EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Sampling and DNA extraction of cotton seeds

Report from the Validation of the "CTAB/Genomic-tip 20" method for DNA extraction from ground cotton seeds

Method development and single laboratory validation:
Dow AgroSciences LLC
GeneScan Analytics GmbH

Method testing and validation:
Joint Research Centre – European Commission
Biotechnology & GMOs Unit
Community Reference Laboratory for GM Food and Feed

Contents

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1. Introduction

The purpose of the DNA extraction method described is to serve as a method to provide DNA for subsequent PCR based detection methods. The method does not only have to yield DNA of sufficient quality and quantity but is also required to be suitable for routine use in terms of ease of operations, sample throughput and costs. This Report describes the method and validation experiments including results.

2. Description of the method

Sampling

For sampling of seeds of 281-24-236/3006-210-23 cotton, the Applicant refers 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) No 1830/2003.

Scope and applicability

The "CTAB/Genomic-tip 20" method for DNA extraction described below is suitable for the isolation of genomic DNA from a wide variety of matrices. However, validation data presented here are restricted to ground cotton seeds. Application of the method to other matrices may require adaptation and needs specific validation.

Practicability

For DNA extraction using the "CTAB/Genomic-tip 20" method described below only standard equipment for molecular biology work is required, e.g. a centrifuge, an incubator and pipettes (for details, see section 3). Costs for reagents and consumables add up to 8 € per DNA extraction. The whole procedure from sample weighing to the final step of dissolving the DNA pellet in buffer solution takes about 9 hours of time in total with 2.5 hours hands-on time.

Principle

The basic principle of DNA extraction consists of first releasing the DNA present in the matrix into aqueous solution and further, concurrently or subsequently, purifying the DNA from PCR inhibitors. The "CTAB/ Genomic-tip 20" method starts with a thermal lysis step. The CTAB buffer A used in this step contains sufficient NaCl for keeping the DNA released from the sample material in solution. CTAB binds e. g. polysaccharides, cell wall debris and denatured proteins. After lysis, contaminants such as lipophilic molecules, proteins and CTAB/polysaccharide complexes are removed by extraction with chloroform:octanol and a crude DNA extract is generated by precipitation with CTAB buffer B. This second CTAB

buffer used in the procedure does not contain NaCl and therefore lowers the overall NaCl concentration. At a concentration below 0.5 M NaCl CTAB forms an insoluble complex with nucleic acids which can be precipitated by centrifugation while polysaccharides, phenolic components and other enzyme-inhibiting contaminants remain in solution. After dissolving the resulting precipitate, remaining inhibitors are removed by further purifying the crude DNA extract by anion-exchange chromatography using the commercially available gravity-flow column "Genomic-tip 20/G" (QIAGEN). During this procedure, DNA fragments (polyanions) bind under low-salt conditions by electrostatic interactions between the negatively charged phosphate groups of the nucleic acid backbone and the positively charged anion-exchange resin. Subsequent washing steps remove impurities. Finally the DNA is eluted with high-salt buffer, desalted and concentrated by a final isopropanol precipitation step.

Note

Milling/grinding of seeds not only facilitates the lysis by mechanically disrupting cellular structures and increasing the surface area, but is also indispensable for the generation of representative test portions by reducing the particle size. For details concerning requirements of particle size/particle number, generation of the test portions etc. please refer to the literature (e. g. prCEN/TS ISO 21568 sampling).
DNA extractions are recommended to be carried out on at least two test portions. Extraction blanks in duplicate (negative controls; handled identically but without sample material) are mandatory throughout extraction and subsequent PCR.

Lysis / CTAB precipitation

1. Weigh out 1 g*) of homogenized cotton seed material and transfer to a 15 ml conical tube
2. Add 5 ml CTAB buffer A and mix thoroughly
3. Incubate 1 hour at 60 °C with agitation
4. Spin down at room temperature for 10 minutes at 3,000 x *g*
5. Transfer supernatant to a new 15 ml conical tube containing 5 ml chloroform:octanol (24:1)
6. Mix thoroughly, incubate 5 min at room temperature with agitation
7. Spin down at room temperature for 10 minutes at 3,000 x *g*
8. Transfer 2.5 ml supernatant to a new 15 ml conical tube containing 5.5 ml CTAB buffer B
9. Mix thoroughly, incubate 30 min at room temperature
10. Spin down at room temperature for 20 min at 10,000 x *g*
11. Discard the supernatant, spin down again for 5 sec, remove any remaining supernatant and conserve the pellet
12. Resuspend the pellet in 500 µl 1x TE buffer (preheated to 50 °C) by vortexing (at least 10 sec at maximum speed).

Purification using Genomic-tip 20/G

13. Add 2.5 ml G2 buffer (containing 4 µl RNase A and 30 µl Proteinase K) and mix thoroughly
14. Incubate 2 hours at 50 °C with agitation
15. Spin down at room temperature for 5 minutes at 10,000 x *g*
16. Transfer the supernatant in a new 15 ml conical tube
17. Equilibrate a QIAGEN Genomic-tip 20/G with 1 ml QBT
18. Apply the sample to the equilibrated Genomic-tip 20/G (QIAGEN)
19. Wash the Genomic-tip 20/G with 3 x 1 ml buffer QC
20. Elute the genomic DNA with 1 ml QF buffer and collect the DNA in a 2 ml microcentrifuge tube
21. Repeat the elution with 1 ml QF buffer and collect the DNA in a second 2 ml microcentrifuge tube
22. Add 700 µl isopropanol to each tube, invert 10 times
23. Spin down at 4 °C for 30 min at 10,000 x g
24. Discard the supernatant and wash the DNA pellets with 1 ml 70% ethanol each
25. Spin down at 4 °C for 10 min at 15,000 x g
26. Discard the supernatant and air-dry the pellets for 10 min
27. Dissolve DNA pellets in 80 µl 1x TE buffer preheated to 50 °C each, incubate another 10 min at 50 °C
28. Transfer the DNA solution in microcentrifuge tube 1 to microcentrifuge tube 2 and combine both DNA solutions extracted (final volume: about 160 µl).

*) Depending on the particle size of the cotton seed material used for DNA extraction it might be advisable to adapt the amount of starting material. In case of very fine material a reduction of the amount of starting material (e. g. using 0.5 g instead of 1 g) might be advantageous.

3. Equipment / Reagents / Plasticware

3.1. Equipment

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

Equipment	**Example of appropriate apparatus**
Pipettes with adjustable volume	e. g. Eppendorf Research. 2 – 20 µl, 20 – 200 µl. 100 -1000 µl
Incubator with shaker or shaking water bath	e. g. Heraeus Function Line B12 in combination with shaker GFL 3005 (Gesellschaft für Labortechnik mbH)
Balances for the preparation of buffers and solutions and for sample weigh in	e. g. Ohaus Scout II. Ohaus Adventurer
Refrigerated centrifuge with rotors for 15 ml centrifuge tubes and microcentrifuge tubes	e. g. Sigma 4 K 15C with suitable rotors
Thermoblock for 1.5/2 ml microcentrifuge tubes	e. g. Bioblock Scientific 92333
Vortex	e. g. NeoLab Vortex VM-300

3.2. Reagents

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

Reagent	**Specification**
NaCl	p. a. quality or Molecular biology grade
CTAB	p. a. quality or Molecular biology grade
Tris	p. a. quality or Molecular biology grade
EDTA · Na_2-salt	p. a. quality or Molecular biology grade
HCl	p. a. quality
Proteinase K	from *Tritirachium album*, DNAses, Rnases, Exonucleases not detectable, Molecular biology grade
RNase A	from bovine pancreas, salt free, protease free and chromatographically homogeneous, ca. 90 Kunitz units/mg
Chloroform	p. a. quality
Octanol	p. a. quality
Isopropanol	p. a. quality
Ethanol	p. a. quality
Genomic-tip 20/G, 25 columns	10223, QIAGEN
Genomic DNA Buffers Set including G2, QBT, QC, and QF*	19060, QIAGEN

***** Buffers can also be prepared. Please, refer to the "QIAGEN Genomic DNA Handbook" supplied with the Genomic-tip 20/G columns for details.

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

CTAB buffer A

1.4 M NaCl
2% (w/v) CTAB
0.1 M Tris-Base
0.015 M EDTA

For 1 liter CTAB buffer A weigh out 81.8 g NaCl, 20 g CTAB, 12.1 g Tris-Base and 5.84 g EDTA in an appropriate beaker and add about 800 ml H_2O_{deion}. Adjust pH with HCl to pH 8.0, stir until all reagents are dissolved. Adjust volume to 1 l with H_2O_{deion}. Autoclave.
Store at room temperature for up to 1 year.

CTAB buffer B

1% (w/v) CTAB
0.1 M Tris-Base
0.015 M EDTA

For 1 liter CTAB buffer B weigh out 10 g CTAB, 12.1 g Tris-Base and 5.84 g EDTA in an appropriate beaker and add about 800 ml H_2O_{deion}. Adjust pH with HCl to pH 8.0, stir until all reagents are dissolved. Adjust volume to 1 l with H_2O_{deion}. Autoclave.
Store at room temperature for up to 1 year.

Proteinase K

20 mg/ml H_2O
For 10 ml proteinase K solution dissolve 200 mg proteinase K in 10 ml H_2O_{deion}.
Store at -20°C for up to 2 years.

RNase A

91 mg/ml
Dissolve 0,5 g RNase A in 5 ml 0.01 M sodium acetate (pH 5.2), aliquot in 1 ml portions, boil for 15 minutes to inactivate DNases, cool slowly to room temperature and add 100 µl 1M Tris-HCl (pH 7.4) to each aliquot.
Store at -20°C for up to 2 years.

70% (v/v) Ethanol

For 200 ml combine 140 ml 100% ethanol with 60 ml H_2O_{deion}.
Store at room temperature for up to 5 years.

1x TE buffer

10 mM Tris, pH 8.3
1 mM EDTA
For 100 ml 1x TE buffer combine 1 ml 1 M Tris (pH 8.3) and 200 µl 0.5 M EDTA (pH 8.0) and adjust the volume to 100 ml with H_2O_{deion}. Autoclave.
Store at room temperature for up to 2 years.

Chloroform : octanol (24:1)

For 100 ml Chloroform: octanol (24:1) combine 96 ml chloroform and 4 ml octanol. Mix.
Store at room temperature under the fume hood for up to 6 months.

3.3. Plasticware

Note: All plasticware has to be sterile and free from DNases, RNases and nucleic acids.

Item	Specification
15 ml conical tubes	e. g. Sarstedt, 62.554 502
1.5 ml and 2 ml microcentrifuge tube	e. g. Roth, 4182.1
filter tips	fitting the pipette models used

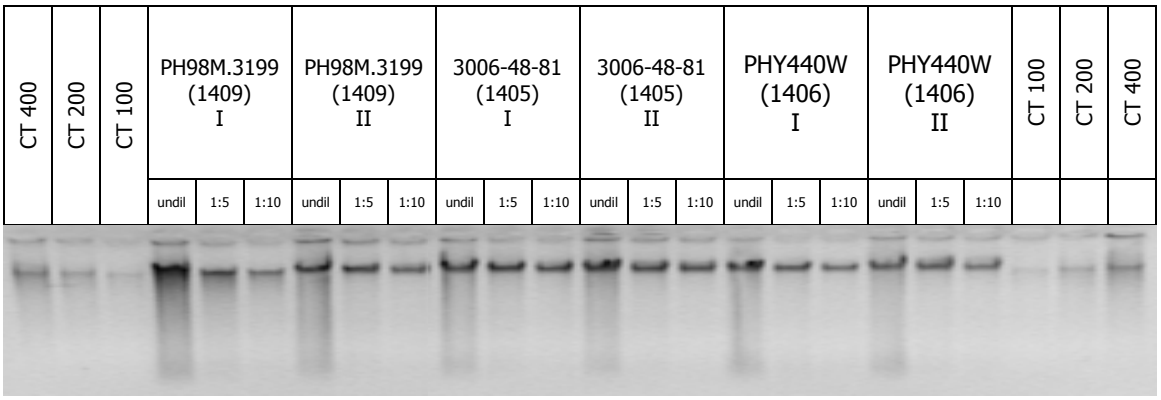
4. Experimental Validation and Results

For experimental validation six different samples of homogenized cotton seeds were extracted using the above described method. Five samples were extracted in duplicate, one sample was extracted six times.
The following method parameters were determined:

- Fragmentation state of DNA
- DNA concentration / yield, repeatability standard deviation RSDr
- Purity / absence of PCR-inhibitors
- Suitability of the resulting DNA for the quantitative, event-specific PCR detection method for 281-24-236/3006-210-23 cotton.

4.1. Fragmentation state of DNA

The fragmentation state of the extracted DNA was evaluated by agarose gel electrophoresis. 5.0 µl of each undiluted DNA and 1:5 and 1:10 dilutions respectively were analyzed on a 1.5% agarose gel (TAE buffer system). Defined amounts of calf thymus DNA (CT) were loaded as DNA quantity standards. After electrophoretic separation the gel was stained in ethidium bromide solution (1 µg/ml in distilled water) for 30 min and the DNA visualized using an UV transilluminator.



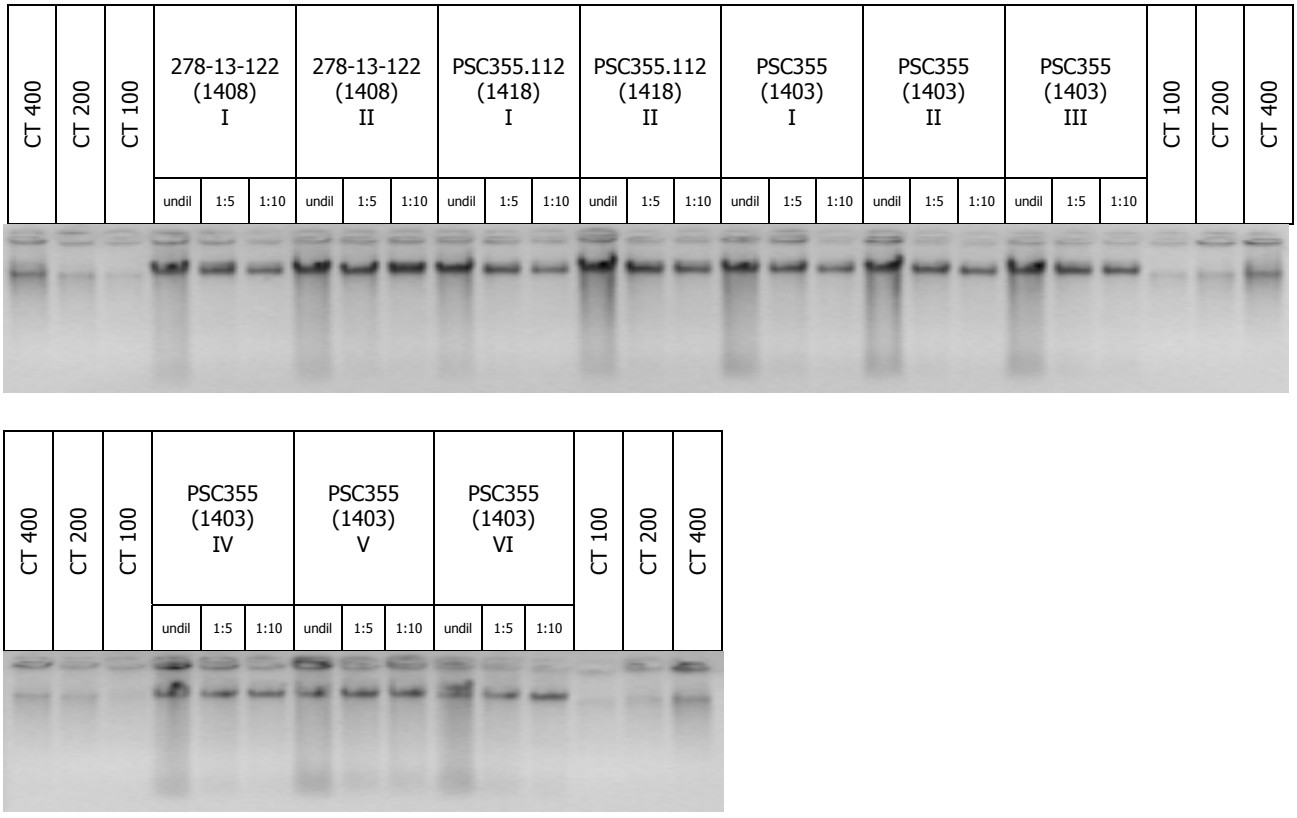


Figure 1. Agarose gel electrophoresis of DNA extracts (undiluted, 1:5 diluted, 1:10 diluted each)

Medium to high molecular weight DNA was observed for all samples.

4.2. DNA concentration / Yield, Repeatability Standard Deviation RSDr

The concentration of the DNA extracts was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). Suitable dilutions of each DNA extract were prepared in triplicates and mixed with the PicoGreen reagent. DNA concentration was determined on the basis of a five-point standard curve (triplicates) ranging from 20 ng/ml to 1000 ng/ml using an ABI PRISM® 7700 as fluorescence detector.

Table 1. DNA concentration / Yield

Cotton Line	Replicate	Concentration (ng/µl)	Mean (ng/µl)	Yield (µg)	Mean (µg)
PH98M.3199 (1409)	I	292	348	47	56
PH98M.3199 (1409)	II	403		64	
3006-48-81 (1405)	I	457	405	73	65
3006-48-81 (1405)	II	353		56	
PHY440W (1406)	I	276	297	44	48
PHY440W (1406)	II	318		51	
278-13-122 (1408)	I	395	349	63	56
278-13-122 (1408)	II	302		48	
PSC355.112 (1418)	I	320	316	51	50
PSC355.112 (1418)	II	311		50	
PSC355 (1403)	I	309	371	49	59
PSC355 (1403)	II	429		69	
PSC355 (1403)	III	333		53	
PSC355 (1403)	IV	360		58	
PSC355 (1403)	V	384		61	
PSC355 (1403)	VI	413		66	

DNA concentration:

Mean of PSC355 (1403), extraction with 6 repeats:	371 ng/µl
Standard deviation of PSC355 (1403), extraction with 6 repeats:	46 ng/µl
Relative standard deviation of extraction with 6 repeats:	12%

Yield:

Mean of PSC355 (1403), extraction with 6 repeats:	59 µg
Standard deviation of PSC355 (1403), extraction with 6 repeats:	7 ng/µl
Relative standard deviation of extraction with 6 repeats:	12%

4.3. Purity / absence of PCR-inhibitors

In order to assess the purity and confirm the absence of PCR inhibitors, the extracted DNA solutions were adjusted to a concentration of 30 ng/µl and spiked with a defined copy number of plasmid molecules carrying a special target sequence (a fragment of the gene coding for the maize High Mobility Group (HMG) Protein A gene).

Subsequently fourfold serial dilutions of each extract were prepared with 0.2x TE buffer (1:4, 1:16, 1:64, 1:256) and analyzed using a real-time PCR system detecting the target sequence on the spike plasmid (maize-specific HMG reference system). 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 (30 ng/µl, 150 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. Evaluation: PCR inhibitors are present if the measured Ct value for the "undiluted" sample is suppressed by > 0.5 cycles from the calculated Ct value.

Table 2. Ct values of "undiluted" (150 ng/reaction) and fourfold serial diluted DNA extracts

DNA extract	Replicate	undiluted	diluted			
		1:1	1:4	1:16	1:64	1:256
PH98M.3199 (1409)*	I	24.83	27.06	29.12	31.01	32.73
PH98M.3199 (1409)	II	24.88	26.91	28.95	31.23	33.24
3006-48-81 (1405)	I	25.93	28.12	30.22	32.26	34.09
3006-48-81 (1405)	II	26.05	28.23	30.17	32.46	34.57
PHY440W (1406)	I	26.13	28.14	30.29	32.43	34.63
PHY440W (1406)	II	25.84	28.05	30.18	32.49	34.51
278-13-122 (1408)	I	26.08	28.16	30.19	32.66	34.75
278-13-122 (1408)	II	26.02	28.01	30.12	32.53	34.76
PSC355.112 (1418)	I	26.06	28.15	30.27	32.27	34.13
PSC355.112 (1418)	II	26.03	28.12	30.20	32.23	34.35
PSC355 (1403)	I	26.09	28.12	30.37	32.37	35.19
PSC355 (1403)	II	25.99	28.24	30.17	32.49	34.77
PSC355 (1403)	III	25.64	27.95	30.22	32.26	34.55
PSC355 (1403)	IV	25.16	27.21	29.63	31.81	34.32
PSC355 (1403)	V	25.87	28.20	30.02	32.17	34.65
PSC355 (1403)	VI	25.93	27.97	30.09	32.23	34.72

***** 200 ng DNA / reaction

Table 3. Comparison of extrapolated Ct values versus measured Ct values

DNA extract	Replicate	Ct extrapol.	Ct experim.	delta Ct *	R^2
PH98M.3199 (1409)	I	25.26	24.83	0.43	0.9984
PH98M.3199 (1409)	II	24.77	24.88	0.11	0.9994
3006-48-81 (1405)	I	26.19	25.93	0.25	0.999
3006-48-81 (1405)	II	26.03	26.05	0.02	0.9991
PHY440W (1406)	I	25.97	26.13	0.16	1.0000
PHY440W (1406)	II	25.89	25.84	0.04	0.9994
278-13-122 (1408)	I	25.88	26.08	0.20	0.9986
278-13-122 (1408)	II	25.69	26.02	0.33	0.9994
PSC355.112 (1418)	I	26.22	26.06	0.16	0.9991
PSC355.112 (1418)	II	26.05	26.03	0.01	0.9999
PSC355 (1403)	I	25.71	26.09	0.38	0.9949
PSC355 (1403)	II	25.94	25.99	0.05	0.9983
PSC355 (1403)	III	25.79	25.64	0.14	0.9995
PSC355 (1403)	IV	24.87	25.16	0.30	0.9993
PSC355 (1403)	V	25.89	25.87	0.01	0.9953
PSC355 (1403)	VI	25.66	25.93	0.28	0.9984

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

All delta Ct values of extrapolated versus measured Ct are < 0.5, indicating absence of PCR inhibitors. R^2 of linear regression > 0.99 for all DNA solutions extracted.

4.4. Suitability of the resulting DNA for quantitative event-specific PCR

In order to demonstrate that the DNA extracted using the DNA extraction method described is suitable for quantitative real-time PCR methods, tests were performed with two quantitative event-specific PCR detection methods for 281-24-236/3006-210-23 hybrid cotton: DNA was extracted from homogenized PSC355 (non-GM) and PHY440W (281-24-236/3006-210-23) cotton seeds according to the protocol described in chapter 2. The concentration of the DNA extracts was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes) as described in section 4.2. After dilution of DNA extracts to 30 ng/µl, absence of PCR inhibitors was confirmed with 150 ng DNA/reaction as described in section 4.3. Subsequently calibration standards were produced by preparing solutions of 20 ng/µl (100 ng/reaction) of total DNA with 10% 281-24-236/3006-210-23 cotton DNA in non-GM cotton DNA (standard 1) and subsequent 1:5 serial dilution with 0.1x TE (standard 2 to 4). Additionally samples with different GMO levels were prepared (0.090%; 0.90%; 5.0%). Each of the three samples prepared was analyzed twice (Replicate 1, Replicate 2). The mean of triplicates is shown in the following tables.

Table 4. 3006-210-23 cotton quantification results

	Reference value (% GMO)		
	0.090%	0.90%	5.0%
Replicate 1			
Quantification results	0.106%	0.98%	5.2%
Relative deviation from true	**17.8%**	**8.9%**	**4.0%**
Replicate 2			
Quantification results	0.081%	0.86%	5.1%
Relative deviation from true	**-10.0%**	**-4.4%**	**2.0%**

Table 5. 281-24-236 cotton quantification results

	Reference value (% GMO)		
	0.090%	0.90%	5.0%
Replicate 1			
Quantification results	0.104%	0.90%	5.1%
Relative deviation from true	**15.6%**	**0.0%**	**2.0%**
Replicate 2			
Quantification results	0.106%	0.94%	5.4%
Relative deviation from true	**17.8%**	**4.4%**	**8.0%**

5. Inter-laboratory transferability

In order to demonstrate inter-laboratory transferability of the small scale DNA extraction method, DNA extraction according to the protocol described above was performed at:
Dow AgroSciences LLC
9330 Zionsville Road, Indianapolis, IN 46268

DNA was extracted, in duplicate, from three different samples of homogenized cotton seeds. After shipping of the extracted DNAs to Genescan, the following method parameters were determined (for details of the experimental procedure please refer to chapter 4.):

- Fragmentation state of DNA
- DNA concentration / yield
- Purity / absence of PCR-inhibitors

5.1. Samples

- PSC355 (1403)
- PHY440W (1406)
- PH00A.303 (1419)

5.2. Fragmentation state of DNA

The fragmentation state of the extracted DNA was evaluated by agarose gel electrophoresis as described in section 4.1.

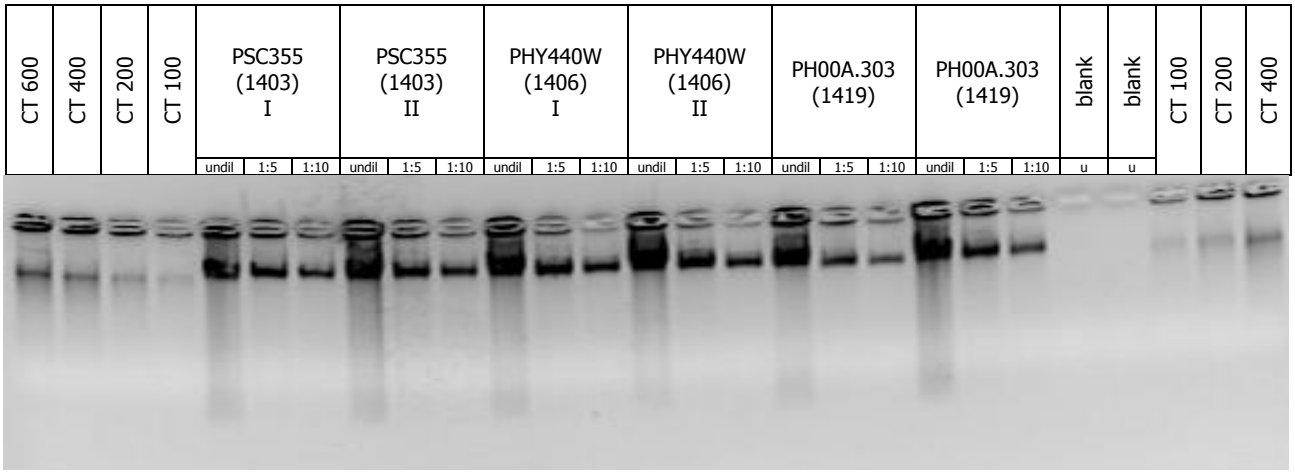


Figure 2. Agarose gel electrophoresis of DNA extracts (undiluted, 1:5 diluted, 1:10 diluted each; blank: extraction negative control)

Medium to high molecular weight DNA was observed for all samples.

5.3. DNA concentration / Yield

The concentration of the DNA extracts was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes) as stated in section 4.2.

Table 6. Concentration / Yield of the DNA extracts

Cotton Line	Replicate	Concentration (ng/µl)	Mean (ng/µl)	Yield (µg)	Mean (µg)
PSC355 (1403)	I	401	381	64	61
PSC355 (1403)	II	361		58	
PHY440W (1406)	I	400	413	64	66
PHY440W (1406)	II	425		68	
PH00A.303 (1419)	I	229	292	37	47
PH00A.303 (1419)	II	354		57	

5.4. Purity/absence of PCR inhibitors

The purity of the extracted DNAs was evaluated as described in section 4.3 except for the fact that the concentration of the DNA extracts was adjusted to 20 ng/µl before spiking.

Table 7. Ct values of "undiluted" (100 ng/reaction) and fourfold serial diluted DNA extracts

DNA extract	Replicate	undiluted	diluted			
		1:1	1:4	1:16	1:64	1:256
PSC355 (1403)	I	25.78	27.86	29.94	32.19	33.99
PSC355 (1403)	II	25.82	27.90	30.14	32.13	34.94
PHY440W (1406)	I	25.69	27.99	30.01	32.11	34.17
PHY440W (1406)	II	25.76	28.01	30.18	32.58	34.52
PH00A.303 (1419)	I	25.95	27.96	29.95	32.03	34.39
PH00A.303 (1419)	II	25.73	27.93	30.12	32.05	34.30

Table 8. Comparison of extrapolated Ct values versus measured Ct values

DNA extract	Replicate	CT extrapolated	CT measured	delta CT	R^2
PH98M.3199 (1409)*	I	25.84	25.78	0.05	0.9982
PH98M.3199 (1409)	II	25.50	25.82	0.32	0.9948
3006-48-81 (1405)	I	25.91	25.69	0.22	0.9999
3006-48-81 (1405)	II	25.84	25.76	0.08	0.9985
PHY440W (1406)	I	25.74	25.95	0.21	0.9984
PHY440W (1406)	II	25.84	25.73	0.11	0.9992

All delta Ct values of extrapolated versus measured Ct are < 0.5, indicating absence of PCR inhibitors. R^2 of linear regression > 0.99 for all DNA solutions extracted.

6. Experimental testing of the DNA extraction method by the Community Reference Laboratory for GM Food and Feed

The aim of the experimental testing is to verify that the method of DNA extraction provides DNA of suitable quantity and quality for the intended purpose.
The DNA extraction method should allow preparation of the analyte in amounts and quality appropriate for the analytical method used to quantify the event-specific analyte versus the reference analyte.
The CRL tested the "CTAB/Genomic-Tip 20" DNA extraction method proposed by the applicant. The experimental testing was carried out on the partially ground seeds cotton from line PSC355 (Lot # 3CA-KC203) provided by the applicant.
To assess the suitability of the extraction method for real-time PCR analysis, the DNA extracted was tested using a qualitative PCR run on the real-time PCR equipment.

6.1 Preparation of samples

About 100 g of seeds of the non-GM cotton line were further ground and homogenized using the GRINDOMIX mixer (Retsch).

6.2 DNA extraction

The DNA was extracted by means of the CTAB/Genomic-Tip 20-based method described above and in-house validated by the applicant.
The DNA extraction was performed on 6 test portions (replicates) and repeated in three different days, giving a total of 18 DNA extracts.

6.3 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 1.5 µl of the DNA solution were analyzed on a 1% agarose gel. The picture of the agarose gel is shown below. In yellow boxes: samples 1-6 extracted on 18/01/06, in green boxes: samples 1-6 extracted on 19/01/06, in blue boxes: samples 1-6 extracted on 23/01/06. A DNA ladder of 1 kb (M) was used.

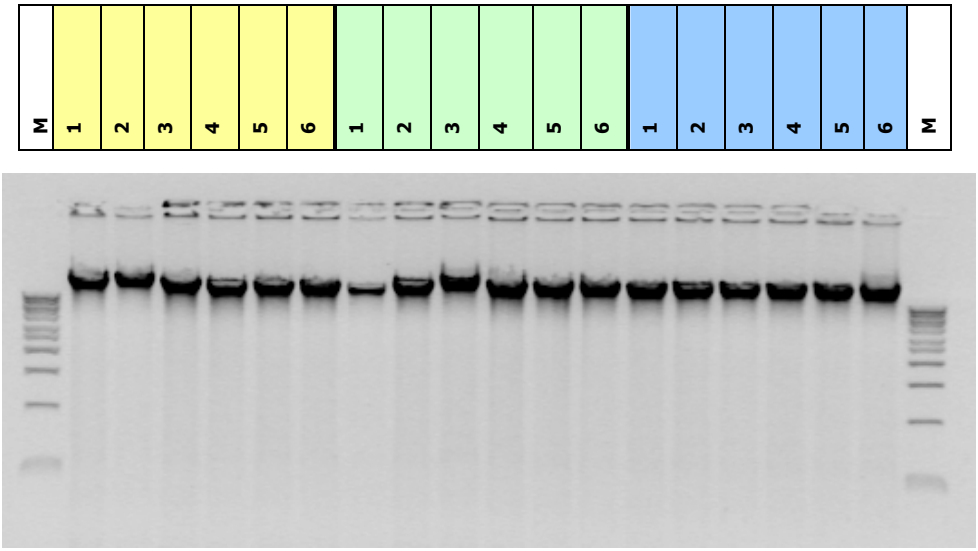


Fig. 3 Agarose gel electrophoresis of DNA extracts.

High molecular weight DNA was observed for all samples.

6.4 DNA concentration / Yield, Repeatability

The DNA concentration of extracted DNA 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 (ng/µl) for all samples (yellow boxes: samples 1-6 extracted on 18/01/06, green boxes: samples 1-6 extracted on 19/01/06, and blue boxes: samples 1-6 extracted on 23/01/06) is reported in the table 9 below.

Table 9. Concentration of DNA extracted

Sample	Concentration (ng/µl)
1	420.4
2	308.4
3	448.9
4	344.5
5	357.5
6	369.4
1	86.2
2	318.6
3	331.3
4	495.6
5	382.5
6	448.2
1	279.1
2	249.5
3	242.6
4	269.8
5	272.8
6	295.3

DNA concentration (ng/µl)

Overall Average of all samples	328.9 ng/µl
Standard deviation	95.12 ng/µl
Coefficient of variation	28.92%

Yield (total volume of DNA solution: 160 µl)

Overall Average of all samples	52.62 µg
Standard deviation	15.22 µg
Coefficient of variation	28.92%

6.5 Purity / Absence of PCR inhibitors

In order to assess purity and absence of PCR inhibitors, all DNA extracts were adjusted to a concentration of 20 ng/µl (successively referred as "undiluted" samples). From these samples fourfold serial dilutions (1:4, 1:16, 1:64, 1:256) were prepared in 1x TE buffer.

All DNA preparations were analyzed applying a real-time PCR system targeting the cotton reference sequence Sah7 (for a validation report on Sah7 system see http://gmo-crl.jrc.it/statusofdoss.htm).

The Ct values obtained for "undiluted" and diluted DNA samples are reported in table 10 below (yellow boxes: 1-6 extracted on 18/01/06, green boxes: samples 1-6 extracted on 19/01/06, and blue boxes: samples extracted on 23/01/06).

Table 10. Ct values of "undiluted" and fourfold serial diluted DNA extracts after amplification of cotton reference gene Sah7.

	Undiluted (20 ng/µl)	**diluted**			
DNA extract	**1:1**	**1:4**	**1:16**	**1:64**	**1:256**
1	21.82	24.10	26.24	28.38	30.74
2	21.91	24.26	26.55	28.66	30.63
3	22.20	24.20	26.58	28.54	30.61
4	22.03	24.21	26.06	28.91	30.94
5	22.29	24.39	26.61	28.74	30.93
6	22.05	24.11	26.18	28.69	30.75
1	22.22	23.91	26.56	28.64	30.61
2	22.18	24.08	26.50	28.65	30.81
3	21.88	23.99	26.63	28.49	30.70
4	22.49	25.00	27.17	29.07	31.27
5	22.25	24.24	26.39	28.30	31.00
6	22.19	24.60	26.41	28.52	30.72
1	22.96	24.96	27.35	29.38	31.10
2	22.79	25.06	26.97	29.29	31.50
3	22.36	24.35	26.58	28.61	30.69
4	22.41	24.77	26.69	29.09	30.94
5	22.34	24.14	26.54	28.68	31.12
6	22.40	24.20	n.d.*	28.33	30.73

*** n.d.** = value not determined

Table 11. Comparison of extrapolated Ct values versus measured Ct values

DNA extract	R^2	Slope*	Ct extrapolated	C_T measured	delta Ct**
1	0.997	-3.66	21.85	21.82	0.032
2	0.990	-3.52	22.22	21.91	0.318
3	0.993	-3.51	22.19	22.20	0.011
4	0.990	-3.82	21.78	22.03	0.257
5	0.998	-3.61	22.23	22.29	0.062
6	0.995	-3.72	21.82	22.05	0.228
1	0.994	-3.68	21.88	22.22	0.340
2	0.991	-3.71	21.93	22.18	0.255
3	0.989	-3.65	21.95	21.88	0.071
4	0.998	-3.44	22.95	22.49	0.463
5	0.993	-3.69	21.93	22.25	0.320
6	0.997	-3.40	22.45	22.19	0.257
1	0.994	-3.40	23.09	22.96	0.123
2	0.998	-3.59	22.80	22.79	0.007
3	0.999	-3.50	22.29	22.36	0.065
4	0.995	-3.47	22.65	22.41	0.242
5	0.997	-3.83	21.85	22.34	0.487
6	0.996	-3.59	21.99	22.40	0.408

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

All delta Ct values of extrapolated Ct versus measured Ct are < 0.5, indicating absence of PCR inhibitors. R^2 of linear regression > 0.99 for all DNA solutions extracted, except one (0.989).

7. Conclusion

The data reported confirm that the method provides DNA of suitable quantity and quality for subsequent PCR based detection applications. The method is therefore considered fit for the intended purpose.

8. Literature

Sambrook. J., Fritsch. E. F. and Maniatis. T. (1989) Molecular Cloning: a laboratory manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

9. Abbreviations

CTAB	cetyltrimethylammoniumbromide
PCR	polymerase chain reaction
Tris	tris(hydroxymethyl)aminomethane
EDTA	ethylenediaminetetraacetic acid
TAE	tris-acetate
CT	calf thymus
TE	tris-EDTA
RNase A	ribonuclease A