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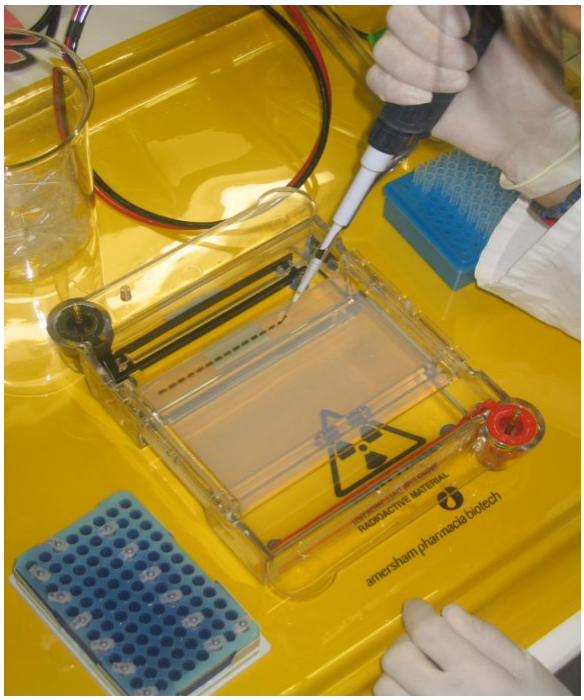
Event-specific Method for the Quantification of Cotton MON 88913 Using Real-time PCR

Validation Report and Protocol

C. Delobel, E. Luque-Perez, G.Pinski, A. Bogni, M. Mazzara, G. Van den Eede



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European Commission
Joint Research Centre
Institute for Health and Consumer Protection

Contact information
Address: Molecular Biology and Genomics Unit
E-mail: JRC-BGMO@ec.europa.eu
Tel.: +39 0332 789379
Fax: +39 0332 786159

http://ihcp.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

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Event-specific Method for the Quantification of Cotton MON 88913 Using Real-time PCR

Validation Report

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Joint Research Centre
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Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the MON 88913 transformation event in cotton DNA (unique identifier MON-88913-8). The collaborative trial was conducted according to internationally accepted guidelines [1, 2].

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed" and with Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003", Monsanto provided the detection method and the samples: genomic DNA from cotton seeds harbouring the MON 88913 event (line ST 4664) and from conventional cotton seeds (line ST 474). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from nine European countries.

The results of the international collaborative trial met the ENGL performance requirements. The method is, therefore, considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Regulation (EC) No 641/2004.

The results of the collaborative study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/ .

Drafted by:
E. Luque-Perez

Report Verification Team:
1) M. Ermolli

2) M. Querci

Scientific and technical approval:
M. Mazzara

Compliance with CRL Quality System:
S. Cordeil

Authorisation to publish:
G. Van den Eede

Address of contact laboratory:

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit
Community Reference Laboratory for GM Food and Feed
Via Fermi 2749, 21027 Ispra (VA) - Italy

Report on Steps 1-3 of the Validation Process

Monsanto submitted the detection method and control samples for cotton event MON 88913 (unique identifier MON-88913-8) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Community Reference Laboratory for GM Food and Feed (CRL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the CRL-GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed for cotton event MON 88913 and positively concluded in August 2007.

Between August 2007 and August 2008, the CRL-GMFF experimentally verified the purity of the control samples provided, and requests for replacement samples were made. The method characteristics were verified by quantifying five blind GM levels within the range 0.09%-8.0% on a genome copy number basis (step 3, experimental testing of samples and methods). The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision of the quantifications were within the limits established by the ENGL. The DNA extraction module of the method was previously tested on samples of food and feed and a report was published on the CRL-GMFF website on 10th June 2008 (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

A Technical Report summarising the results of tests carried out by the CRL-GMFF (step 3) is available on request.

Content

1. Introduction

Monsanto submitted the detection method and control samples for cotton event MON 88913 (unique identifier MON-88913-8) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Joint Research Centre (JRC, Molecular Biology and Genomics Unit of the Institute for Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Regulation (EC) No 1829/2003) organised the international collaborative study for the event-specific method for the detection and quantification of cotton MON 88913. The study involved twelve laboratories, among those listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for detection") of Regulation (EC) No 1981/2006 of 22 December 2006.

Upon reception of method, samples and related data (step 1), the JRC carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3) according to the requirements of Regulation (EC) No 641/2004 and following its operational procedures.

The internal experimental evaluation of the method was carried out between August 2007 and August 2008.

Following the evaluation of the data and the results of the internal laboratory tests, the international collaborative study was organised (step 4) and took place in September 2008.

A method for DNA extraction from seeds and grains followed by PEG precipitation, submitted by the applicant, was evaluated by the CRL-GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on method testing are available at http://gmo-crl.jrc.ec.europa.eu/.

The operational procedure of the collaborative study included the following module:

- Quantitative real-time PCR (Polymerase Chain Reaction). The method is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event MON 88913 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton endogenous gene, *acp1* (reference gene) and the target assay (MON 88913) are performed in separate wells.

The international collaborative study was carried out in accordance with the following internationally accepted guidelines:

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

2. List of participating laboratories

As part of the international collaborative study the method was tested in twelve laboratories to determine its performance.

In August 2008, the CRL-GMFF invited all National Reference Laboratories nominated under Regulation (EC) No 1981/2006 of 22 December 2006 and listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for detection") of that Regulation to express the availability to participate in the validation study of the quantitative real-time PCR method for the detection and quantification of cotton event MON 88913.

Thirty-two laboratories expressed in writing their willingness to participate, two declined the invitation, while thirty-eight did not answer. The CRL-GMFF performed a random selection of twelve laboratories out of those that responded positively to the invitation, making use of a validated software application.

Clear guidance was given to the selected laboratories with regards to the standard operational procedures to follow for the execution of the protocol. The participating laboratories are listed in Table 1.

Table 1. Laboratories participating in the validation of the detection method for cotton line MON 88913.

Laboratory	Country
Central Agricultural Office, Food and Feed Safety Directorate, Central Feed Investigation Laboratory	HU
Central Science Laboratory	UK
Crop Research Institute - Reference Laboratory for GMO Detection and DNA fingerprinting	CZ
Federal Institute for Risk Assessment	DE
Groupement d'Intérêt Public – Groupe d'Etude et de contrôle des Variétés et des Productions Agricoles	FR
Institute for National Investigation for the Health and Veterinarian Nature Saxonia	DE
Institute of Chemical Technology Prague	CZ
LGC Limited	UK
National Centre for Food, Spanish Food Safety Agency	ES
National Institute of Biology	SI
Scientific Institute of Public Health (IPH)	BE
Veterinary Public Health Institute for Lazio and Toscana Regions; National Reference Centre for GMO Analysis	IT

3. Materials

For the validation of the quantitative event-specific method, control samples consisted of:

i) genomic DNA extracted from homozygous cotton seeds harbouring the event MON 88913 (Lot No. GLP-0602-16861-S), and
ii) genomic DNA extracted from conventional cotton seeds (Lot No. GLP-0602-16862-S).

Samples were provided by the applicant in accordance to the provisions of Regulation (EC) No 1829/2003, Art 2.11 [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)].

Samples containing mixtures of 100% cotton MON 88913 and non-GM cotton genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the control samples provided, in a constant amount of total cotton DNA.

Participants received the following materials:

- ✓ Five calibration samples (140 µL of DNA solution each) labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (70 µL of DNA solution each) labelled from U1 to U20.
- ✓ Reaction reagents as follows:
  - universal PCR Master Mix (2x), two bottles: 5 mL each
  - distilled sterile water, one tube: 4 mL

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

  acp1 reference system

  - *acp1* primer 1 (10 µM): 240 µL
  - *acp1* primer 2 (10 µM): 240 µL
  - *acp1* probe (10 µM): 160 µL

  MON 88913 system

  - MON 88913 primer 1 (10 µM): 800 µL
  - MON 88913 primer 2 (10 µM): 800 µL
  - MON 88913 probe (10 µM): 320 µL

4. Experimental design

Twenty unknown samples (labelled from U1 to U20), representing five GM levels, were used in the validation study (Table 2). On each PCR plate, the samples were analysed for the MON 88913 specific system and for the *acp1* reference system. Two plates were run per participating laboratory with two replicates for each GM level. In total, four replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. Participating laboratories carried out the determination of the GM% according to the instructions provided in the protocol and using the electronic tool provided (Excel spreadsheet).

Table 2. MON 88913 GM contents

MON 88913 GM% (GM copy number/cotton genome copy number x 100)
0.09
0.30
0.90
3.00
8.00

5. Method

Description of operational steps followed

For the specific detection of event MON 88913 genomic DNA, a 94-bp fragment of the region that spans the 5' plant-to-insert junction in cotton MON 88913 event is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM as reporter dye at its 5' end and MGBNFQ as quencher dye at its 3' end.

For the relative quantification of event MON 88913 DNA, a cotton-specific reference system amplifies a 76-bp fragment of *acp1*, a cotton endogenous gene encoding an acyl carrier protein, using two *acp1* gene-specific primers and an *acp1* gene-specific probe labelled with FAM dye and TAMRA as quencher dye.

Standard curves are generated for both the MON 88913 and the *acp1* specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For relative quantification of event MON 88913 DNA in a test sample, the MON 88913 copy number is divided by the copy number of the cotton reference gene (*acp1*) and multiplied by 100 to obtain the percentage value (GM% = MON 88913/*acp1* x 100).

Calibration sample S1 was prepared by mixing the appropriate amount of MON 88913 DNA in control non-GM cotton DNA to obtain a 10% GM MON 88913. Sample S2 was prepared by two-fold dilution from the S1 sample; sample S3 was prepared by five-fold dilution from S2 sample; sample S4 was prepared by three-fold dilution of sample S3 and sample S5 was prepared by four-fold dilution from the S4 sample.

The absolute copy numbers in the calibration curve samples are determined by dividing the sample DNA weight (nanograms) by the published average 1C value for cotton genome (2.33 pg) [(3)]. The copy number values used in the quantification, the GM contents of the calibration samples and total DNA quantity used in PCR are provided in Table 3.

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

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction	200	100	20	6.68	1.68
Cotton genome copies	85837	42919	8584	2861	715
MON 88913 GM cotton copies	8584	4292	858	286	72

6. Deviations reported

Seven laboratories reported no deviations from the protocol.

Two laboratories reported a different plate set-up from the protocol, with no consequences in the analysis since the results were correctly attributed to the corresponding samples.

One laboratory performed PCR reactions in 20 µL of total volume because a 384-well plate configuration of the ABI 7900HT instrument was used. Final concentrations of PCR reagents remained unchanged.

One laboratory reported a minor amplification in a reagent control sample, as consequence of an experimental error, introducing a tip containing the U12 unknown sample in the control sample well already filled. However, this had no effect in the results.

One laboratory reported not centrifuging the plates prior to PCR analysis, as established in the protocol.

7. Summary of results

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula ((10^(-1/slope))-1) x 100) of the reference curve and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for the MON 88913 system and the *acp1* reference system are summarised in Table 4.

The mean PCR efficiency was 105% for the MON 88913 system and 101% for the *acp1* system, with both values within the ENGL acceptance criteria. The linearity of the method was 0.99 and 1.00 for MON 88913 and *acp1* systems, respectively.

Data reported confirm the appropriate performance characteristics of the method tested in terms of efficiency and linearity.

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2)

		MON 88913			*acp1*		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.38	98	0.99	-3.29	101	1.00
	B	-3.32	100	1.00	-3.32	100	1.00
2	A	-3.31	101	1.00	-3.41	97	1.00
	B	-3.31	100	1.00	-3.36	99	1.00
3	A	-3.37	98	1.00	-3.31	100	1.00
	B	-3.41	96	1.00	-3.30	101	1.00
4	A	-3.20	105	1.00	-3.32	100	1.00
	B	-3.26	103	0.99	-3.23	104	1.00
5	A	-3.43	96	1.00	-3.34	99	1.00
	B	-3.40	97	1.00	-3.35	99	1.00
6	A	-3.30	101	0.99	-3.22	104	1.00
	B	-3.40	97	0.99	-3.24	103	1.00
7	A	-3.24	103	1.00	-3.33	100	1.00
	B	-3.34	99	1.00	-3.36	98	1.00
8	A	-3.32	100	1.00	-3.32	100	1.00
	B	-3.30	101	1.00	-3.14	108	0.99
9	A	-3.26	103	1.00	-3.29	101	1.00
	B	-3.30	101	0.99	-3.24	104	1.00
10	A	-3.12	109	0.98	-3.22	105	0.99
	B	-2.85	125	0.99	-3.35	99	1.00
11	A	-2.67	137	0.94	-3.19	106	1.00
	B	-2.65	139	0.89	-3.22	105	1.00
12	A	-3.31	100	0.99	-3.44	95	0.99
	B	-2.87	123	0.99	-3.14	108	1.00
	Mean	-3.22	105	0.99	-3.29	101	1.00

GMO quantification

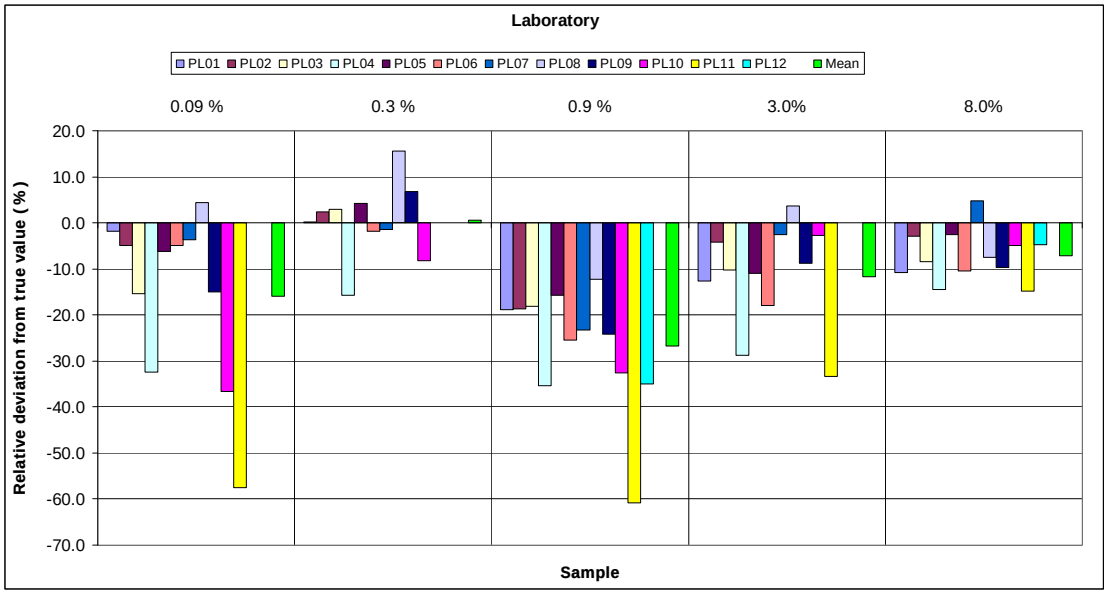
Table 5 shows the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

Table 5. GM% mean values determined by laboratories for unknown samples.

	GMO content (GMO% = GMO copy number/cotton genome copy number x 100)																			
LAB	0.09				0.3				0.9				3.0				8.0			
	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	0.11	0.09	0.07	0.08	0.30	0.32	0.26	0.33	0.74	0.81	0.64	0.73	2.47	2.94	2.53	2.54	6.99	8.08	7.27	6.21
2	0.07	0.10	0.09	0.09	0.31	0.33	0.28	0.30	0.69	0.76	0.67	0.81	2.98	2.80	2.91	2.82	7.45	8.02	7.47	8.11
3	0.08	0.08	0.08	0.07	0.30	0.31	0.32	0.30	0.79	0.76	0.69	0.70	2.65	2.94	2.85	2.33	6.95	7.52	7.73	7.13
4	0.07	0.05	0.06	0.05	0.27	0.21	0.24	0.28	0.63	0.52	0.57	0.60	1.74	2.05	2.00	2.76	6.55	6.43	5.85	8.57
5	0.09	0.08	0.09	0.09	0.29	0.30	0.34	0.32	0.72	0.75	0.79	0.76	2.77	2.69	2.63	2.58	7.36	7.57	7.36	8.88
6	0.10	0.09	0.08	0.08	0.29	0.32	0.26	0.31	0.74	0.66	0.57	0.72	2.59	2.49	2.30	2.47	7.11	6.89	7.72	6.98
7	0.09	0.09	0.09	0.08	0.30	0.34	0.26	0.29	0.73	0.73	0.64	0.67	3.39	2.62	2.72	2.97	8.63	8.23	8.44	8.21
8	0.07	0.10	0.09	0.11	0.37	0.38	0.33	0.31	0.93	0.78	0.73	0.72	3.78	2.74	2.81	3.10	7.31	7.25	7.52	7.53
9	0.08	0.08	0.08	0.07	0.35	0.32	0.29	0.32	0.76	0.60	0.65	0.72	2.89	2.79	2.64	2.62	6.82	6.83	7.36	7.91
10	0.06	0.07	0.06	0.04	0.33	0.26	0.22	0.29	0.71	0.70	0.47	0.53	2.50	3.15	3.11	2.92	6.65	6.93	7.24	9.58
11	0.03	0.03	0.05	0.04	0.08	0.20	0.10	0.13	0.41	0.21	0.34	0.46	2.35	1.64	2.08	1.93	5.90	5.33	9.50	6.50
12	0.02	0.02	0.11	0.11	0.11	0.11	0.37	0.27	0.47	0.39	0.73	0.75	3.10	1.29	1.24	2.52	7.86	6.21	8.07	8.33

In Figure 1, the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the relative GM quantification obtained by the participating laboratories; the green bar represents the overall mean for each GM level.

Figure 1. Relative deviation (%) from the true value of MON 88913 for all laboratories



As observed in Figure 1, the mean relative deviations from the true values are negative for most GM levels apart from 0.3% GM level, where it is positive. This means that the GM content tends to be under-estimated at most GM levels. For 0.9% GM level, five laboratories showed deviation from the true value above 25%, with an overall average relative deviation of 27%. For samples at 0.09% and 3.0% GM levels, some laboratories also showed deviations from the true value above 25%, however, the average relative deviations for those levels were well below 25%.

Overall, the average relative deviation is within the acceptance criterion at all GM levels tested, except for 0.9% GM level for which the value was slightly above the acceptance limit (27%), indicating a satisfactory trueness of the method.

8. Method performance requirements

Among the performance criteria established by ENGL and adopted by the CRL-GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm, see also Annex 1), repeatability and reproducibility are assessed through an international collaborative trial, carried out with the support of twelve ENGL laboratories (see Table 1).

Table 6 illustrates the estimation of repeatability and reproducibility at various GM levels, according to the range of GM percentages tested during the collaborative trial. The relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 33% 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 6, the method satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R is 25% at the 0.09% GM level, thus well within the acceptance criterion.

Table 6. MON 88913: summary of validation results.

unknown sample GMO %	Expected value (GMO%)				
	0.09	0.3	0.9	3.0	8.0
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	1	2	0	1	0
Reason for exclusion	1 C	1 C 1G	-	1 C	-
Mean value	0.08	0.30	0.66	2.65	7.42
Relative repeatability standard deviation, RSD_r (%)	13	10	13	11	12
Repeatability standard deviation	0.01	0.03	0.09	0.28	0.89
Relative reproducibility standard deviation, RSD_R (%)	25	12	21	16	12
Reproducibility standard deviation	0.02	0.04	0.14	0.42	0.89
Bias (absolute value)	-0.01	0.001	-0.24	-0.35	-0.58
Bias (%)	-16	0.5	-27	-12	-7.2

C = Cochran's test; G = Grubbs' 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 6 further documents the relative repeatability standard deviation (RSD_r), as estimated for each GM level. In order to accept methods for collaborative study evaluation, the CRL requires that the RSD_r value is below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" http://gmo-

crl.jrc.ec.europa.eu/guidancedocs.htm). As can be observed from the values reported, the method showed a repeatability standard deviation below 25% at all GM levels, with the highest value of RSD_r (%) of 13% at 0.09% and 0.9% 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. In this case, the method satisfies this requirement across the dynamic range tested, apart from the 0.9% GM level that shows a bias of -27%.

9. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (as detailed at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). The method acceptance criteria were reported by the applicant and used to evaluate the method prior to the international collaborative study (see Annex 1 for a summary of method acceptance criteria and method performance requirements).

The results obtained during the collaborative study, indicate that the analytical module of the method submitted by the applicant mostly complies with ENGL performance criteria.

Therefore, the method is considered applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32232) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)].

11. References

1. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem.*, 67, 331-343.
2. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
3. Arumuganathan K. and Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9, 208-218.

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: the description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: the applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: the ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: the practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

Definition: property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: the method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: the range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: the dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: the closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: the accuracy should be within ± 25% of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: the rate of amplification that leads to a theoretical slope of – 3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $[10^{(-1/slope)}] - 1$

Acceptance Criterion: the average value of the slope of the standard curve should be in the range of ($-3.1 \geq slope \geq -3.6$)

R^2 Coefficient

Definition: the R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: the average value of R^2 should be ≥ 0.98.

Repeatability Standard Deviation (RSD,)

Definition: the standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: the relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Note: estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

Limit of Quantitation (LOQ)

Definition: the limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than $1/10^{th}$ of the value of the target concentration with an $RSD_r \leq 25\%$. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

Limit of Detection (LOD)

Definition: the limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

Acceptance Criterion: LOD should be less than $1/20^{th}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq$ 5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Robustness

Definition: the robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance Criterion: the response of an assay with respect to these small variations should not deviate more than $\pm$ 30%. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements

Dynamic Range

Definition: in the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: the dynamic range of the method should include the 1/10 and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD_R)

Definition: the standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: the relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An RSD_R < 50% is acceptable for concentrations below 0.2%.

Trueness

Definition: the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: the trueness should be within $\pm$ 25% of the accepted reference value over the whole dynamic range.

Event-specific Method for the Quantification of Cotton Event MON 88913 Using Real-time PCR

Protocol

5 May 2009

Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Method development:

Monsanto Company

Method validation:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Molecular Biology and Genomics Unit

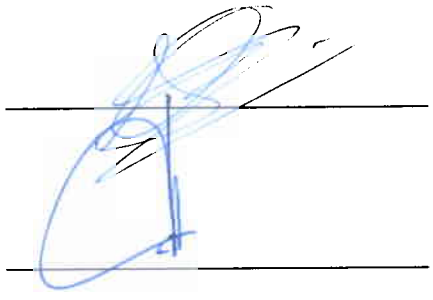
Drafted by
E. Luque-Perez

Report Verification Team
1) M. Querci

2) M. Ermolli

Scientific and technical approval
M. Mazzara

Compliance to CRL Quality System
S. Cordeil



Authorisation to publish
G. Van den Eede

Address of contact laboratory:
European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 2749, 21027 Ispra (VA) - Italy

Content

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of cotton event MON 88913 DNA to total cotton DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels.

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 88913 DNA, a 94-bp fragment of the region that spans the 5′ plant-to-insert junction in cotton MON 88913 event is amplified using two 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, as a reporter at its 5′ end, and with the non-fluorescent quencher MGBNFQ at its 3′ end.

For the relative quantification of cotton event MON 88913 DNA, a cotton-specific reference system amplifies a 76-bp fragment of *acp1*, a cotton endogenous gene encoding an acyl carrier protein, using two *acp1* gene-specific primers and an *acp1* gene-specific probe labelled with FAM as reporter dye at its 5′ end, and with the non-fluorescent quencher TAMRA at its 3′ end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event MON 88913 DNA in a test sample, cotton MON 88913 and *acp1* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of cotton event MON 88913 DNA to total cotton DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional cotton seeds.

The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GMO contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve participating laboratories in September 2008.

Each participant received twenty blind samples containing cotton MON 88913 genomic DNA at five GM contents, ranging from 0.09% to 8.0%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of event MON 88913 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.045% in 200 ng of total 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 0.09% in 200 ng of total cotton DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.09%.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence at the junction of the insert and the genomic DNA flanking the insert. According to the method developer, the sequence is specific to cotton event MON 88913 and thus imparts event-specificity to the detection method.

The specificity of event-specific and the cotton-specific assays were experimentally tested by the applicant in real-time PCR against DNA extracted from plant materials containing the specific targets of maize GA21, NK603, MON 810, MON 863, MON 88017, LY038, MON 88034 (tested only for the cotton-specific assay) and conventional corn; canola RT73, RT200 and conventional canola; soybean 40-3-2 and conventional soybean; wheat MON 71800 and conventional wheat; lentils, sunflower, nuts, buck wheat, rye berries, peanuts (shelled), pinenuts, quinoa and millet; cotton lines MON 531, MON 757, MON 15986, MON 1445 and conventional cotton.

According to the method developer, the MON 88913 system did not react with any of the plant materials tested, except the positive control cotton line MON 88913; the cotton-specific reference system reacted only with conventional cotton and with all the cotton GM varieties tested.

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 used should be sterilised prior to use and any residue of DNA has to be 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 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 88913

3.2.1 General

The PCR set-up for the taxon specific target sequence (*acp1*) and for the GMO (event MON 88913) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 50 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% MON 88913 in non-GM cotton DNA for a total of 200 ng of DNA (corresponding to approximately 85837 cotton genome copies with one genome assumed to correspond to 2.33 pg of haploid cotton genomic DNA) [(1)]. The other four standards are prepared by serial dilution.

A calibration curve is produced by plotting the Ct 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 sequence detection system 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 gently and centrifuge the required amount of components needed for the run. Keep thawed reagents on ice.
2. In two reaction tubes (one for the MON 88913 system and one for the *acp1* system) on ice, add the following components (Table 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 88913 specific system.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
MON 88913 primer 1 (10 µM)	500 nM	2.5
MON 88913 primer 2 (10 µM)	500 nM	2.5
MON 88913 probe (5 µM)	100 nM	1
Nuclease free water	#	15
Template DNA (max 200 ng)	#	4
Total reaction volume:		50

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the cotton *acp1* reference system.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
acp1 primer 1 (10 µM)	150 nM	0.75
acp1 primer 2 (10 µM)	150 nM	0.75
acp1 probe (5 µM)	50 nM	0.5
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4
Total reaction volume:		50

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the cotton MON 88913 and one for the *acp1* reaction mix) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of reaction mix (e.g. 46 µL x 3 = 138 µL reaction mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 4 µL x 3 = 12 µL DNA for three PCR repetitions). Vortex each tube for approx. 10 s. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
6. 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 room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 3.

Table 3. Cycling program for MON 88913 specific system and for the cotton *acp1* reference system.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	UNG		50	120	No	1
2	Initial denaturation		95	600	No	1
3	Amplification	Denaturation	95	15	No	45
		Annealing & Extension	60	60	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 system (e.g. MON 88913) 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 Ct values. 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 geometric 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 Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *acp1* system).

e) Save the settings and export all the data to a text file 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 Ct-values for each reaction.

The standard curves are generated both for the *acp1* and the MON 88913 specific systems by plotting the Ct 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 numbers in the unknown sample.

For the determination of the amount of event MON 88913 DNA in the unknown sample, the MON 88913 copy number is divided by the copy number of the cotton reference gene (*acp1*) and multiplied by 100 to obtain the percentage value (GM% = MON 88913/*acp1* x 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
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437.

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
MON 88913 target sequence	
MON 88913 primer 1	5' - GGC TTT GGC TAC CTT AAG AGA GTC - 3'
MON 88913 primer 2	5' - CAA ATT ACC CAT TAA GTA GCC AAA TTA C - 3'
MON 88913 probe	6-FAM - 5' - AAC TAT CAG TGT TTG ACT ACA T - MGBNFQ - 3'
Reference gene *acp1* target sequence	
acp1 primer 1	5' - ATT GTG ATG GGA CTT GAG GAA GA - 3'
acp1 primer 2	5' - CTT GAA CAG TTG TGA TGG ATT GTG - 3'
acp1 probe	6-FAM - 5' - ATT GTC CTC TTC CAC CGT GAT TCC GAA - TAMRA -3'

5. References

1. Arumuganathan K., Earle E.D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter,* 9: 208-218.

Report on the Validation of a DNA Extraction Method for Cotton Seeds and Grains

19 June 2008

Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit

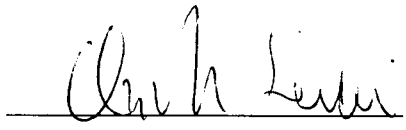
Method development:

Monsanto Company

Method testing and single laboratory validation:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Biotechnology & GMOs Unit

Drafted by:
C. Savini

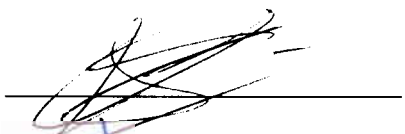


Report Verification Team:
1) M. Querci

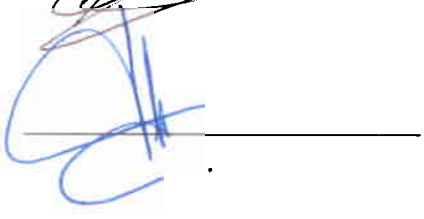
2) M. Ermolli

Scientific and technical approval:
M. Mazzara

Compliance with CRL Quality System:
S. Cordeil



Authorisation to publish:
G. Van den Eede



Address of contact laboratory:
European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 1, 2749, 21027 Ispra (VA) - Italy

Content

1. Introduction

This report describes the validation of a DNA extraction protocol derived from the publicly available "CTAB" method [(1)] and its applicability on the samples of food and feed provided by the applicant. This protocol can be used for the extraction of DNA from cotton seeds and grains.

The purpose of the DNA extraction method described is to provide DNA with purity and quantity suitable for real-time PCR based detection methods.

This protocol is recommended to be executed only by skilled laboratory personnel as the procedures comprise the use of hazardous chemicals and materials. It is strongly advised to take particular notice of products safety recommendations and guidelines.

2. Materials (Equipment/Chemicals/Plasticware)

2.1. Equipment

The following equipment is used in the DNA extraction procedure described (equivalents may be used):

1. Centrifuge (Beckman Coulter Avanti J-251)
2. Shaker (LabLine Enviro 3527)
3. Thermometer (VWR Cat. No. 61222-504)
4. Vacufuge (Eppendorf 5301 22 82 010-9)
5. Water bath (Precision Cat. No. 51220046)
6. Microcentrifuge (any appropriate model)

2.2. Chemicals

The following chemicals are used in the DNA extraction procedure described (equivalents may be used):

1. 24:1 chloroform:isoamyl alcohol (Sigma Cat. No. C-0549)
2. 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma Cat. No. P-3803)
3. Ammonium acetate 7.5 M (Sigma Cat. No. A-2706)
4. CTAB (Sigma Cat. No. H-6269)
5. 0.5 M EDTA, pH 8.0 (GibcoBRL Cat. no. 15575-038)
6. 100% ethanol (AAPER)
7. NaCl (Sigma Cat. No. S-5150)
8. 2-mercaptoethanol (Bio-Rad Cat. no. 161-0710)
9. RNase A (Roche Cat. No. 10 109 196 001)

10. Isopropanol (EM Science Cat. No. PX1835-9)
11. 1 M Tris HCl pH 8.0 (Sigma Cat. No. T-3038)
12. Proteinase K (Roche Cat. No. 03 115 836 001)
13. Polyethylene Glycol (MW 8000) (Sigma Cat. No. P2139)

2.3. Solutions

The following buffers and solutions are used in the DNA extraction procedure described:

1. CTAB Extraction Buffer (2%) (store at room temperature)

- 2% w/v CTAB
- 100 mM Tris HCl pH 8.0
- 20 mM EDTA pH 8.0
- 1.4 M NaCl

2. Tris-EDTA buffer (TE 1X) (store at room temperature)

- 10 mM Tris HCl pH 8.0
- 1 mM EDTA pH 8.0

3. Proteinase K (10 mg/ml) (store at -20 ˚C)

4. RNase A (10 mg/ml) (store at -20 ˚C)

5. PEG Precipitation Buffer (20% w/v) (store at room temperature)

6. Ethanol (70% v/v) (store at room temperature)

7. Ethanol (80% v/v) (store at room temperature)

2.4. Plasticware

1. 50 ml conical tubes (Corning Cat. No. 430290)
2. 13 ml Sarstedt tubes (Sarstedt Cat. No. 60.540)
3. 1.5 ml microcentrifuge tubes
4. filter tips

Note: All plasticware should be sterile and free of DNases, RNases and nucleic acids.

2.5. Precautions

- Phenol, chloroform, isoamyl alcohol, and isopropanol are hazardous chemicals; therefore, all manipulations have to be performed according to safety guidelines, under

fume hood.

- It is recommended to use clean containers for Waring blenders for grinding the seed bulk samples.
- All tubes and pipette tips have to be discarded as biological hazardous material.

2.6 Abbreviations

EDTA	ethylenediaminetetraacetic acid
PCR	polymerase chain reaction
RNase A	ribonuclease A
TE	Tris EDTA
Tris	Tris(hydroxymethyl)aminomethane

3. Description of the methods

3.1 Sampling

For sampling methods, it is referred to the technical guidance documents and protocols described in Commission Recommendation 2004/787/EC on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) N. 1830/2003.

3.2 Scope and applicability

The method for DNA extraction described below is suitable for the isolation of genomic DNA from a wide variety of cotton tissues and derived matrices. However, validation data presented here are restricted to ground cotton seeds and grains. Application of the method to other matrices may require adaptation and possible further specific validation.

3.3 Principle

The basic principle of the DNA extraction consists of first releasing the DNA present in the matrix into aqueous solution and further purifying the DNA from PCR inhibitors. The present method starts with a lysis step (thermal lysis in the presence of Tris HCl, EDTA, CTAB and β-mercaptoethanol) followed by removal of contaminants such as lipophilic molecules and proteins by extraction with phenol and chloroform.
A DNA precipitate is then generated by using isopropanol. The pellet is dissolved in TE buffer. Remaining inhibitors are removed by PEG precipitation and re-suspension in TE buffer.

3.4 Tissues grinding procedure

Tissues should be processed prior to extraction procedure. Possible methods of processing include a mortar and pestle with liquid nitrogen (leaf) or commercial blender (grain or seed).

3.5 Extraction of genomic DNA from cotton seeds/grains

1. Weight out 6 g of processed tissue into a 50 mL conical tube appropriate for centrifugation. Note: For unprocessed tissue, weighing may occur prior to processing as long as entire processed sample is transferred to the conical tube.
2. For each 6 g sample add 25 mL of a solution consisting of 24.25 mL, pre-warmed CTAB extraction buffer, 0.5 mL 2-mercaptoethanol (2-ME), and 0.25 mL of 10 mg/mL proteinase K for a final concentration of 2% (2-ME) and 100 µg/mL (proteinase K).
3. Mix the tube vigorously by inversion for 45-60 seconds.
4. Incubate for 60 minutes at 55 °C and mix the tube vigorously for 40-60 seconds every 20 minutes. Cool the tube on bench for 10 minutes.
5. Add 20 mL of phenol:choloroform:isoamyl alcohol (PCI 25:24:1, pH 6.7). Cap the tube and mix vigorously by inversion at least for 1 minute.
6. Centrifuge for 10 minutes at 13000 x g at room temperature to separate the aqueous and organic phases. Transfer upper aqueous phase to a clean 50 mL conical tube.
7. Repeat extraction twice for a total of three extractions (step 5-6).
8. Transfer upper aqueous phase to a new tube, add 2/3 volume of -20 °C isopropanol and gently mix the tube by inversion.
9. To precipitate the DNA place the tube at -20 °C for 30 minutes. (DNA may be stored as isopropanol precipitate at -20 °C for up to 1 year).
10. To pellet the DNA centrifuge the tubes at approximately 13000 x g for 20 minutes at 4 °C. Carefully pour off isopropanol or remove by pipetting, and then perform a quick spin in the centrifuge to bring down the isopropanol from the side of the tube. Remove remaining isopropanol by pipette and ensure all residual isopropanol is removed before proceeding to the next step without overdrying the pellet.
11. Re-dissolve the pellet in 4 mL of TE pH 8.0. Note: it may be necessary to incubate the tube at 60°C to resuspend the pellet.
12. Transfer the resuspended pellet to a 13 mL tube, add 40 µL of 10 mg/ml RNase and then incubate at 37 °C for 30 minutes.
13. To extract the DNA add 4 mL of chloroform:isoamyl alcohol (CIA 24:1), mix vigorously by inversion for 40-60 seconds and centrifuge for 10 minutes at 13000 x g at room temperature. Transfer the upper aqueous phase to a clean tube.
14. Repeat step 13 once, then add half volume of 7.5 M ammonium acetate, gently mix by inversion and add 2 volumes of 100% ethanol. Mix by inversion and place at -20 °C for 30 minutes. DNA may be stored as ethanol precipitate at -20 °C for up to 1 year.
15. Centrifuge at 13000 x g for 20 minutes at 4 °C to pellet the DNA.
16. Rinse the DNA pellet twice with 10 mL of 70 % ethanol loosening the pellet from the side of the tube and remove residual ethanol by vacuum.

17. Re-suspend DNA in 1 mL TE, pH 8.0 and incubate at 65 °C for at least 1 hour with periodic gentle mixing.
18. Centrifuge the DNA solution at 16000 x g for 10 minutes at 4 °C. Transfer the aqueous portion to a clean tube without disturbing the pellet and store at 4 °C.
19. Add equal volume of 20% PEG precipitation buffer (~1 mL) to the extracted DNA solution. Mix well by inversion.
20. Incubate the PEG/DNA mixture for 15 minutes at 37 °C.
21. Centrifuge the PEG/DNA mixture for 15 minutes at 15000 x g at room temperature.
22. Pour off supernatant. Wash the walls of the tube and DNA pellet with 1 mL of 80% ethanol loosening the pellet from the tube. Carefully pour off ethanol, centrifuge briefly and remove by pipetting any residual ethanol.
23. Repeat wash (step 22) for a total of two washes.
24. Completely dry any residual ethanol by vacufuge at low heat.
25. Re-suspend the pellet in 1 mL TE or H_2O. If necessary, incubate the sample at 60°C to dissolve the pellet.
26. Centrifuge the re-suspended DNA solution at 15000 x g for 15 minutes.
27. Transfer DNA solution to a clean tube without disturbing the pellet and store DNA at 4° C.

4. Testing of the DNA extraction method by the Community Reference Laboratory for GM Food and Feed

The aim of the experimental testing was to verify that the DNA extraction method provides DNA of suitable quantity and quality for the intended purpose. The DNA extraction method should allow preparation of the analyte in quality and quantity appropriate for the analytical method used to quantify the event-specific analyte versus the reference analyte.

The CRL-GMFF tested the method proposed by the applicant on samples of food and feed consisting of ground cotton seeds provided by the applicant.

To assess the suitability of the DNA extraction method for real-time PCR analysis, the extracted DNA was tested using a qualitative PCR run on the real-time PCR equipment.

4.1 Preparation of samples

About 200 g of cotton seed material were ground using a GRINDOMIX GM 200 (Retsch GmbH) mixer.

4.2 DNA extraction

DNA was extracted following method described above (see paragraph 3. “Description of the methods”); the DNA extraction was carried out on 6 test portions (replicates) and repeated over three days, giving a total of 18 DNA extractions.

4.3 DNA concentration, yield and repeatability

Concentration of the DNA extracted was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). Each DNA extract was measured twice, and the two values were averaged. DNA concentration was determined on the basis of a five point standard curve ranging from 1 to 500 ng/µL using a Biorad VersaFluor fluorometer.

The DNA concentration for all samples is reported in the Table 1 below.

Table 1. DNA concentration (ng/µL) of eighteen samples extracted in three days: yellow boxes for samples extracted on day 1, green boxes for samples extracted on day 2 and blue boxes for samples extracted on day 3.

Sample	Concentration (ng/µL)
1	346.8
2	359.4
3	297.9
4	265.2
5	479.2
6	326.2
1	346.0
2	322.4
3	308.6
4	465.9
5	302.2
6	329.9
1	568.1
2	427.8
3	501.5
4	442.6
5	383.8
6	395.8

- ✓ DNA concentration (ng/µL)

Overall average	381.6 ng/µL
Standard deviation of all samples	82.7 ng/µL
Coefficient of variation	21.7 %

- ✓ Yield (total volume of DNA solution: 18000 µl)

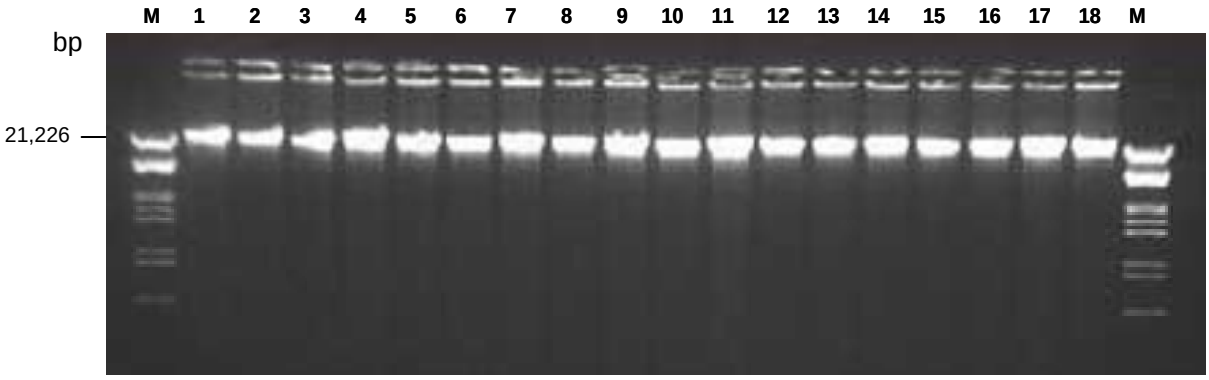
Overall average	381.6 µg
Standard deviation	82.7 µg
Coefficient of variation	21.7 %

4.4 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 8 µL of the DNA solution were analysed on a 1.0% agarose gel (Figure 1).

The eighteen genomic DNA samples extracted as described above appeared as distinct fluorescent banding patterns migrating through the gel corresponding to high molecular weight DNA. None of the DNA samples showed indication of significant degradation ('smearing').

Figure 1. Agarose gel electrophoresis of eighteen genomic DNA samples extracted from cotton seeds. Lanes 2-7: samples extracted on day 1; lanes 8-13 samples extracted on day 2; lanes 14-19 samples extracted on day 3; lanes 1 and 20: Lambda DNA/EcoRI+HindIII molecular weight marker (M).



4.5 Purity / Absence of PCR inhibitors

In order to assess the purity and to confirm the absence of PCR inhibitors, the extracted DNA solutions were adjusted to a concentration of 50 ng/µL (hereafter referred as "undiluted" samples).

Subsequently fourfold serial dilutions of each extract were prepared with 0.2x TE buffer (1:4, 1:16, 1:64, 1:256) and analysed using a real-time PCR system detecting the target sequence of the endogenous gene *acyl carrier protein 1* (*acp 1*). The Ct values obtained for "undiluted" and diluted DNA samples are reported in the Table 2.

Table 2. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of cotton gene *acp 1*. Yellow boxes for samples extracted on day 1, green boxes for samples extracted on day 2 and blue boxes for samples extracted on day 3.

	Undiluted	Diluted			
DNA extract	(40 ng/µL)	1:4	1:16	1:64	1:256
1	22.60	24.85	26.21	28.26	30.23
2	22.57	24.52	25.91	28.03	30.37
3	22.73	25.48	26.13	28.27	30.21
4	22.85	25.59	26.38	28.20	30.37
5	22.69	24.48	26.15	28.23	30.43
6	22.72	25.30	26.35	28.41	30.40
1	22.44	24.37	25.98	28.14	30.42
2	22.28	24.55	25.85	28.19	30.30
3	22.60	24.68	25.97	28.07	30.25
4	22.63	24.60	26.38	28.40	30.40
5	22.48	25.39	26.49	28.44	30.62
6	22.77	24.55	26.21	28.18	30.27
1	22.85	24.79	26.51	28.43	30.55
2	22.80	24.87	26.28	28.17	30.46
3	22.82	24.58	26.15	28.35	30.51
4	22.73	24.71	26.26	28.22	30.39
5	22.87	25.45	26.25	28.48	30.44
6	22.66	25.23	26.24	28.29	30.43

Table 3 below reports the comparison of extrapolated Ct values versus measured Ct values for all samples and the values of linearity (R^2) and slope of all measurements.

To measure inhibition, the Ct values of the four diluted samples were plotted against the logarithm of the dilution and the Ct value for the “undiluted” sample (50 ng/µL) was extrapolated from the equation calculated by linear regression.

Subsequently the extrapolated Ct for the “undiluted” sample was compared with the measured Ct. The evaluation is carried out considering that PCR inhibitors are present if the measured Ct value for the “undiluted” sample is suppressed by > 0.5 cycles from the calculated Ct value. In addition, the slope of the curve should be between -3.6 and -3.1.

Table 3. Comparison of extrapolated Ct values versus measured Ct values (amplification of cotton gene *acp 1*)

DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**
1	0.99	-3.02	22.84	22.60	0.24
2	0.98	-3.26	22.29	22.57	0.28
3	0.96	-2.71	23.44	22.73	0.71
4	0.96	-2.68	23.60	22.85	0.74
5	0.99	-3.31	22.33	22.69	0.35
6	0.98	-2.88	23.27	22.72	0.55
1	0.99	-3.37	22.15	22.44	0.29
2	0.99	-3.25	22.33	22.28	0.04
3	0.98	-3.22	22.34	22.60	0.26
4	0.99	-3.22	22.59	22.63	0.03
5	0.98	-2.93	23.32	22.48	0.84
6	0.99	-3.17	22.52	22.77	0.25
1	0.99	-3.19	22.77	22.85	0.08
2	0.99	-3.10	22.78	22.80	0.02
3	0.99	-3.32	22.40	22.82	0.42
4	0.99	-3.22	22.52	22.73	0.21
5	0.97	-2.86	23.36	22.87	0.49
6	0.97	-2.93	23.14	22.66	0.47

Note: In yellow boxes samples from 1 to 6 extracted on day 1; in green boxes samples from 1-6 extracted on day 2; in blue boxes samples from 1-6 extracted on day 3.

***The expected slope for a PCR with 100% efficiency is -3.32**

****delta Ct = abs (Ct extrapolated - Ct measured)**

All ΔCt values of extrapolated versus measured Ct are < 0.5, with four exceptions: the sample number 3, 4 and 6 extracted on day 1, with a value of 0.71, 0.74 and 0.55 respectively and sample number 5 extracted on day 2 with a value of 0.84.

R^2 of linear regression is > 0.98 for all DNA samples except samples 3 and 4 and six extracted on day 1 (0.96, 0.96 and 0.978), sample 5 extracted on day 2 (0.978) and samples 5 and 6 extracted on day 3 (0.97 and 0.97). The slope of the curve are between -3.1 and -3.6, with exception of samples 1, 3, 4 and 6 extracted on day 1, sample 5 extracted on day 2, samples 5 and 6 extracted on day 3.

4.5.1 Additional PEG precipitation

All samples that failed to pass the test to assess absence of PCR inhibitors were processed with a second PEG precipitation, following steps from 19 to 27 of the extraction procedure (see paragraph 3. “Description of the methods”).

In order to assess the purity and to confirm the absence of PCR inhibitors, the extracted DNA solutions were treated as described previously, solutions were adjusted to a concentration of 50 ng/µL and fourfold serial dilutions of each extract were prepared with 0.2x TE buffer (1:4, 1:16, 1:64, 1:256) and analysed using a real-time PCR system detecting the target sequence of the endogenous gene *acyl carrier protein 1* (*acp 1*). The Ct values obtained for "undiluted" and diluted DNA samples are reported in the Table 4.

Table 4. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of cotton gene *acp 1*.

	Undiluted	Diluted			
DNA extract	(40 ng/µL)	1:4	1:16	1:64	1:256
1	22.48	24.68	26.54	28.84	30.94
3	22.61	24.58	26.65	28.65	31.02
4	22.63	24.56	26.59	28.62	31.06
6	22.67	24.54	26.59	28.75	30.95
5	22.56	24.63	26.67	28.69	31.08
5	22.58	24.53	26.59	28.63	30.87
6	22.55	24.66	26.35	28.49	30.94

To measure inhibition, the Ct values of the four diluted samples were plotted against the logarithm of the dilution and the Ct value for the "undiluted" sample (50 ng/µL) was extrapolated from the equation calculated by linear regression.

Subsequently the extrapolated Ct for the "undiluted" sample was compared with the measured Ct. The evaluation is carried out considering that PCR inhibitors are present if the measured Ct value for the "undiluted" sample is suppressed by > 0.5 cycles from the calculated Ct value. In addition, the slope of the curve should be between -3.6 and -3.1.

Table 5. Comparison of extrapolated Ct values versus measured Ct values (amplification of cotton gene *acp 1*).

DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**
1	0.99	-3.51	22.48	22.48	0.00
3	0.99	-3.54	22.39	22.61	0.22
4	0.99	-3.57	22.33	22.63	0.30
6	0.99	-3.55	22.36	22.67	0.31
5	0.99	-3.55	22.42	22.56	0.14
5	0.99	-3.50	22.39	22.58	0.19
6	0.99	-3.49	22.36	22.55	0.19

After the second PEG precipitation all ΔCt values of extrapolated versus measured Ct are < 0.5; R^2 of linear regression is > 0.99 for all DNA samples; the slopes of the curves are between -3.1 and -3.6.

5. Conclusion

The data reported confirm that the extraction method, applied to cotton seeds provided by the applicant, produces DNA of suitable quantity and quality for subsequent PCR based detection applications.

In some cases a second PEG precipitation step may be needed to improve the quality of the DNA extracted.

The method is consequently applicable to samples of Cotton seeds provided as samples of food and feed in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

6. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32232) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)]

7. References

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Abstract

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the MON 88913 transformation event in cotton DNA (unique identifier MON-88913-8). The collaborative trial was conducted according to internationally accepted guidelines [1, 2].

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 “on genetically modified food and feed” and with Regulation (EC) No 641/2004 of 6 April 2004 “on detailed rules for the implementation of Regulation (EC) No 1829/2003”, Monsanto provided the detection method and the samples: genomic DNA from cotton seeds harbouring the MON 88913 event (line ST 4664) and from conventional cotton seeds (line ST 474). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from nine European countries.

The results of the international collaborative trial met the ENGL performance requirements. The method is, therefore, considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Regulation (EC) No 641/2004.

The results of the collaborative study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/ .

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