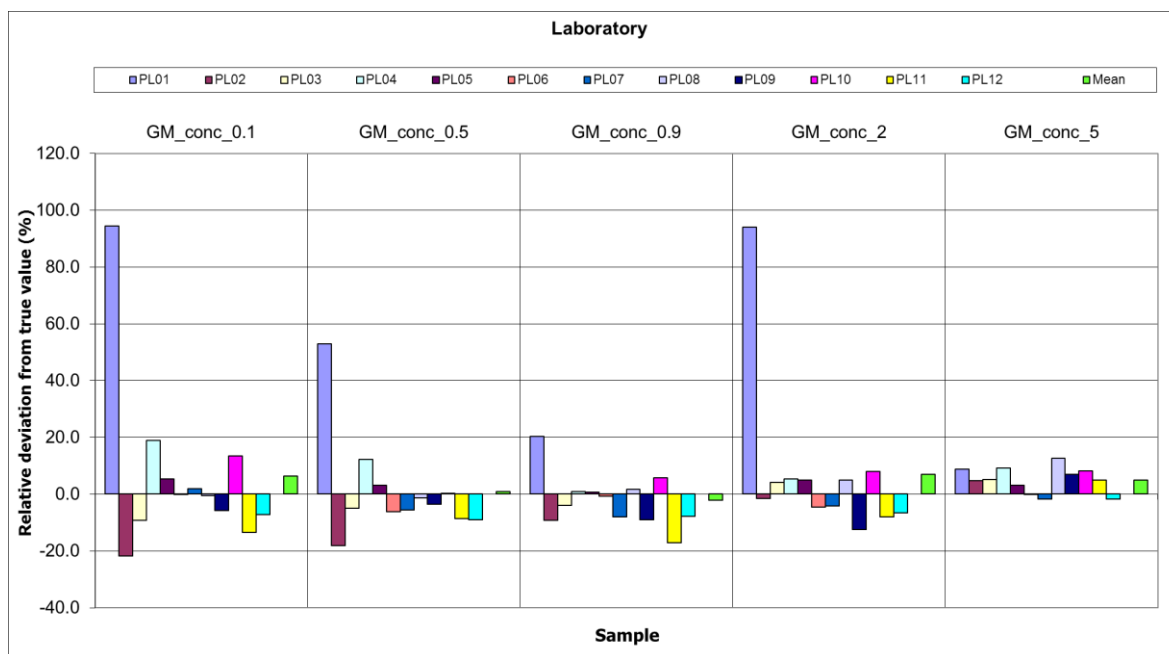
LAB	GMO content (%) *																			
	0.10				0.50				0.90				2.0				5.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.14	0.38	0.14	0.13	0.66	1.0	0.72	0.68	0.99	1.7	0.89	0.73	1.2	1.1	2.0	1.6	5.5	4.7	5.5	6.1
2	0.08	0.08	0.07	0.08	0.37	0.42	0.45	0.40	0.81	0.84	0.82	0.80	1.8	1.9	2.1	2.0	5.2	5.3	5.5	4.9
3	0.08	0.10	0.10	0.09	0.49	0.42	0.49	0.50	0.75	0.87	0.91	0.93	2.1	2.1	2.1	2.1	5.2	5.4	5.2	5.2
4	0.12	0.14	0.11	0.11	0.50	0.52	0.71	0.52	0.85	1.0	0.87	0.87	2.2	2.3	1.8	2.1	6.0	5.1	5.7	5.0
5	0.10	0.11	0.11	0.10	0.56	0.49	0.50	0.52	0.91	0.85	0.92	0.95	2.0	2.2	2.1	2.1	5.1	5.3	5.1	5.0
6	0.09	0.09	0.11	0.11	0.48	0.45	0.43	0.52	0.90	0.81	1.0	0.87	1.8	1.9	2.1	1.9	5.0	4.9	5.2	4.8
7	0.11	0.08	0.09	0.13	0.48	0.47	0.43	0.51	0.84	0.85	0.83	0.79	1.8	2.0	1.9	2.0	5.3	4.7	4.9	4.7
8	0.09	0.09	0.10	0.12	0.48	0.47	0.52	0.50	0.93	0.87	0.95	0.91	2.1	1.9	2.3	2.2	5.9	5.6	5.8	5.3
9	0.10	0.10	0.09	0.09	0.62	0.42	0.44	0.44	0.82	0.81	0.84	0.81	1.7	1.8	1.8	1.8	4.6	4.7	4.8	7.4
10	0.14	0.10	0.09	0.12	0.46	0.43	0.55	0.56	1.1	0.87	1.0	0.80	1.9	2.5	2.2	2.0	4.6	5.5	5.7	5.9
11	0.08	0.10	0.07	0.10	0.40	0.43	0.56	0.44	0.73	0.72	0.78	0.74	1.9	1.9	1.6	2.0	4.8	5.4	6.0	4.8
12	0.10	0.09	0.09	0.09	0.45	0.46	0.41	0.49	0.87	0.80	0.83	0.82	1.9	1.8	1.9	1.9	5.2	4.6	5.1	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 for each GM level before eliminating outliers.

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



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 5.0 % twelve laboratories were within the limit; at GM-levels 0.10 %, 0.50 % and 2.0 % eleven laboratories were within the limit. One laboratory overestimated GM-levels 0.10 %, 0.50 % and 2.0 % by more than 25 %. No clear trend for over- or underestimation was observed.

4.2.3 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 16 % at the 0.10 % GM level, thus within the acceptance criterion.

Table 13. Summary of validation results for the DAS-81910-7 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	1	1	1	1	1
Reason for exclusion*	C	C	C	C	C
Mean value	0.10	0.48	0.86	2.0	5.2
Relative repeatability standard deviation, RSD_r (%)	12	12	7.4	7.3	6.9
Repeatability standard deviation	0.01	0.06	0.06	0.14	0.36
Relative reproducibility standard deviation, RSD_R (%)	16	13	9.5	9.3	7.5
Reproducibility standard deviation	0.02	0.06	0.08	0.18	0.39
Bias** (absolute value)	-0.002	-0.02	-0.04	-0.02	0.24
Bias (%)	-1.7	-3.8	-4.3	-0.92	4.8

* 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 12 % at the 0.10 % and 0.50 % GM levels.

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 4.8 % at the 5.0 % GM level.

5. Compliance of the method for detection and quantification of event DAS-81910-7 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 mass fraction of GM-material) was 14.0 %, 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 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 the RSD_r of the method was 12 % at the level of 0.1 % related to mass fraction of GM-material, therefore also below 25 % and 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 DAS-81910-7 at or around 0.1 % level related to mass fractions of GM material.

Source	RSD_r %	GM %
Applicant's method optimisation	14 %	0.1 %
EURL GMFF tests	11 - 18 %	0.1 %
Collaborative study	12 %	0.1 %

Based on the results of the EURL GMFF in-house verification and of the international collaborative study, it is concluded that the RSD_r % is below 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.

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 3.4.3), 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.

7. 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. Plant DNA C-values Database, <http://data.kew.org/cvalues/>

Annex 1: Event-specific Method for the Quantification of Cotton DAS-81910-7 by Real- time PCR

Validated Method

Method development:

Dow AgroSciences 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 DAS-81910-7 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 DAS-81910-7, a 120 bp fragment of the region spanning the 5' plant-to-insert junction in cotton DAS-81910-7 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 (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of GM event DAS-81910-7, a cotton taxon-specific system amplifies a fragment of a cotton *Sinapis Arabidopsis Homolog 7* (*SAH7*) endogenous gene (Accession number, GeneBank: FN610856.1), using *SAH7* gene-specific primers and an *SAH7* gene-specific probe labelled with FAM as reporter dye at its 5' end and TAMRA as quencher dye at its 3' end.

The *SAH7* gene is present not only in the A-subgenome, but also in the D-subgenome of *Gossypium hirsutum*. The A-subgenome specific copy differs from the D-subgenome specific copy by several single or double nucleotide substitutions and small deletions/insertions. The primers and probe of the cotton-specific reference PCR system match perfectly without any single mismatch to both subgenome gene copies. However, due to sequence differences within the amplified region, the size of the amplicons resulting from the A- and D-subgenomes differs slightly, being respectively 115 bp and 123 bp of length.

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 DAS-81910-7 DNA in a test sample, Cq values for the DAS-81910-7 and the *SAH7* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DAS-81910-7 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 October-November 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 0.035 % (copies GM/total haploid genome copies) 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 % (copies GM/total haploid genome copies) in 150 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' plant-to-insert junction in cotton DAS-81910-7 and is therefore event-specific for the event DAS-81910-7.

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 DAS-81910-7

3.2.1 General

The real-time PCR set-up for the taxon (*SAH7*) and the GMO (event DAS-81910-7) 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 DAS-81910-7) and the taxon (*SAH7*) assay with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curves are established on four calibration samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % cotton DAS-81910-7 DNA in a total of 175 ng of cotton DNA (corresponding to 75107 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 to be prepared by serial dilutions (dilution factor 5 for samples S2-S3 and dilution factor 8 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)*	175	35	7	0.88
Cotton haploid genome copies	75107	15021	3004	376
DAS-81910-7 copies	7511	1502	300	38

* 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 DAS-81910-7 cotton specific system (Table 2) and the *SAH7* reference gene system (Table 3). According to the method developer, the ROX concentration has to be adjusted depending on the real-time PCR instrument used (§ <https://biotium.com/faqs/what-rox-concentration-should-i-use-in-my-qpcr-reaction/>).

The three options (No ROX, 0.1x ROX and 1x ROX) are reported as follows and are indicated in Tables 2 and 3:

- *'No ROX' applies to instruments:*
 - BioRad: iCycler™, MyiQ™, MiQ™2, iQ™5, CFX-96 Touch™, CFX-384 Touch™, Chromo4™, MiniOpticon™
 - Qiagen: Rotor-Gene® Q, Rotor-Gene® 3000, Rotor-Gene® 6000
 - Eppendorf: Mastercycler® Realplex
 - Illumina: Eco™ RealTime PCR System
 - Cepheid: SmartCycler®
 - Roche: LightCycler® 480, LightCycler® 2.0
- *'0.1x ROX' applies to instruments:*
 - ABI: 7500, 7500 Fast, ViiA 7™, QuantStudio™
 - Stratagene: MX4000P, MX3000P, MX3005P
- *'1x ROX' applies to instruments:*
 - ABI: 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne®, StepOne Plus®

Please note that additional volume is included 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 DAS-81910-7 assay.

Component	Final concentration	µL/reaction
Jumpstart™ Taq ReadyMix™ (2x)	1x	12.5
10x ROX	No ROX / 0.1x / 1x*	0 / 0.25 / 2.5*
MgCl ₂ (100 mM)	3.5 mM	0.88
1706-f2 (10 µM)	300 nM	0.75
1706-r3 (10 µM)	300 nM	0.75
1706-p3** (10 µM)	150 nM	0.38
Nuclease free water	-	4.75 / 4.5 / 2.25*
DNA	-	5
Total reaction volume:		25 µL

* see 3.2.3 for the selection of the ROX concentration according to the instrument used

** 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
Jumpstart™ Taq ReadyMix™ (2x)	1x	12.5
10x ROX	No ROX / 0.1x / 1x*	0 / 0.25 / 2.5*
MgCl ₂ (100 mM)	3.5 mM	0.88
Sah7-uni-f1 (10 µM)	350 nM	0.88
Sah7-uni-r1 (10 µM)	250 nM	0.63
Sah7-uni-s1** (10 µM)	175 nM	0.44
Nuclease free water	-	4.69 / 4.44 / 2.19*
DNA	-	5
Total reaction volume:		25 µL

* see 3.2.3 for the selection of the final ROX concentration according to the instrument used

** TaqMan® probe is labelled with 6-FAM 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 DAS-81910-7 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 DAS-81910-7 cotton system and 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.

6. Spin down the tubes. Aliquot 25 µL for the DAS-81910-7 system and for the *SAH7* 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 (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 DAS-81910-7 and for the *SAH7* reference system. Define TAMRA as quencher dye for the DAS-81910-7 specific system and for the *SAH7* reference system. Select ROX as the passive reference dye. Enter the correct reaction volume (25 µL).
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 DAS-81910-7/*SAH7* assays.

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

* 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 Cq 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 Cq = 25, set the baseline crossing at Cq = 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 Cq values for each reaction.

The standard curves are generated both for the *SAH7* and the DAS-81910-7 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 DAS-81910-7 DNA in the unknown sample, the DAS-81910-7 copy number is divided by the cotton haploid genome copy number measured with the endogenous gene *SAH7* and multiplied by 100 ($GM\% = \text{DAS-81910-7} / \text{SAH7} \times 100$).

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

- JumpStart™ Taq ReadyMix™ Sigma-Aldrich®, catalogue number P2893
- Nuclease-free Water (e.g. HPLC Gradient Grade) Ambion®, product number AM9937
- 1 M MgCl₂ Sigma-Aldrich®, catalogue number M1028
- ROX Reference Dye Invitrogen, catalogue number 12223012

4.3 Primers and Probes

Table 5. Primers and probes for the DAS-81910-7 and *SAH7* methods

	Name	DNA Sequence (5' to 3')	Length (nt)
DAS-81910-7			
Forward primer	1706-f2	AAG CTT AGG TGA TTT CGA TGA TG	23
Reverse primer	1706-r3	GAC CTC AAT TGC GAG CTT TC	20
Probe	1706-p3	FAM - CAC ACC AAA AGT TAG GCC CG - TAMRA	20
<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	FAM - AAA CAT AAA ATA ATG GGA ACA ACC ATG ACA TGT - TAMRA	33

FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.

5. References

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

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