



## **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  
Biotechnology & GMOs Unit**

#### **Method development and single laboratory validation:**

Bayer CropScience GmbH

#### **Method testing and confirmation:**

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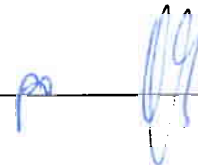
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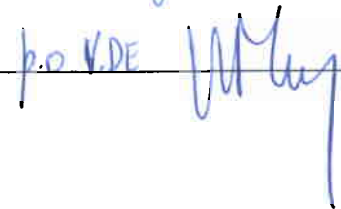
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## 1. Introduction

This report describes a plant DNA extraction protocol derived from the publicly available "CTAB" method <sup>(1)</sup>. 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. Na<sub>2</sub>-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 chloroform: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/ $\mu$ l) and DNA extraction efficiency

DNA extraction	Sample mass (gr)	[DNA] (ng/ $\mu$ l)	DNA extraction efficiency ( $\mu$ g 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  $1659 \pm 397$  ng/ $\mu$ l.

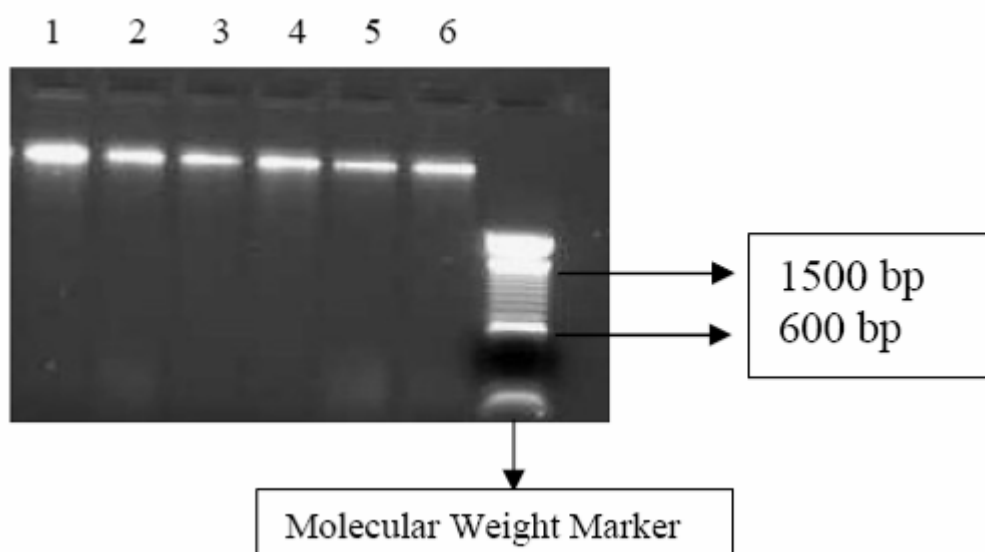
The average DNA extraction efficiency was  $265.4 \pm 63.6$   $\mu$ g DNA/g of sample, the relative Repeatability Standard Deviation (RSDr) was 24.0%.

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  $\mu$ l 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 <sub>measured</sub>	Ct <sub>extrapolated</sub>	$\Delta$ Ct (Ct <sub>measured</sub> - Ct <sub>extrapolated</sub> )
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/ $\mu$ l)**

Overall average of all samples:	286 ng/ $\mu$ l
Standard deviation of all samples	19.7 ng/ $\mu$ l
Coefficient of variation	6.9 %

**Yield (total volume of DNA solution: 160  $\mu$ l)**

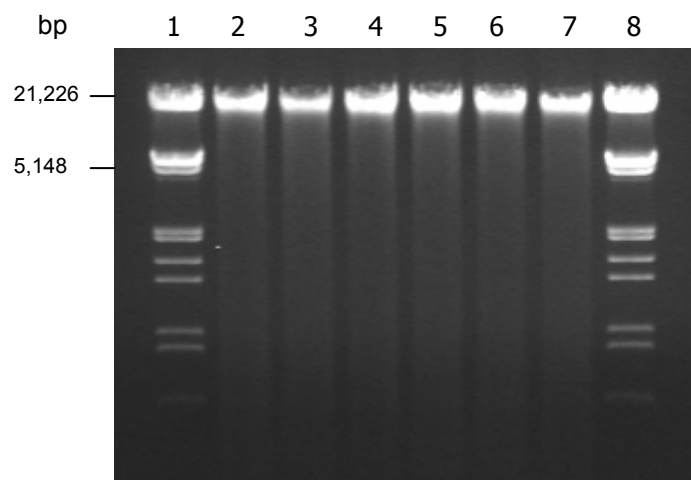
Overall average of all samples:	45.7 $\mu$ g
Standard deviation	3.15 $\mu$ g
Coefficient of variation	6.9 %

**5.4 Fragmentation state of DNA**

The size of the six extracted DNA was evaluated by agarose gel electrophoresis; 8  $\mu$ l 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/ $\mu$ 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 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*

DNA extract	Undiluted (40 ng/μl)	Diluted			
	1:1	1:4	1:16	1:64	1:256
<b>1</b>	20.98	23.00	25.05	27.18	29.30
<b>2</b>	21.22	22.93	24.88	26.98	29.45
<b>3</b>	21.02	23.14	24.96	27.16	29.27
<b>4</b>	21.09	22.95	25.06	27.04	29.29
<b>5</b>	21.09	23.11	25.15	27.13	29.35
<b>6</b>	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	$\Delta Ct^{**}$
<b>1</b>	0.999	-3.49	20.87	20.98	0.11
<b>2</b>	0.999	-3.51	21.82	22.40	0.58
<b>3</b>	0.996	-3.42	20.98	21.02	0.04
<b>4</b>	0.999	-3.49	20.83	21.09	0.25
<b>5</b>	0.998	-3.44	21.01	21.09	0.09
<b>6</b>	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

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