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Event-specific Method for the Quantification of Cotton Line GHB614 Using Real-time PCR

Validation Report and Protocol

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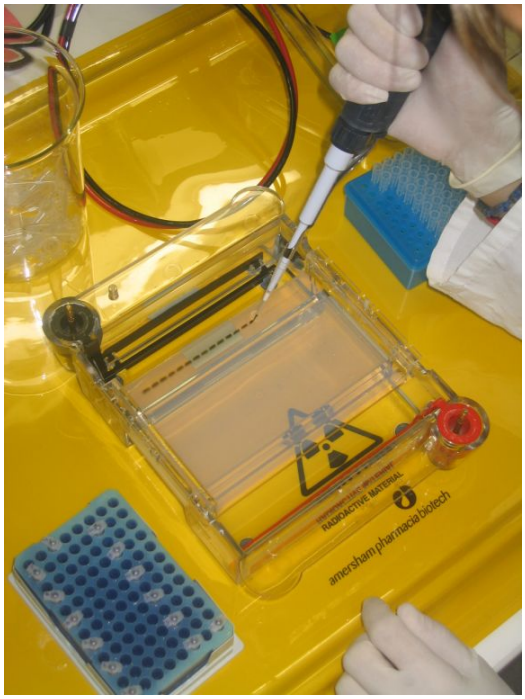

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Event-specific Method for the Quantification of Cotton Line GHB614 Using Real-time PCR

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 GHB614 transformation event in cotton DNA (unique identifier BCS-GHØØ2-5). 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, Bayer CropScience provided the detection method and the samples (genomic DNA from cotton plants containing the transformation event GHB614 and from conventional cotton plants). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from ten 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 Commission Regulation (EC) No 641/2004.

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

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Report on Steps 1-3 of the Validation Process

Bayer CropScience submitted the detection method and control samples for cotton event GHB614 (unique identifier BCS-GHØØ2-5) under Article 8 and 20 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.it/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.it/guidancedocs.htm) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed and requests of complementary information addressed to the applicant. Upon reception of complementary information, the scientific evaluation of the detection method for event GHB614 was positively concluded in March 2008.

In January 2008, the CRL-GMFF verified the purity of the control samples provided and in March 2008 the CRL-GMFF verified experimentally the method characteristics (step 3, experimental testing of samples and methods) by quantifying five blind GM levels within the range 0.09%-4.5% on a genome copy number basis. 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 14th March 2007 (http://gmo-crl.jrc.it/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

Bayer CropScience submitted the detection method and control samples for cotton event GHB614 (unique identifier BCS-GHØØ2-5) under Article 8 and 20 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, Biotechnology and GMOs Unit of the Institute for Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Commission Regulation EC (No) 1829/2003) organised the international collaborative study for the event-specific method for the detection and quantification of GHB614 cotton. 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 Commission 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 Commission Regulation (EC) No 641/2004 and following its operational procedures.

The internal experimental evaluation of the method was carried out between January and March 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 April 2008.

A method for DNA extraction from cotton seeds, 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 is available at http://gmo-crl.jrc.it/.

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 GHB614 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton *adhC* (*alcohol dehydrogenase C*) endogenous assay (reference gene) and the target assay (GHB614) 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 November 2007 the CRL-GMFF invited all National Reference Laboratories nominated under Commission 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 maize GM event GHB614.

Thirty-eight laboratories expressed in writing their willingness to participate, two declined the invitation, while thirty-one 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 GHB614.

Laboratory	Country
Austrian Agency for Health and Food Safety, Competence Centre Biochemistry	AT
Central Agricultural Office, Food and Feed Safety Directorate, Central Feed Investigation Laboratory - National Reference Laboratory	HU
Central Agricultural Office, Food and Feed Safety Directorate, Lab. for GMO food	HU
Danish Plant Directorate, Laboratory for Diagnostics in Plants, Seed, and Feed	DK
Federal Institute for Risk Assessment	DE
Finnish Customs Laboratory	FI
Institute of Biochemistry and Biophysics Polish Academy of Sciences, Genetic Modifications Analysis Laboratory	PL
National Centre for Food, Spanish Food Safety Agency	ES
National Institute of Biology	SI
National Institute of Engineer, Technology and Innovation – Food Industry Laboratory	PT
State Institute of Chemical and Veterinarian Analysis	DE
Walloon Agricultural Research Centre (CRA-W) - Dept. of Quality of Agricultural Products	BE

3. Materials

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

i) genomic DNA extracted from leaves of cotton plants harbouring the event GHB614 homozygously (32RRMM0225)
and
ii) genomic DNA extracted from leaves of a near-isogenic non-GM cotton plants (32RRMM0393)

Samples were provided by the applicant in accordance to the provisions of Commission 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% GHB614 cotton 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 (200 µL of DNA solution each) labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (80 µL of DNA solution each) labelled from U1 to U20.
- ✓ Reaction reagents as follows:
  - ❑ Universal PCR Master Mix, two bottles: 5 mL each
  - ❑ Distilled sterile water, one tube: 4 mL

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

  adhC reference system

  - ❑ KVM157 (10 µM): 160 µL
  - ❑ KVM158 (10 µM): 160 µL
  - ❑ TM012 (10 µM): 160 µL

  GHB614 system

  - ❑ SHA007 (10 µM): 320 µL
  - ❑ SHA008 (10 µM): 320 µL
  - ❑ TM072 (10 µM): 160 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 GHB614 specific system and for the *adhC* reference system. In total, two plates were run per participating laboratory and 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. GHB614 GM contents

GHB614 GM% (GM copy number/cotton genome copy number x 100)
0.09
0.40
0.90
2.00
4.50

5. Method

Description of operational steps followed

For the specific detection of event GHB614 DNA, a 119-bp fragment of the integration region of the construct inserted into the plant genome (3' insert-to-plant junction) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM dye and TAMRA as quencher dye.

For the relative quantification of event GHB614 DNA, a cotton-specific reference system amplifies a 73-bp fragment of the cotton endogenous gene *adhC* (*alcohol dehydrogenase C*), using two *adhC* gene-specific primers and an *adhC* gene-specific probe labelled with VIC dye and TAMRA as quencher dye.

Standard curves are generated for both the GHB614 and the *adhC* 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 GHB614 DNA in a test sample, the GHB614 copy number is divided by the copy number of the cotton reference gene (*adhC*) and multiplied by 100 to obtain the percentage value (GM% = GHB614 / *adhC* x 100).

Calibration sample S1 was prepared by mixing the appropriate amount of GHB614 DNA in control non-GM cotton DNA to obtain a 10% GM GHB614 in a total of 200 ng cotton DNA. Samples S2 was prepared by three-fold dilution from the S1 sample; sample S3 was prepared by five-fold dilution from S2 sample; sample S4 was prepared by four-fold dilution of sample S3 and sample S5 was prepared by five-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 (% GM calculated considering the 1C value for cotton genome as 2.33 pg) [(3)].

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

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction	300	100	20	5	1
Cotton genome copies	128750	42917	8583	2146	429
GHB614 GM cotton copies	12875	4292	858	215	43

6. Deviations reported

Nine laboratories reported no deviations from the protocol.

One laboratory inverted the S2 and S5 samples on one plate with no consequences since sample label was correctly attributed in subsequent analysis.

One laboratory inverted the loading position for the reference and GM-specific systems in one plate. This had no consequences in terms of data analysis.

One laboratory performed PCR reactions in 20 µL of total volume because only a 384-well plate configuration of the ABI 9700HT instrument was available. Final concentrations of PCR reagents remained unchanged.

The regression parameters of the measured versus true values for one laboratory were inconsistent. Based on the F statistics by Dent and Blackie (1979), the hypothesis of intercept and slope simultaneously equal to 0 and 1 was rejected ($F = 789.36$, $p = 8.26011E^{-05}$); therefore this laboratory was excluded from data analysis.

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 GHB614 system and the *adhC* reference system are summarised in Table 4.

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

		GHB614			*adhC*		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.40	97	1.00	-3.49	93	1.00
	B	-3.57	91	0.99	-3.53	92	1.00
2	A	-3.47	94	1.00	-3.59	90	1.00
	B	-3.50	93	1.00	-3.60	89	1.00
3	A	-	-	-	-	-	-
	B	-	-	-	-	-	-
4	A	-3.44	95	1.00	-3.44	95	1.00
	B	-3.57	91	0.99	-3.54	92	1.00
5	A	-3.50	93	1.00	-3.39	97	1.00
	B	-3.53	92	1.00	-3.52	93	1.00
6	A	-3.22	105	0.98	-3.64	88	0.99
	B	-3.24	104	0.99	-3.63	89	0.99
7	A	-3.55	91	1.00	-3.56	91	1.00
	B	-3.62	89	1.00	-3.51	92	1.00
8	A	-3.63	88	0.99	-3.53	92	1.00
	B	-3.56	91	1.00	-3.53	92	1.00
9	A	-3.53	92	1.00	-3.46	95	1.00
	B	-3.37	98	1.00	-3.52	92	1.00
10	A	-3.49	94	1.00	-3.49	93	1.00
	B	-3.48	94	1.00	-3.47	94	1.00
11	A	-3.51	93	1.00	-3.39	97	1.00
	B	-3.47	94	1.00	-3.49	93	1.00
	A	-3.64	88	1.00	-3.57	90	1.00
	B	-3.51	93	1.00	-3.58	90	1.00
	Mean	-3.49	94	1.00	-3.52	92	1.00

The mean PCR efficiency was 94% for the GHB614 system and 92% for the *adhC* system, with both values within the ENGL acceptance criteria. The linearity of the method was 1.00 for both systems.

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

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.

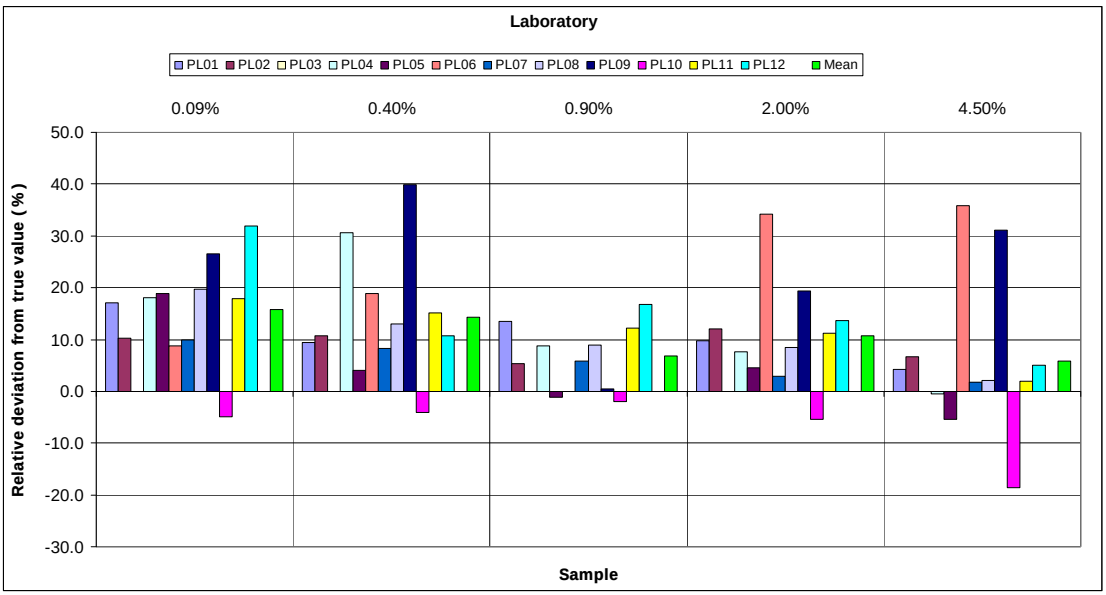
	GMO content (GMO% = GMO copy number/cotton genome copy number x 100)																			
LAB	0.1				0.4				0.9				2.0				4.5			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.10	0.09	0.11	0.12	0.41	0.48	0.39	0.47	1.04	0.96	1.02	1.05	2.22	2.26	2.13	2.17	4.68	4.77	4.53	4.77
2	0.10	0.11	0.09	0.10	0.45	0.48	0.42	0.42	1.08	0.94	0.86	0.91	2.33	2.35	2.14	2.15	4.81	4.97	4.64	4.78
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	0.10	0.11	0.11	0.10	0.45	0.74	0.42	0.48	0.99	1.03	0.90	1.00	2.10	2.28	2.16	2.06	4.46	4.71	4.57	4.15
5	0.12	0.09	0.12	0.11	0.41	0.43	0.38	0.44	0.88	0.93	0.82	0.92	2.04	2.09	2.08	2.16	4.38	4.53	3.91	4.22
6	0.15	0.06	0.09	0.09	0.46	0.50	0.60	0.34	1.43	1.60	2.30	0.79	2.84	3.44	2.41	2.05	7.16	7.58	4.61	5.11
7	0.10	0.09	0.11	0.10	0.44	0.41	0.48	0.40	0.94	0.93	0.98	0.96	2.03	2.09	2.11	2.00	4.57	4.23	4.62	4.88
8	0.11	0.11	0.11	0.10	0.45	0.42	0.47	0.47	0.98	1.01	0.97	0.96	2.23	2.18	2.12	2.15	4.60	4.47	4.55	4.76
9	0.09	0.10	0.18	0.08	0.70	0.61	0.48	0.45	1.06	0.57	0.99	1.00	2.43	2.88	2.14	2.10	7.74	6.82	4.52	4.51
10	0.09	0.10	0.09	0.07	0.39	0.37	0.36	0.41	1.01	0.76	0.80	0.95	2.23	1.70	1.75	1.89	3.60	2.98	4.05	4.01
11	0.12	0.10	0.11	0.09	0.49	0.48	0.42	0.45	1.03	1.03	1.01	0.97	2.13	2.27	2.30	2.20	4.81	4.63	4.40	4.51
12	0.12	0.13	0.12	0.11	0.41	0.41	0.47	0.48	1.10	0.94	1.07	1.09	2.35	2.26	2.26	2.22	4.66	4.86	4.65	4.74

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.

As observed in Figure 1, the mean relative deviations from the true values are positive for all GM levels, meaning that the GM content tends to be over-estimated at all GM levels. Only two laboratories showed a deviation from the true values above 25% at the GM levels of 0.09%, 0.4%, 2.0% and 4.5%.

Overall, the average relative deviation is within the acceptance criterion at all GM levels tested, indicating a satisfactory trueness of the method.

Figure 1. Relative deviation (%) from the true value of GHB614 for all laboratories



8. Method performance requirements

Among the performance criteria established by ENGL and adopted by the CRL-GMFF (http://gmo-crl.jrc.it/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 values of RSD_R (%) is 17% at the 0.4% GM level, thus well within the acceptance criterion.

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.it/guidancedocs.htm).

Table 6. GHB614: summary of validation results.

	Expected value (GMO %)				
unknown sample GMO %	**0.09**	**0.4**	**0.9**	**2.0**	**4.5**
Laboratories having returned valid results	11	11	11	11	11
Samples per laboratory	4	4	4	4	4
Number of outliers	2	0	2	3	3
Reason for exclusion	2C	-	2C	3C	2C, 1G
Mean value	0.10	0.46	0.97	2.18	4.59
Relative repeatability standard deviation, RSDr (%)	9.4	15	6.8	3.3	4.1
Repeatability standard deviation	0.010	0.070	0.065	0.072	0.190
Relative reproducibility standard deviation, RSDR (%)	12	17	8.3	4.4	5.1
Reproducibility standard deviation	0.012	0.078	0.080	0.097	0.235
Bias (absolute value)	0.014	0.057	0.068	0.175	0.089
Bias (%)	15	14	7.5	8.8	2.0

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.

As can be observed from the values reported in Table 6, the method has a repeatability standard deviation below 25% at all GM levels, with the highest value of RSD_r (%) of 15% at the 0.4% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. In this case the method fully satisfies this requirement across the entire dynamic range tested; in fact, the highest deviation from true value (bias %) is 15% at the 0.09% level, thus well within the acceptance criterion.

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.it/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 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

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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 ≥ slope ≥ - 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_r)

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 Line GHB614 Using Real-time PCR

Protocol

5 September 2008

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

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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 GHB614 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 GHB614 DNA, a 119-bp fragment of the integration region of the construct inserted into the plant genome (located at the 3' plant DNA region) 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 TAMRA at its 3' end.

For the relative quantification of cotton event GHB614 DNA, a cotton-specific reference system amplifies a 73-bp fragment of the cotton endogenous alcohol dehydrogenase C gene (*adhC*), using two specific primers and an *adhC* gene-specific probe labelled with VIC as a reporter 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 GHB614 DNA in a test sample, cotton GHB614 and *adhC* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of cotton event GHB614 DNA to total cotton DNA.

2. Validation status and performance characteristics

2.1 General

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

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 April 2008.

Each participant received twenty blind samples containing cotton GHB614 genomic DNA at five GM contents, ranging from 0.09% to 4.5%.

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 GM GHB614 in four unknown samples. Four replicates of each GM level were analysed on the same PCR plate.

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

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.023% in 200 ng of total cotton DNA. The relative LOD was not assessed in a collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.08% in 200 ng of total cotton DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.09%.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to cotton event GHB614 and thus imparts event-specificity to the 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 rice LLRice62, maize T25, MON810, Bt11, GA21, NK603, oilseed rape Ms1, Ms8, Rf1, Rf2, Rf3, Topas 19-2, T45, OXY-235, RT73, soybean A2704-12, A5547-127, Round-Up Ready® and cotton lines LLCotton25, T303-3, T304-40, GHB623, GHB119, GHB714, MON1445 and conventional cotton.

According to the applicant, the GHB614 system did not react with any of the plant materials tested, except the positive control cotton line GHB614; 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 GHB614

3.2.1 General

The PCR set-up for the taxon specific target sequence (*adhC*) and for the GMO (event GHB614) 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 25 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% GHB614 in non-GM cotton DNA for a total of 300 ng of DNA (corresponding to approximately 128,750 cotton genome copies with one genome assumed to correspond to 2.33 pg of haploid cotton genomic DNA) [(1)].

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 e.g. 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 GHB614 system and one for the *adhC* 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 GHB614 specific system.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
SHA007 primer (10 µM)	400 nM	1
SHA008 primer (10 µM)	400 nM	1
TM072 TaqMan® probe (10 µM)	200 nM	0.5
Nuclease free water	#	5
Template DNA (max 200 ng)	#	5.0
Total reaction volume:		25

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

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM157 primer (10 µM)	200 nM	0.5
KVM158 primer (10 µM)	200 nM	0.5
TM012 TaqMan® probe (10 µM)	200 nM	0.5
Nuclease free water	#	6
Template DNA (max 200 ng)	#	5.0
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the cotton GHB614 and one for the *adhC* reaction mixes) 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. 20 x 3 = 60 µL reaction mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 x 3 = 15 µL DNA for three PCR repetitions). Vortex each tube for approx. 10 sec. 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 cycling conditions described in Table 3.

Table 3. Cycling program for GHB614 specific system and for the cotton *adhC* reference system

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG		50°C	120	No	1
2	Initial denaturation		95°C	600	No	1
3	Amplification	Denaturation	95°C	15	No	45
		Annealing & Extension	60°C	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. GHB614) 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. *adhC* 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 *adhC* and the GHB614 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 copy numbers in the unknown sample DNA.

For the determination of the amount of event GHB614 DNA in the unknown sample, the GHB614 copy number is divided by the copy number of the cotton reference gene (*adhC*) and multiplied by 100 to obtain the percentage value (GM% = GHB614/*adhC* 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
- 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')
GHB614 target sequence	
SHA007	5' – CAA ATA CAC TTG GAA CGA CTT CGT – 3'
SHA008	5' – GCA GGC ATG CAA GCT TTT AAA – 3'
TM072 (probe)	6 – FAM 5' – CTC CAT GGC GAT CGC TAC GTT CTA GAA TT– 3' TAMRA
Reference gene *adhC* target sequence	
KVM157	5' – CAC ATG ACT TAG CCC ATC TTT GC – 3'
KVM158	5' – CCC ACC CTT TTT TGG TTT AGC – 3'
TM012 (probe)	VIC 5' – TGC AGG TTT TGG TGC CAC TGT GAA TG – 3' TAMRA

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Community Reference Laboratory
CRL
GM Food and Feed

Cotton Seeds Sampling and DNA Extraction

Report on the Validation of DNA Extraction Method from Cotton Seeds

14 March 2007

Directorate General Joint Research Centre
Institute for Health and Consumer Protection
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Content

1. Introduction

This report describes a plant DNA extraction protocol derived from the publicly available "CTAB" method [(1)]. This protocol can be used for the extraction of DNA from cotton seeds and grains ground to powder with a Waring™ blender or with any other appropriate seed crushing device.
The procedure includes the use of hazardous chemicals and materials: it should be executed only by skilled laboratory personnel. It is also 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. Waring blender, model 7010S/7010G/7010HS/7010HG or equivalent
2. 70 mm Blender Base (Eberbach Corp. Cat.No. 8495) for Waring blender or equivalent
3. Micro centrifuge with 18,000 x g for Eppendorf tubes
4. Table centrifuge (swinging buckets) with 3,000 x g for Falcon tubes
5. Water bath adjustable to 60°C
6. Fluostar Galaxy type 0403 from BMG LabTechnologies
7. PC with Fluostar software (Fluo32)

2.2. Chemicals

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

1. Na2-EDTA: Titriplex III (Merck Cat. No. 1.08418.1000)
2. Tris-HCl: Tris(hydroxymethyl)aminomethane hydrochloride (USB Cat. No. 22676)
3. NaCl: sodium chloride (Duchefa Cat. No. S0520)
4. CTAB p.a. (Merck Cat. No. 1.02342.0100)
5. RNase A (Roche Cat.No. 0109-142)
6. Proteinase K (Promega Cat. No. V3021)
7. Ethanol p.a. (Merck Cat. No. 1.00983.1000)
8. Isopropanol p.a. (Merck Cat. No. 1.09634.2500)
9. Chloroform p.a. (Merck Cat. No. 1.02445.2500)
10. Octanol p.a. (Fluka Cat. No. 74850)
11. Genomic-tip 20/G (Qiagen, Cat. No. 10223)
12. Genomic DNA Buffers set including G2, QBT, QC and QF (Qiagen, Cat.No. 19060)

2.3. Solutions

The buffers and solutions used in the DNA extraction procedure are described in the 'CTAB/Genomic-tip 20" DNA extraction method' from Dow AgroSciences LLC (http://gmo-crl.jrc.it/statusofdoss.htm).

2.4. Precautions

- Octanol, chloroform 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

2.5 Abbreviations:

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

3. Description of the methods

Sampling:
Sampling approaches for seeds and grains are referred to in technical guidance documents and protocols described in:

- International Organization for Standardizations, Switzerland: ISO standard 6644, ISO standard 13690, ISO standard 5725;
- International Rules for Seed Testing (2004) International Seed Testing Association (ISTA), Switzerland. ISBN 3-906549-38-0;
- USDA-GIPSA (2001) Sampling grains for the detection of Biotech grains http://www.usda.gov/gipsa/.

Scope and applicability:
The "CTAB/Genomic-tip 20" method for DNA extraction is suitable for the isolation of genomic DNA from a wide variety of matrices. However, validation data included in the present document are restricted to ground cotton seeds. Application of the method to other matrices may require adaptation and possible further specific validation.

Principle:
The basic principle of DNA extraction consists of first releasing the DNA present in the matrix into aqueous solution and further purifying the DNA from PCR inhibitors. The "CTAB/Genomic-tip 20"" method starts with a lysis step (thermal lysis in the presence of Tris HCl, EDTA and CTAB) followed by removal of contaminants such as lipophilic molecules and proteins by extraction with choloroform:octanol and by the generation of a crude DNA extract by precipitation with CTAB buffer B. The resulting precipitate is then dissolved and remaining inhibitors are removed by anion-exchange chromatography using the commercially available gravity-flow column "Genomic-tip 20/G".

Seed crushing procedure:

- Seeds and grains are crushed using a Waring blender
- Grind in intervals of 10 seconds for 4 times at maximum speed. Shake between intervals until all powder is loose to improve crushing-procedure
- Seeds should be crushed until a fine powder is obtained and a homogenous powder is obtained
- Prevent cross-contamination by dust particles between the samples

Cotton seed DNA extraction protocol

The "CTAB/Genomic-tip 20" protocol from Dow AgroSciences LLC (http://gmo-crl.jrc.it/statusofdoss.htm) for DNA extraction of cotton seed and grain was applied.

4. Testing of the DNA extraction method by the method developer

Experimental data are provided from an in-house validation in which the analytical module has been applied to the relevant matrix in the context of the application for authorisation. A seed bulk sample was ground to fine powder. Six replicated independent DNA extractions were performed from 1 gram flour samples.

4.1. DNA concentration, yield and repeatability

The concentration of the extracted DNA was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). 1:50 dilutions of each DNA sample were prepared in duplicate and mixed with the PicoGreen reagent. The DNA concentration was determined on the basis of a nine-point standard curve ranging from 10 ng/ml to 200 ng/ml, using a Fluostar for fluorescence detection. Each concentration was obtained as an average of two readings per sample (Table 1).

Table 1. Results of the 6 DNA extractions: DNA concentration (ng/µl) and DNA extraction efficiency

DNA extraction	Sample mass (gr)	[DNA] (ng/µl)	DNA extraction efficiency (µg DNA/gram of sample)
A1	1.0	1313.8	210.2
A2	1.0	1372.6	219.6
A3	1.0	1637.0	261.9
A4	1.0	1850.0	296.0
A5	1.0	2361.8	377.9
A6	1.0	1419.2	227.1

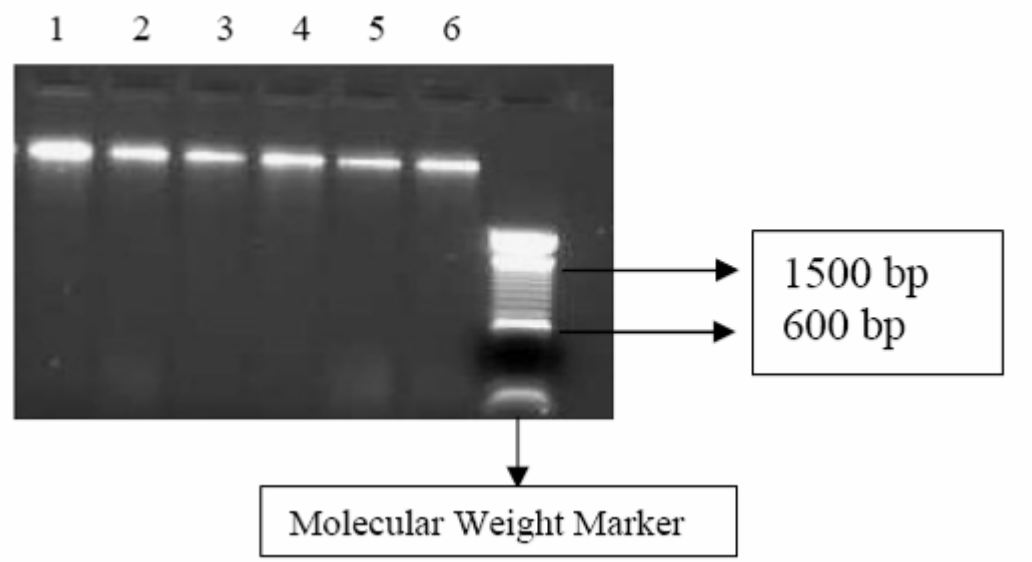
Table 1 lists the DNA concentrations and DNA yield of all 6 DNA extractions. The average DNA concentration was 1659 ± 397 ng/µl.
The average DNA extraction efficiency was 265.4 ± 63.6 µg DNA/g of sample, the relative Repeatability Standard Deviation (RSDr) was 24.0%.

Therefore, the cotton DNA extraction procedure is sufficiently efficient and yields adequate amounts of genomic DNA with a sufficient level of repeatability.

4.2. Analysis of DNA fragmentation

Analysis of DNA fragmentation was performed by ethidium bromide-stained agarose gel electrophoresis. Twenty five microliters of a 50x dilution of each DNA sample were analysed on a 1% agarose gel (TBE buffer), including a molecular weight marker (Figure 1).

Figure 1. Agarose gel electrophoresis of six genomic DNA samples extracted from cotton seeds (25 µl of 50x diluted DNA).



The 6 genomic DNA samples extracted appeared as distinct fluorescent banding patterns migrating through the gel corresponding to high molecular weight DNA. None of the 6 genomic DNA samples showed indications of significant degradation.

4.3. Evidence of the absence of PCR-inhibitory compounds

The absence of PCR inhibitory compounds in the DNA preparations was demonstrated by Real-time PCR using the oligonucleotides directed to the endogenous control gene Alcohol dehydrogenase, *AdhC* on serial dilutions of the DNA preparations.

All DNA extracts were adjusted to a concentration of 40 ng/µl. From this sample (named undiluted sample), a fourfold serial dilution series was prepared with water (1:4, 1:16, 1:64, 1:256).
To assess the presence of inhibitors, the Ct values (y axis) of the dilution series were plotted against the logarithm of the DNA amount (x axis). By linear regression, a trend line (y = ax + b) was calculated, as well as a correlation coefficient, r^2, as a measure of linearity (Table 2).
The ideal slope value, 'a', (optimal PCR efficiency) then becomes a = -3.32 (typically values between -3.1 and -3.6 indicate excellent PCR efficiencies).

Correlation coefficients of r^2 >0.98 indicate an excellent linear relationship, and thus equally efficient PCR amplification over the measured dynamic range.
The Ct value for the undiluted sample (40 ng/µl, 200 ng/reaction) was extrapolated from the equation calculated by linear regression. Subsequently the extrapolated Ct for the undiluted sample was compared with the measured Ct. Differences between measured and extrapolated Ct of < 0.5 are indicative of low level or absence of inhibition.

The results reported in Table 2 show no evidence of the presence of PCR-inhibitory compounds; in fact all 6 Ct values are < 0.5, suggesting absence of PCR inhibitors.

Table 2. Results of the analysis for PCR inhibitory compounds; comparison of extrapolated Ct values versus measured Ct values.

DNA	Slope, a	Intercept, b	Linearity, R^2	$Ct_{measured}$	$Ct_{extrapolated}$	ΔCt ($Ct_{measured}$-$Ct_{extrapolated}$)
A1	-3.3574	32.0841	0.9990	24.38	24.36	0.02
A2	-3.4347	32.3183	0.9994	24.44	24.41	0.03
A3	-3.4794	32.4502	0.9998	24.47	24.44	0.03
A4	-3.5702	32.8232	0.9999	24.74	24.61	0.13
A5	-3.3920	32.4542	0.9984	24.69	24.65	0.04
A6	-3.4376	32.4653	0.9997	24.54	24.56	-0.01

5. Experimental 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 "CTAB/Genomic-tip 20" 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.

5.1 Preparation of samples

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

5.2 DNA extraction

DNA was extracted following the "CTAB/Genomic-tip 20" method described at http://gmo-crl.jrc.it/statusofdoss.htm; the DNA extraction was carried out on 6 test portions (replicates).

5.3 DNA concentration, yield and repeatability

DNA 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 3 below.

Table 3. DNA concentration (ng/µl) of six DNA extractions from samples of cotton seeds

Sample	Concentration (ng/µl)
1	293
2	260
3	304
4	304
5	292
6	263

DNA concentration (ng/μl)

Overall average of all samples:	286 ng/μl
Standard deviation of all samples	19.7 ng/μl
Coefficient of variation	6.9 %

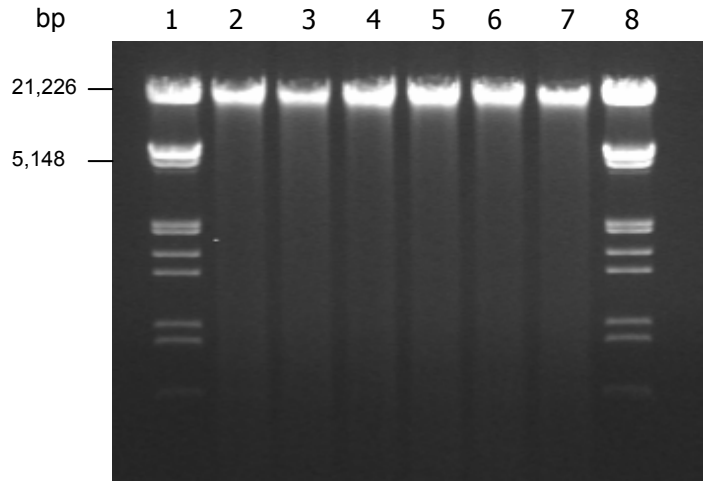
Yield (total volume of DNA solution: 160 μl)

Overall average of all samples:	45.7 μg
Standard deviation	3.15 μg
Coefficient of variation	6.9 %

5.4 Fragmentation state of DNA

The size of the six extracted DNA was evaluated by agarose gel electrophoresis; 8 µl of the DNA solution were analysed on a 1.0% agarose gel (Figure 2).
A Lambda DNA/EcoRI+HindIII Marker (M) was used.

Figure 2. Agarose gel electrophoresis of genomic DNA samples extracted from cotton seeds (lanes 2-7); lanes 1 and 8: Lambda DNA/EcoRI+HindIII Marker.



The six 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 indications of significant degradation ('smearing').

5.4 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 40 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 control gene Alcohol dehydrogenase, *AdhC*. The Ct values obtained for "undiluted" and diluted DNA samples are reported in the Table 4 below:

Table 4. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of cotton *Alcohol dehydrogenase* gene, *AdhC*

	Undiluted (40 ng/µl)	**Diluted**			
DNA extract	**1:1**	**1:4**	**1:16**	**1:64**	**1:256**
1	20.98	23.00	25.05	27.18	29.30
2	21.22	22.93	24.88	26.98	29.45
3	21.02	23.14	24.96	27.16	29.27
4	21.09	22.95	25.06	27.04	29.29
5	21.09	23.11	25.15	27.13	29.35
6	21.49	23.49	25.51	27.78	30.08

Table 5 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.

Table 5. Comparison of extrapolated Ct values versus measured Ct values (amplification of cotton Alcohol dehydrogenase gene, *AdhC*)

DNA extraction	**R^2**	**Slope***	**Ct extrapolated**	**mean Ct measured**	**ΔCt****
1	0.999	-3.49	20.87	20.98	0.11
2	0.999	-3.51	21.82	22.40	0.58
3	0.996	-3.42	20.98	21.02	0.04
4	0.999	-3.49	20.83	21.09	0.25
5	0.998	-3.44	21.01	21.09	0.09
6	0.999	-3.46	21.92	21.99	0.07

***The expected slope for a PCR with 100% efficiency is -3.32**
****delta Ct = abs (Ct extrapolated - Ct measured)**

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 (40 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.

R^2 of linear regression is > 0.99 for all DNA samples.

6. Conclusion

The data reported confirm that the extraction method provides DNA of suitable quantity and quality for subsequent PCR based detection applications. The method is therefore applicable to the samples of food and feed provided in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

7. 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)]

8. 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 GHB614 transformation event in cotton DNA (unique identifier BCS-GHØØ2-5). The collaborative trial was conducted according to internationally accepted guidelines.

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, Bayer CropScience provided the detection method and the samples (genomic DNA from cotton plants containing the transformation event GHB614 and from conventional cotton plants). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from ten 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 Commission Regulation (EC) No 641/2004

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

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