



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

06 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	4
2. MATERIALS (EQUIPMENT/CHEMICALS/PLASTICWARE)	4
2.1. EQUIPMENT	4
2.2. CHEMICALS.....	4
2.3. SOLUTIONS.....	5
2.4. PLASTICWARE.....	5
2.5. PRECAUTIONS.....	5
2.6 ABBREVIATIONS	6
3. DESCRIPTION OF THE METHODS	6
3.1 SAMPLING:	6
3.2 SCOPE AND APPLICABILITY	6
3.3 PRINCIPLE	6
3.4 TISSUES GRINDING PROCEDURE	7
3.5 EXTRACTION OF GENOMIC DNA FROM COTTON SEEDS/GRAINS	7
4. TESTING OF THE DNA EXTRACTION METHOD BY THE COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED	8
4.1 PREPARATION OF SAMPLES	8
4.2 DNA EXTRACTION	8
4.3 DNA CONCENTRATION, YIELD AND REPEATABILITY	9
4.4 FRAGMENTATION STATE OF DNA	10
4.5 PURITY / ABSENCE OF PCR INHIBITORS.....	10
4.5.1 <i>Additional PEG precipitation</i>	12
5. CONCLUSION	14
6. QUALITY ASSURANCE	14
7. REFERENCES	14

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:chloroform: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 H₂O. 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/ μ L using a Biorad VersaFluor fluorometer.

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

Table 1. DNA concentration (ng/ μ L) 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/ μ L)
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/ μ L)

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

✓ Yield (total volume of DNA solution: 18000 μ l)

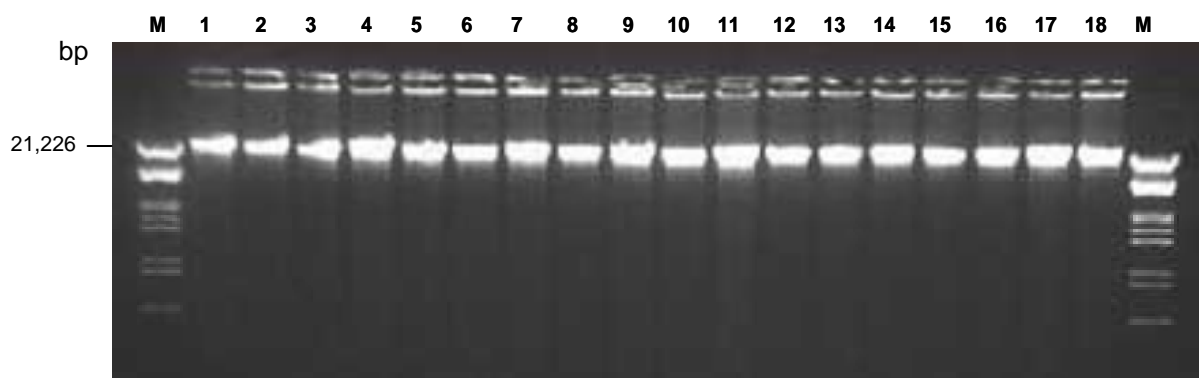
Overall average	381.6 μ g
Standard deviation	82.7 μ g
Coefficient of variation	21.7 %

4.4 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 8 μ L 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/ μ L (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.

DNA extract	Undiluted (40 ng/ μ L)	Diluted			
		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/ μ L) 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.

R² 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/ μ L 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*.

DNA extract	Undiluted (40 ng/ μ L)	Diluted			
		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/ μ L) 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	Δ Ct**
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

1. Murray M.G., Thompson W.F., 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* 8, 4321-4325.
2. Sambrook J., Russell D.W., 2001. *Molecular Cloning. A laboratory manual*. Third edition. Cold Spring Harbor Laboratory Press.