

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Event-specific Method for the Quantification of Cotton MON 88701 Using Real-time PCR

Validation report

European Union Reference Laboratory for
Genetically Modified Food and Feed

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Event-specific Method for the Quantification of Cotton MON 88701 Using Real-time PCR

Validation Report

24 June 2016

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate¹ the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting and quantifying cotton event MON 88701 (unique identifier MON-887Ø1-3). The validation study was conducted according to the EURL GMFF validation procedure (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>) and internationally accepted guidelines⁽¹⁻⁵⁾.

In accordance with current EU legislation², Monsanto Company provided the detection method and the positive and negative control samples (genomic DNA extracted from cotton seeds harbouring the MON 88701 event as positive control DNA, genomic DNA extracted from conventional cotton seeds as negative control DNA). The EURL GMFF verified the performance data provided by the applicant, where necessary experimentally, prepared the validation samples (calibration samples and blind samples at different GM percentage [MON 88701-DNA mass/total cotton-DNA mass]), organised an international collaborative study, and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method, in line with the provisions of Annex III-3.C.2 to Regulation (EU) No 503/2013, meets most of the method performance requirements, as established by the EURL GMFF and the ENGL, and it fulfils the analytical requirements of Regulation (EU) No 619/2011³. In agreement with the Steering

¹ Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed".

² Regulation (EC) No 503/2003 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".

³ 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.

Committee of the ENGL, the EURL GMFF concludes that the method is by all means fit for purpose and can be used for regulatory control of food and feed. Laboratories using it for the purposes of Regulation (EU) No 619/2011 are asked to provide the EURL GMFF with their experimental data and results in order to allow further verification of the performance of the method in that part of the dynamic range.

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR)]

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

The EURL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

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

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1. INTRODUCTION

In line with Regulation (EC) No 1829/2003, Monsanto Company provided the EURL GMFF with an event-specific method for detection and quantification of cotton event MON 88701 (unique identifier MON-887Ø1-3) together with genomic DNA as positive and negative control samples.

The dossier was found to be complete (step1) and the scientific dossier evaluation (step 2) concluded that the method meets the ENGL method acceptance criteria⁴.

In step 3 of the procedure (experimental testing), the EURL GMFF verified the purity of the control samples and conducted an in-house testing of the method provided and concluded that the method performance was adequate for organising an international collaborative study.

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

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

Documentation and the data provided 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.

2.1 Bioinformatics analysis

The detection method spans the junction between the transgenic insert and the 3' genomic region (the forward primer anneals to the T-DNA border region, the reverse primer anneals to cotton genomic sequence, and the probe spans the two). The sequence-tagged site (STS) of MON 88701 insert was analysed by BLAST (NCBI)⁶ against the "nt" and "patents" databases, and no significant similarity was found with any other published sequence (except the first part corresponding to the *A. tumefaciens* T border sequence that is found in many vector sequences). In addition, the primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System of the JRC, as well as the whole genomes of *Brassica rapa*, *Glycine max*, *Oryza indica*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays* using the e-PCR prediction tool (NCBI), and no potential amplicon was identified. The amplicon size is expected to be 84 bp, consistent to what reported by the applicant. On the basis

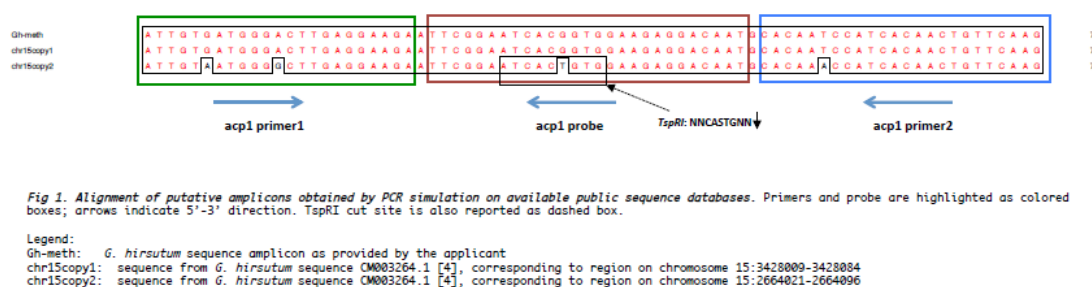
⁴ EURL GMFF/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>).

of the bioinformatics analysis, based on sequence information provided by the applicant, it can be concluded that the event specific assay is indeed event specific.

The specificity of the cotton taxon-specific *acyl carrier protein* (*acp1*) system was previously validated by the EURL GMFF and it was confirmed *in-silico* by the EURL GMFF using updated sequence information of the cotton genome available in public databases.

GMO quantification in copy numbers requires that the number of copies of the reference gene, against which the GM content is established, is known, and ideally a reference gene should only exist in single copy per genome. The allotetraploid cotton species *Gossypium hirsutum*, subject of the present application, derives from interspecific hybridization between *G. arboreum* and *G. raimondii*, carrying the A- and D-subgenome, respectively. The *G. hirsutum* ploidy is AADD, $2n = 52$ and because of such a complexity, its genomic sequence was determined only recently ⁽⁷⁾, while the genomic sequences of its progenitors were disclosed and published previously ⁽⁸⁻¹⁰⁾.

An *in silico* PCR simulation was performed on the recent genomic sequence by scanning the cotton contig sequences. Two potential amplicons were found, both located on chromosome 15 (GenBank ID CM003264.1, see figure below). The sequence of one amplicon (chr15copy1 in the figure below) is identical to the sequence submitted by the applicant (Gh-meth) and the sequence of the other amplicon (chr15copy2) has three mismatches in the region covered by *acp1* primers and one mismatch in the region covered by *acp1* probe, generating one *TspRI* restriction site as shown in the figure. Therefore the proposed cotton taxon-specific method (*acp1*) can potentially target these two regions on chromosome 15 and for this reason, as further explained in paragraph 3.2, the validation exercise has been executed in mass fraction of GM DNA.



2.2 ENGL method acceptance criteria

The parameters of the calibration curves (slope, R^2 coefficient) were determined by the applicant by quantifying three different GM levels (10%, 1% and 0.085%), each in fifteen replicates, expressed in mass fraction of GM material (mass/mass), i.e. relative content of MON 88701 DNA to total cotton DNA (see Table 1).

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

| | MON 88701 | <i>acp1</i> |
|-------------------------|------------------|--------------------|
| Slope | -3.49 | -3.43 |
| R^2 | 1.00 | 1.00 |

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall be within the range of -3.1 to -3.6 , and the R^2 shall be ≥ 0.98 .

Table 1 indicates that the slope and R^2 coefficient of the standard curves for the GM-system (MON 88701) and the cotton-specific (*acp1*) system, as established by the applicant, were within the ENGL acceptance criteria.

Also precision and trueness of the method were established by the applicant based on fifteen values for each of three GM levels (expressed as mass fraction of GM-material). Table 2 reports mean values of precision and trueness for the three GM-levels as provided by the applicant. Both parameters were within the ENGL acceptance criteria (trueness $\pm 25\%$, RSDr $\leq 25\%$ across the entire dynamic range).

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

| Expected GMO % | Test results | | |
|-----------------------|---------------------|------------|-----------|
| | 0.085 | 1.0 | 10 |
| Measured mean % | 0.083 | 1.0 | 9.7 |
| Precision (RSDr %) | 12 | 4.2 | 5.9 |
| Trueness (bias %) | -2.5 | 2.9 | -3.3 |

3. Step 3 and step 4 (experimental testing of samples and methods)

3.1 DNA extraction

Genomic DNA was isolated by the applicant from MON 88701 and non-GM cotton seeds using the CTAB method followed by PEG precipitation. That DNA extraction method was validated by the EURL GMFF in the context of the application for cotton MON1445 and was found to function suitably for the assessed matrix. The assessment report is published at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

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 MON 88701.

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 MON 88701 DNA to total cotton DNA. The procedure consists of two simplex systems, in which a cotton specific assay (*acp1*) and the GM target assay (MON 88701) are performed in separate wells.

For the detection of GM event MON 88701, an 84-bp fragment of the region spanning the 3' insert-to-plant junction in cotton MON 88701 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 at its 3' end.

For the relative quantification of GM event MON 88701, a cotton taxon-specific reference system amplifies a 76-bp fragment of *acp1*, a cotton endogenous gene encoding an *acyl carrier protein*, using *acp1* gene-specific primers and an *acp1* gene-specific probe labelled with FAM as reporter dye at its 5' end, and TAMRA as quencher at its 3' end.

The EURL GMFF normally designs the validation trial in terms of copy numbers of GM material and total cotton genome material.

However, this exercise requires ideally the use of a single copy reference system or, at least, of a reference system whose copy number is exactly known. Given the knowledge built in the recent years in genome sequences, the bioinformatics analyses revealed (Chapter 2) that the taxon-specific method (*acp1*) might target two distinct regions on *G. hirsutum* chromosome 15; for this reason the validation exercise is here expressed in mass fraction of GM DNA, given the fact that the available CRM is certified in mass.

Standard curves are generated for both the MON 88701 and the *acp1* systems by plotting the C_q values measured for the calibration points against the logarithm of the amount of MON 88701 DNA and by fitting a regression line into these data. Thereafter, the amount of MON 88701 DNA in the test sample is estimated by interpolation from the standard curves.

The amount of MON 88701 DNA is divided by the amount of the cotton reference gene (*acp1*) and multiplied by 100 to obtain the percentage value (GM% = MON 88701/ *acp1* x 100).

The GM contents of the calibration samples and the total DNA quantity used in PCR are provided in Table 3.

Note: Numerical values presented in the tables of this report were rounded keeping two digits for values ≤ 1 , one digit for values between 1 and 10 and no digit for values ≥ 10 . The calculations in the MS Excel files however were done over not rounded data. This approach might generate small inconsistencies in the numerical values reported in the tables.

Table 3. GM% values of the standard curve samples.

| Sample code | S1 | S2 | S3 | S4 | S5 |
|--------------------------------------|-----------|-----------|-----------|-----------|-----------|
| Amount of total DNA in reaction (ng) | 200 | 67 | 22 | 5.6 | 1.4 |
| % GM-DNA | 10 | 10 | 10 | 10 | 10 |
| Amount of GM DNA (ng) | 20 | 6.7 | 2.2 | 0.56 | 0.14 |

3.3 EURL GMFF experimental testing (step 3)

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

The method performance characteristics were verified by the EURL-GMFF by quantifying five blinded test samples containing a range of MON 88701 levels (10%-0.1% in mass fraction of GM material). The experiments were performed under repeatability conditions first on an ABI 7900 real-time platform and then repeated on the Roche LC480 equipment, and followed the protocol provided by the applicant. Test samples with GM levels 10%, 5.0%, 2.0% and 0.9% were tested in two real-time PCR runs with two replicates for each GM level on each plate (total of four replicates per GM level). The test sample with GM level 0.1% was tested in 15 replicates in an additional run. 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.

In order to assess the method compliance with Regulation (EU) No 619/2011, the EURL GMFF estimated the method precision (RSDr %) on 15 replicates at 0.1% GM level in mass fraction.

3.4 International collaborative study (step 4)

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

- i. The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) ⁽¹⁾

- ii. 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 assess in 12 laboratories the performance of the PCR analytical method provided by the applicant and in-house verified by the EURL GMFF.

3.4.1 List of participating laboratories

The 12 participants in the MON 88701 validation study were randomly selected from the 26 National Reference Laboratories (NRLs) that offered to participate.

The selected laboratories received a detailed validation protocol and were instructed to strictly follow this protocol. Any deviation had to be reported back. The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the international collaborative study of the detection method for cotton MON 88701.

| Laboratory | Country |
|--|----------------|
| Institute of Chemical Technology Prague | CZ |
| Plant Health Laboratory | FR |
| Food and Environment Research Agency | UK |
| Laboratory of DNA analysis - Department of Gene Technology - Tallinn University of Technology | EE |
| RIKILT Institute of Food Safety | NL |
| Environment Agency Austria | AT |
| National Centre for Food, Spanish Food Safety Agency and Nutrition | ES |
| National Food Agency, Science Department | SE |
| Scientific Institute of Public Health | BE |
| Plant Breeding and Acclimatization Institute – National Research Institute, GMO Controlling Laboratory | PL |
| Agricultural Institute of Slovenia | SI |
| State Sanitary and Epidemiological Station, Regional Laboratory of Genetically Modified Food | PL |

3.4.2 Real-time PCR equipment used in the study

The laboratories involved in the collaborative study used a range of real-time PCR equipment: seven laboratories used the ABI 7500, two used the ABI 7900, one used the ABI StepOne Plus, one used the Stratagene Mx3005P and one used BioRad CFX-96.

The variability of equipment, with its known potential influence on PCR results, reflects the real situation in the control laboratories and the fact that in this case it did not significantly influence the performance of the method provides additional assurance that the method is useable 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:

- genomic DNA extracted by the applicant from homozygous cotton seeds harbouring the event MON 88701, and
- genomic DNA extracted by the applicant from conventional cotton seeds genetically similar to those harbouring the MON 88701 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 2.11⁵.

These control samples were used by the EURL GMFF to prepare calibration samples (of known GMO content) and test samples (of undisclosed GM content = blinded samples) by mixing MON 88701 cotton DNA and non-GM cotton DNA.

The calibration sample S1 was prepared by mixing the appropriate amount of MON 88701 DNA with control non-GM cotton DNA to obtain a 10% GM-sample (in mass fraction of GM-material). Calibration samples S2 and S3 were prepared by serial three-fold dilution from the S1 sample and samples S4 and S5 were prepared by serial four-fold dilution from the S3 sample.

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

Five calibration samples with known concentrations of GM-event (140 µL of DNA solution each) labelled from S1 to S5 (Table 3).

Twenty blinded test DNA samples (70 µL of DNA solution each at 45 ng/µL) labelled from U1 to U20, representing five GM levels expressed in mass fractions of GM DNA (Table 5).

⁵ 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. MON 88701 content of test samples

| MON 88701 GM% (MON 88701-DNA mass/total cotton DNA mass) |
|--|
| 10 |
| 5.0 |
| 2.0 |
| 0.90 |
| 0.10 |

Reaction reagents:

TaqMan[®] Universal PCR Master Mix UNG (2x), two vials: 8 mL
sterile distilled water, one tube: 12 mL

Primers and probes (1 tube each) as follows:

acp1 taxon-specific method

acp1 primer 1 (10 µM): 250 µL
acp1 primer 2 (10 µM): 250 µL
acp1 probe (10 µM): 80 µL

MON 88701 method

MON 88701 primer 1 (10 µM): 500 µL
MON 88701 primer 2 (10 µM): 500 µL
MON 88701 probe (10 µM): 400 µL

3.4.4 Design of the collaborative study

Participating laboratories received a detailed validation protocol including the exact design of the PCR plates to ensure that on each PCR plate all samples are analysed in parallel for the MON 88701 and the *acp1* specific systems. In total, two plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for the MON 88701 and *acp1* assays in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per determined plate lay-out.

The amplification reactions 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

Seven laboratories reported no deviations from the protocol. One laboratory repeated one run due to the erroneous application of one standard while performing the run and one other laboratory repeated one run due to a pipetting error for the NTC.

One laboratory encountered some technical difficulties during the analyses (bad PCR replicates). Two laboratories set the reaction volume to a total of 30 μ L instead of 50 μ L.

4. Results

4.1 EURL GMFF experimental testing

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

Test samples with GM levels 10%, 5.0%, 2.0% and 0.9% in mass fraction of GM material were tested by the EURL GMFF in two real-time PCR runs (run A and B on ABI 7900 and run D and E on Roche LC480) with two replicates for each GM-level on each plate (total of four replicates per GM-level). The sample with a 0.1% GM level was tested in 15 replicates in one run (run A to C on ABI 7900 and run D to F on Roche LC480). The corresponding standard curve parameters are shown in Tables 7a and 7b and in Tables 8a and 8b.

Table 7a. Standard curve parameters of the real-time PCR testing carried out on ABI 7900

| | MON 88701 method | | | acp1 reference method | | |
|-------|------------------|-----------------|----------------|-----------------------|-----------------|----------------|
| | Slope | PCR efficiency* | R ² | Slope | PCR efficiency* | R ² |
| Run A | -3.43 | 96 | 1.00 | -3.42 | 96 | 1.00 |
| Run B | -3.34 | 99 | 1.00 | -3.39 | 97 | 1.00 |
| Run C | -3.49 | 93 | 0.99 | -3.44 | 95 | 1.00 |

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

Table 7b. Standard curve parameters of the real-time PCR testing carried out on Roche LC[®]480

| | MON 88701 method | | | acp1 reference method | | |
|-------|------------------|-----------------|----------------|-----------------------|-----------------|----------------|
| | Slope | PCR efficiency* | R ² | Slope | PCR efficiency* | R ² |
| Run D | -3.46 | 94 | 1.00 | -3.45 | 95 | 1.00 |
| Run E | -3.44 | 95 | 1.00 | -3.41 | 96 | 1.00 |
| Run F | -3.48 | 94 | 1.00 | -3.44 | 95 | 1.00 |

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

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R² coefficient shall be ≥ 0.98 .

Tables 7a and 7b document that the slopes of the standard curves, and the R² coefficients were in all cases within the limits established by the ENGL.

Table 8a. Outcome of in-house tests, with regards to the quantification of the five test samples. Tests carried out on ABI 7900

| Target GM-level %* | Measured GM-level % | Bias % of the target GM-level | Precision (RSDr %) |
|--------------------|---------------------|-------------------------------|--------------------|
| 10 | 9.5 | -4.8 | 4.9 |
| 5.0 | 4.3 | -14 | 12 |
| 2.0 | 1.9 | -6.8 | 8.2 |
| 0.90 | 0.78 | -13 | 13 |
| 0.10 | 0.09 | -12 | 15 |

* mass fraction GM DNA

Table 8b. Outcome of in-house tests, with regards to the quantification of the five test samples. Tests carried out on Roche LC[®]480

| Target GM-level % | Measured GM-level % | Bias % of the target GM-level | Precision (RSDr %) |
|-------------------|---------------------|-------------------------------|--------------------|
| 10 | 10 | 1.3 | 2.9 |
| 5.0 | 4.6 | -7.3 | 3.3 |
| 2.0 | 2.0 | -1.0 | 3.8 |
| 0.90 | 0.90 | -0.46 | 3.0 |
| 0.10 | 0.09 | -6.29 | 3.3 |

* mass fraction GM DNA

According to the ENGL method acceptance criteria the method's trueness, measured as bias %, should be within $\pm 25\%$ of the target value over the entire dynamic range. The method's precision, estimated as RSDr % (relative repeatability standard deviation), should be $\leq 25\%$ over the dynamic range. Tables 8a and 8b document that trueness and precision of quantification were within the limits established by the ENGL for both PCR machines used.

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 curves, reported by participating laboratories are displayed in Table 9. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

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

Table 9 indicates that the efficiency of amplification for the MON 88701 system ranges from 78% to 111% and the linearity from 0.97 to 1.00; the amplification efficiency for the cotton specific system ranges from 78% to 90% and the linearity from 0.99 to 1.00. The mean PCR efficiency is 87% for the MON 88701 assay and 85% for the *acp1* assay.

Table 9. Values of slope, PCR efficiency and R² obtained during the validation study

| Lab | Plate | MON 88701 | | | acp1 | | |
|------|-------|-----------|--------------------|----------------|-------|--------------------|----------------|
| | | Slope | PCR Efficiency (%) | R ² | Slope | PCR Efficiency (%) | R ² |
| 1 | A | -3.93 | 80 | 0.99 | -3.96 | 79 | 1.00 |
| | B | -3.65 | 88 | 0.99 | -3.75 | 85 | 1.00 |
| 2 | A | -3.64 | 88 | 1.00 | -3.67 | 87 | 1.00 |
| | B | -3.70 | 86 | 1.00 | -3.69 | 87 | 1.00 |
| 3 | A | -3.78 | 84 | 1.00 | -3.83 | 82 | 0.99 |
| | B | -3.80 | 83 | 0.99 | -3.75 | 85 | 0.99 |
| 4 | A | -3.78 | 84 | 1.00 | -3.81 | 83 | 1.00 |
| | B | -3.70 | 86 | 1.00 | -3.72 | 86 | 1.00 |
| 5 | A | -3.85 | 82 | 1.00 | -3.87 | 81 | 1.00 |
| | B | -3.87 | 81 | 1.00 | -3.89 | 81 | 1.00 |
| 6 | A | -3.50 | 93 | 1.00 | -3.69 | 86 | 1.00 |
| | B | -3.60 | 90 | 1.00 | -3.68 | 87 | 1.00 |
| 7 | A | -3.85 | 82 | 1.00 | -3.70 | 86 | 1.00 |
| | B | -3.67 | 87 | 0.99 | -3.73 | 85 | 1.00 |
| 8 | A | -3.61 | 89 | 1.00 | -3.62 | 89 | 1.00 |
| | B | -3.63 | 89 | 1.00 | -3.66 | 87 | 1.00 |
| 9 | A | -3.08 | 111 | 0.97 | -3.80 | 83 | 1.00 |
| | B | -4.00 | 78 | 0.98 | -3.99 | 78 | 0.99 |
| 10 | A | -3.57 | 90 | 0.99 | -3.59 | 90 | 0.99 |
| | B | -3.69 | 87 | 1.00 | -3.57 | 90 | 0.99 |
| 11 | A | -3.61 | 89 | 0.99 | -3.70 | 86 | 1.00 |
| | B | -3.57 | 91 | 1.00 | -3.73 | 85 | 1.00 |
| 12 | A | -3.89 | 81 | 1.00 | -3.80 | 83 | 1.00 |
| | B | -3.88 | 81 | 1.00 | -3.86 | 82 | 1.00 |
| Mean | | -3.70 | 87 | 0.99 | -3.75 | 85 | 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 .

Both values, -3.70 for the GM system and -3.75 for the reference system, were outside the ENGL method acceptance criteria; the average R² for the MON 88701 and the *acp1* assays is respectively 0.99 and 1.00.

The EURL GMFF carefully investigated the results of the international collaborative study in terms of slope values and the experimental tests are outlined in Annex 2. A clear explanation for the slightly unsatisfactory slopes was finally not found but there are indications that the genomic DNA might have been damaged during the delivery of the samples for the ring trial, which impacted the PCR efficiency negatively.

4.2.2 GMO quantification

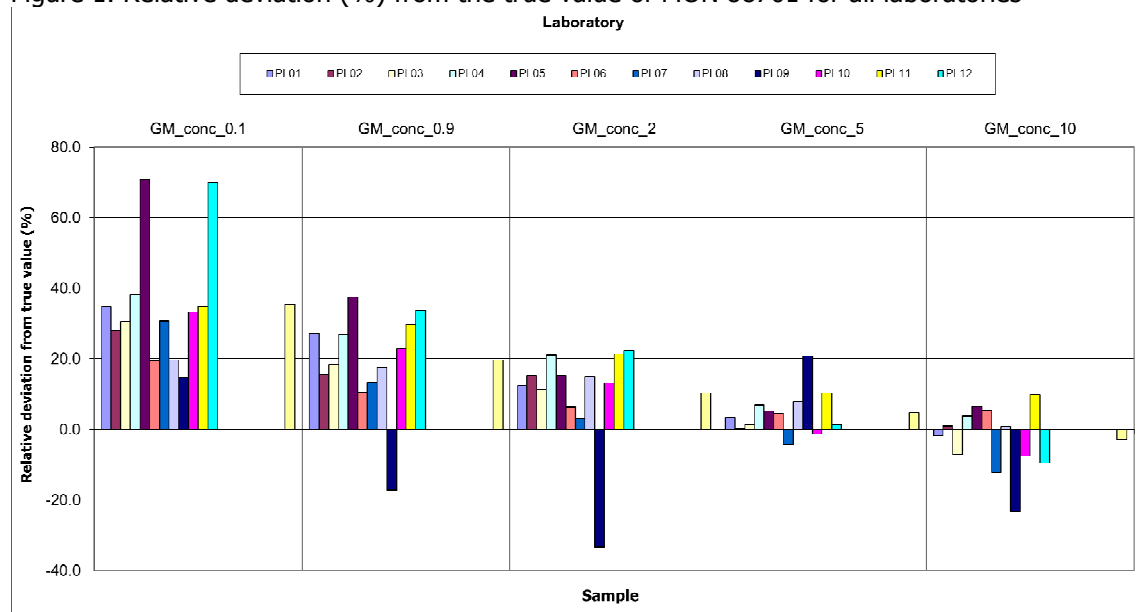
Table 10 reports the values of the four replicates for each GM level as provided by all laboratories. This is expressed in terms of mass fraction of GM material (mass/mass).

Table 10. GM% values determined by laboratories for the test samples, including outliers.

| LAB | GMO content (%) | | | | | | | | | | | | | | | | | | | |
|-----------|-----------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| | 0.1 | | | | 0.9 | | | | 2.0 | | | | 5.0 | | | | 10 | | | |
| | 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 | 0.15 | 0.12 | 0.12 | 0.14 | 1.03 | 0.93 | 1.46 | 1.16 | 2.13 | 2.01 | 2.34 | 2.51 | 5.09 | 5.09 | 5.21 | 5.30 | 8.95 | 9.02 | 10.88 | 10.49 |
| 2 | 0.13 | 0.12 | 0.14 | 0.12 | 0.98 | 1.06 | 1.12 | 1.00 | 2.35 | 2.33 | 2.28 | 2.27 | 5.24 | 5.22 | 5.00 | 4.60 | 9.68 | 9.80 | 9.81 | 11.07 |
| 3 | 0.13 | 0.11 | 0.15 | 0.13 | 1.04 | 1.05 | 1.01 | 1.16 | 2.07 | 2.27 | 2.33 | 2.24 | 4.90 | 5.44 | 5.46 | 4.49 | 8.83 | 8.83 | 10.76 | 8.75 |
| 4 | 0.15 | 0.13 | 0.13 | 0.14 | 1.04 | 1.16 | 1.23 | 1.14 | 2.37 | 2.45 | 2.51 | 2.35 | 5.27 | 5.48 | 5.36 | 5.29 | 10.55 | 10.35 | 10.50 | 10.10 |
| 5 | 0.18 | 0.15 | 0.18 | 0.18 | 1.14 | 1.36 | 1.28 | 1.16 | 2.47 | 2.52 | 1.98 | 2.25 | 5.19 | 5.86 | 5.11 | 4.88 | 10.49 | 11.01 | 10.21 | 10.92 |
| 6 | 0.10 | 0.14 | 0.13 | 0.10 | 1.04 | 0.97 | 1.01 | 0.96 | 2.17 | 2.19 | 2.00 | 2.15 | 5.24 | 5.29 | 5.15 | 5.22 | 10.82 | 9.97 | 10.71 | 10.61 |
| 7 | 0.13 | 0.12 | 0.13 | 0.14 | 0.98 | 1.01 | 1.12 | 0.96 | 1.94 | 1.97 | 2.24 | 2.10 | 4.91 | 4.99 | 4.73 | 4.50 | 8.60 | 8.71 | 9.26 | 8.62 |
| 8 | 0.11 | 0.13 | 0.13 | 0.11 | 1.13 | 1.00 | 1.06 | 1.04 | 2.38 | 2.32 | 2.25 | 2.25 | 5.17 | 5.39 | 5.73 | 5.27 | 10.32 | 10.05 | 9.76 | 10.18 |
| 9 | 0.05 | 0.25 | 0.13 | 0.03 | 1.34 | 0.80 | 0.44 | 0.41 | 1.74 | 1.90 | 0.90 | 0.81 | 5.14 | 8.25 | 3.98 | 6.79 | 9.41 | 4.34 | 11.46 | 5.48 |
| 10 | 0.14 | 0.14 | 0.13 | 0.12 | 0.96 | 1.24 | 1.10 | 1.12 | 2.53 | 1.91 | 2.22 | 2.40 | 5.10 | 4.99 | 4.92 | 4.73 | 9.56 | 8.95 | 9.16 | 9.34 |
| 11 | 0.14 | 0.11 | 0.14 | 0.15 | 1.04 | 1.22 | 1.19 | 1.22 | 2.38 | 2.67 | 2.31 | 2.35 | 5.52 | 5.67 | 5.24 | 5.63 | 11.22 | 10.66 | 11.46 | 10.61 |
| 12 | 0.17 | 0.15 | 0.18 | 0.18 | 1.27 | 1.25 | 1.17 | 1.12 | 2.49 | 2.45 | 2.48 | 2.36 | 5.42 | 5.02 | 4.70 | 5.16 | 8.68 | 9.81 | 8.72 | 8.98 |

A graphical representation of the data reported in Table 10 is provided in Figure 1, where the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the deviation of the GM level measured by the respective laboratory in % of the true GM level; the light yellow bar on the right represents the mean relative deviation over all twelve participating laboratories for each true GM level.

Figure 1. Relative deviation (%) from the true value of MON 88701 for all laboratories



*PL: participating laboratory

A trend can be observed overestimating the GM content at the lowest levels. All laboratories overestimated the GM content of the sample at 0.1%.

All data were retained to feed the statistical analysis and tests of outliers (Cochran and Grubbs) whose results are reported in Table 11.

4.2.3 Method performance requirements

Among the method 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 11 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study.

According to the ENGL method performance requirements, the relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 35% over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range. As it can be observed in Table 11, the method satisfies this requirement at all GM levels tested. Indeed, the highest value of RSD_R is 14% at the 0.1% GM level, thus within the acceptance criterion.

Table 11. Summary of validation results for the MON 88701 detection and quantification method, expressed in terms of mass fraction of GM materials (mass/mass).

| | Test Sample Expected GMO % | | | | |
|--|----------------------------|------|------|------|-------|
| | 0.1 | 0.9 | 2.0 | 5.0 | 10 |
| Laboratories having returned valid results | 12 | 12 | 12 | 12 | 12 |
| Samples per laboratory | 4 | 4 | 4 | 4 | 4 |
| Number of outliers | 2 | 2 | 1 | 1 | 1 |
| Reason for exclusion | C+DG | 2C | C | C | C |
| Mean value (%) | 0.13 | 1.1 | 2.3 | 5.2 | 9.9 |
| Relative repeatability standard deviation, RSD_r (%) | 10 | 7.0 | 6.8 | 5.1 | 5.6 |
| Repeatability standard deviation | 0.01 | 0.08 | 0.15 | 0.26 | 0.56 |
| Relative reproducibility standard deviation, RSD_R (%) | 14 | 9.6 | 7.9 | 6.1 | 8.7 |
| Reproducibility standard deviation | 0.02 | 0.11 | 0.18 | 0.31 | 0.86 |
| Bias (absolute value) | 0.03 | 0.20 | 0.29 | 0.16 | -0.10 |
| Bias (%) | 34 | 23 | 14 | 3.3 | -0.95 |

C= Cochran's test; G= Grubbs tests; DG= double Grubbs; 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 11 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for a collaborative study, the EURL GMFF requires the RSD_r value to be below 25%, as indicated by the ENGL (see "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 RSD_r is below 25% at all GM levels, with the highest value of 10% at the 0.1% GM level. This confirms the information provided by the applicant concerning the RSD_r , and verified by the EURL GMFF during step 3 of the method assessment.

The trueness of the method is estimated using the measurements of the method-bias for each GM level. According to ENGL method performance requirements, trueness should be $\pm 25\%$ across the entire dynamic range. In this case, the method satisfies this requirement across the tested dynamic range and, notably, including the 0.9% GM level (legal threshold for labelling of adventitious presence of GM material, as per Reg. (EC) No 1829/2003); the method overestimates the true GM content at the 0.1% level, with a bias of 34%.

5. Compliance of the method for detection and quantification of event MON 88701 with the requirements of Regulation (EU) No 619/2011

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

- at step 2 of the validation process (scientific assessment of the dossier), the EURL GMFF concluded that it could accept the applicant's data on method performance. Indeed, the RSDr at the level of 0.085% expressed in mass fraction of GM material resulted to be 12% calculated from 15 replicates (Table 2), hence below the acceptance criterion of 25%;
- at step 3 of the validation process (in-house verification of the method), the EURL GMFF determined the RSDr % value at the level of 0.1% in mass fraction of GM material. The experiments were carried out under repeatability conditions on fifteen replicates. The RSDr resulted to be 15% when the method was tested on the ABI 7900 and 3.3% when it was tested on the Roche LC[®]480 (Tables 7a and 7b, respectively), hence below 25%;
- further to the conclusion of step 4 of the validation process (collaborative study), the EURL GMFF analysed the data generated by the twelve participating laboratories for determining the method performance parameters. The RSDr of the method at the level of 0.1% of mass fraction of GM material was 10%, therefore below the limit of 25%.

Table 12. Precision of the event-specific method for quantitative detection of MON 88701 at or around 0.1% level related to mass fractions of GM material.

| Source | RSDr % | GM % |
|---------------------------------|--|--------|
| Applicant's method optimisation | 12 % | 0.08 % |
| EURL GMFF in-house verification | 15 % ^a and 3.3 % ^b | 0.1 % |
| Collaborative study | 10 % | 0.1 % |

^a ABI 7900 real-time PCR

^b Roche LC[®]480 real-time PCR

Based on the results of the EURL GMFF in-house verification and of the international collaborative study, it is concluded that the method RSDr 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. Conclusions

A method for detection, identification and quantification of GM event MON 88701 was provided by the applicant. The method has been fully validated in accordance to the EURL GMFF validation scheme, respecting all requirements of the relevant EU legislation and international standards for method validation.

The validation study confirmed that the method is applicable to the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex I-2.C.2 to Regulation (EC) No 641/2004 and (EU) No 619/2011 and meets all method performance requirements established by the ENGL down to the 0,9% GM level. At 0.1% GM level the method shows a trueness value slightly outside the required range but at that low levels this is deemed to be acceptable for the following reasons: a) the precision is within the limits b) the positive bias would determine an overestimation of the GM content and not an underestimation which would instead cause a problem c) the true value falls within the 95% CI around the mean (0.13%+- 0.036).

The method is therefore valid to be used for regulatory purposes, including the quantification of low level presence [0.1% (mass/mass)] of the GM event. It can be assumed that it is applicable to any appropriately extracted cotton genomic DNA.

Regarding the method performance requirements that were not met by the results of the collaborative study, i.e. trueness at the 0.1% level and mean slopes of the standard curves, the EURL GMFF, in collaboration with the European Network of GMO Laboratories (ENGL), will conduct a monitoring by asking laboratories using the methods for the purpose of Regulation (EU) No 619/2011 to provide their experimental data to the EURL GMFF in order to clarify if the found minor deviations are confirmed in practice and of any significance.

7. References

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Annex 1: Event-specific Method for the Quantification of Cotton MON 88701 Using Real-time PCR

Validated Method

24 June 2016

Method development:

Monsanto Comapany

Method validation:

European Union Reference Laboratory for GM Food and Feed (EURL GMFF)

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 MON 88701 (unique identifier MON-88701-3) 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 assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of cotton event MON 88701, an 84-bp fragment of the region spanning the 3' insert-to-plant junction in cotton MON 88701 event is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein) as a reporter at its 5' end, and TAMRA (6-carboxytetramethylrhodamine) as quencher at its 3' end.

For the relative quantification of cotton event MON 88701 DNA, a cotton-specific reference system amplifies a 76-bp fragment of *acyl carrier protein 1* (*acp1*), a cotton endogenous gene (GeneBank accession number: U48777.1), using *acp1* gene-specific primers and a *acp1* gene-specific probe labelled with FAM as reporter dye at its 5' end, and TAMRA as quencher at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Cq" value. For quantification of the amount of MON 88701 DNA in a test sample, Cq values for the MON 88701 and *acp1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON 88701 DNA to total cotton DNA.

2. Validation and performance characteristics

2.1 General

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

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).

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 0.04% (related to mass fraction of GM material) in 200 ng of total cotton DNA. The relative LOD was not assessed by the EURL GMFF in the collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.09% (related to mass fraction of GM material) in 200 ng of total 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 3' insert-to-plant junction in cotton MON 88701; the sequence is specific to event MON 88701 and thus imparts event-specificity to the method.

The specificity of the event-specific assay was assessed by the applicant in real-time PCR using genomic DNA samples (200 ng), extracted from MON 88701 (positive control sample) and from maize NK603, MON 863, MON 810, MON 88017, MON 89034, MON 87460, MON 87427, conventional maize; cotton MON 531, MON 1445, MON 15985, MON 88913, conventional cotton; soybean MON 89788, GTS 40-3-2, MON 87708, MON 87705, MON 87769, MON 87701, MON 87712, and conventional soybean; canola RT73, MON 88302 and conventional canola; wheat MON 71800 and conventional wheat, lentil, sunflower, peanut, quinoa, millet.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the TaqMan® probe of the MON 88701 event showed no amplification signals following quantitative PCR analysis (45 cycles).

The specificity of the cotton taxon-specific assay, previously validated by the EURL GMFF was demonstrated by the method developer contextually to the event-specific assay, on maize NK603, MON 863, MON 810, MON 88017, MON 89034, MON 87460, MON 87427, conventional maize; cotton MON 531, MON 1445, MON 15985, MON 88913, conventional cotton; soybean MON 89788, GTS 40-3-2, MON 87708, MON 87705, MON 87769, MON 87701, MON 87712, and conventional soybean; canola RT73, MON 88302 and conventional canola; wheat MON 71800 and conventional wheat, lentil, sunflower, peanut, quinoa, millet.

Specificity was further verified and confirmed *in silico* by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

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 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 frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps, unless specified otherwise, should be carried out at 0 – 4 °C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of cotton event MON 88701

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*acp1*) and for the GMO (event MON 88701) target sequence has to be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated and is therefore not recommended.

The method is developed and validated for a total volume of 50 µL per reaction mixture with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

To establish the calibration curve five samples should be prepared and analysed. The range of GM contents in the calibration curve should be equal or included in the range validated during the international collaborative trial.

For the collaborative trial, the calibration curve was established on the basis of five samples. The first point of the calibration curve contained 10% (mass/mass) cotton MON 88701 DNA in a total of 200 ng of cotton DNA.

Standards S2 and S3 were prepared by serial three-fold dilution of the S1 sample. Standards S4 and S5 were prepared by serial four-fold dilution of the standard S3.

The GM contents of the calibration samples and the total DNA quantity used in PCR are provided in Table 1.

Table 1. GM% values of the standard curve samples.

| Sample code | S1 | S2 | S3 | S4 | S5 |
|--------------------------------------|-----|-----|-----|------|------|
| Amount of total DNA in reaction (ng) | 200 | 67 | 22 | 5.6 | 1.4 |
| % GM-DNA | 10 | 10 | 10 | 10 | 10 |
| Amount of GM DNA (ng) | 20 | 6.7 | 2.2 | 0.56 | 0.14 |

A calibration curve is produced by plotting the C_q values for the calibration points against the logarithm of the amount of MON 88701 DNA. This can be done by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the software of the real-time PCR equipment.

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. To prepare the amplification reaction mixtures, add the following components (Table 2 and 3) in two reaction tubes (one for the MON 88701 assay and one for the *acp1* assay) on ice and in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the **MON 88701** assay.

| Component | Final concentration | µL/reaction |
|---|---------------------|-------------|
| TaqMan® Universal PCR Master Mix UNG (2x) | 1x | 25 |
| MON 88701 primer 1 (10 µM) | 300 nM | 1.50 |
| MON 88701 primer 2 (10 µM) | 300 nM | 1.50 |
| MON 88701 probe (10 µM) | 250 nM | 1.25 |
| Nuclease free water | / | 16.75 |
| DNA | / | (4) |
| Total reaction volume: | | 50 µL |

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the cotton *acp1* assay.

| Component | Final concentration | µL/reaction |
|---|---------------------|-------------|
| TaqMan® Universal PCR Master Mix UNG (2x) | 1x | 25 |
| <i>acp1</i> primer 1 (10 µM) | 150 nM | 0.75 |
| <i>acp1</i> primer 2 (10 µM) | 150 nM | 0.75 |
| <i>acp1</i> probe (10 µM) | 50 nM | 0.25 |
| Nuclease free water | / | 19.25 |
| DNA | / | (4) |
| Total reaction volume: | | 50 µL |

- Mix well and centrifuge briefly.
- Prepare two reaction tubes (one for the cotton MON 88701 and one for the *acp1* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of reaction mix for 3.5 PCR repetitions (e.g. 161 µL for the *acp1* reference system and 161 µL for the MON 88701 cotton system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (e.g. 14 µL DNA). The additional 0.5 repetition included will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
- Spin down the tubes in a micro-centrifuge. Aliquot 50 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4 °C) to spin down the reaction mixture.
- Place the plate into the instrument.
- Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for MON 88701/*acp1* methods.

| Step | Stage | T °C | Time (sec) | Acquisition | Cycles |
|------|----------------------|-----------------------|------------|-------------|--------|
| 1 | UNG | 50 °C | 120 | No | 1x |
| 2 | Initial denaturation | 95 °C | 600 | No | 1x |
| 3 | Amplification | Denaturation | 95 °C | No | 45x |
| | | Annealing & Extension | 60 °C | Yes | |

3.3 Data analysis

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

- a) Set the threshold: display the amplification curves of one assay (e.g. MON 88701) 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: 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 other system (e.g. *acp1*).
- 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 *acp1* and the MON 88701 specific assays by plotting the Cq values measured for the calibration points against the logarithm of the amount of MON 88701 DNA (ng) and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the amount of MON 88701 DNA in the unknown samples.

The amount of event MON 88701 is divided by the amount of the cotton reference gene (*acp1*) and multiplied by 100 to obtain the percentage value ($GM\% = \text{MON 88701} / \text{acp1} \times 100$).

4 Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge

- Micropipettes
- Centrifuge for PCR plates
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix, UNG. Applied Biosystems Cat. 4304437.

4.3 Primers and Probes

| Oligonucleotides | Name | DNA Sequence (5' to 3') | Length (nt) |
|--------------------|----------------------|---|-------------|
| MON 88701 | | | |
| Forward primer | MON 88701 primer 1 | 5' CAT ACT CAT TgC TgA TCC Atg TAg A 3' | 25 |
| Reverse primer | MON 88701 primer 2 | 5' AgT gTT AAA CAA gTT Atg TTC TAg AgC 3' | 27 |
| Probe | MON 88701 probe | 5'-6FAM- TTC CCg gAC Atg Aag CCT TAA TTC AAT – TAMRA-3' | 27 |
| <i>acp1</i> | | | |
| Forward primer | <i>acp1</i> primer 1 | 5' ATT gTg ATg ggA CTT gAg gAA gA 3' | 23 |
| Reverse primer | <i>acp1</i> primer 2 | 5' CTT gAA CAg TTg TgA Tgg ATT gTg 3' | 24 |
| Probe | <i>acp1</i> probe | 5'-6FAM- ATT gTC CTC TTC CAC CgT gAT TCC gAA –TAMRA-3' | 27 |

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

Annex 2: Troubleshooting

After analysis of the results of the collaborative study additional in-house experiments were performed with the aim to further investigate the slope values and the bias at the 0.1% level obtained during the collaborative study. During in-house testing of the GM and reference systems conducted before the collaborative study, the EURL GMFF did not identify specific problems, both in terms of slope values and bias % (4.1.1). In fact, all parameters were within ENGL acceptance criteria.

One experiment, consisting in a repetition of the GM quantification on the samples used for the collaborative trial, showed acceptable values of trueness and precision with a slight worsening in the slopes for both the GM and the reference system (slope -3.6). For this reason, the loss of efficiency reported for the reference amplification system was further investigated.

Two following experiments performed in November 2013, aiming at evaluating the effect of the storage temperature and the influence of the PCR instrument, gave indication of a possible effect of the storage temperature on the efficiency of PCR amplification. Mean values were anyway within the ENGL acceptance criteria.

In January-February 2014, the EURL GMFF conducted additional tests to determine the impact of various factors (temperature, plastic ware and addition of background DNA) on DNA short term stability (3 weeks) but these tests (data not shown) did not produce clear indication on the causes of the method underperformance at the 0.1% level.

Based on these elements the EURL GMFF decided to recall the left-over samples from the laboratories that participated to the collaborative study, to test the samples in-house and to further investigate this issue. Results obtained on samples recalled from the collaborative study are shown in Table 13.

Table 13. Comparison between slope values obtained during the collaborative study and slope values obtained at the EURL GMFF after recall of samples.

| Slope values of the <i>acp1</i> reference system | Collaborative study | Tests using recalled validation samples | Tests using validation counter-samples* and recalled primers/probes |
|---|----------------------------|--|--|
| PL1 | -3.86 | -3.45 | - |
| PL4 | -3.77 | -3.60 | -3.27 |
| PL5 | -3.88 | -4.00 | -3.40 |
| PL6 | -3.69 | -3.89 | - |
| PL8 | -3.64 | -3.36 | - |
| PL11 | -3.72 | -3.48 | - |
| PL12 | -3.83 | -3.89 | -3.30 |

* stored at the EURL GMFF

After evaluation of these results on samples recalled from the collaborative study, it appeared that in about 50% of cases the returned samples produced results in line with the performance demonstrated at the EURL GMFF during step 3; in the other cases an efficiency similar to the one obtained during the validation study was obtained by the EURL GMFF after re-testing the samples recalled. However, the amount of samples recalled was not sufficient to repeat the experiment and further investigate the issue. Although the collected information is therefore not conclusive, we hypothesise that genomic DNA may have been damaged during the delivery of the ring-trial kits to the participating laboratories.

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