Report on the Verification of Performance of a DNA Extraction Method for Maize Grains

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Joint Research Centre
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Biotechnology & GMOs Unit

Method development and single laboratory validation:

Syngenta Seeds S.A.S.

Method testing and confirmation:

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1. Introduction

This report describes the verification of the performance of a DNA extraction method derived from the publicly available "CTAB" method [1], and its applicability on the samples of food and feed provided by the applicant. The method was already fully evaluated for its suitability on maize materials (see http://gmo-crl.jrc.it/statusofdoss.htm; MIR 604, DNA extraction, CRLVL-04/05XP).

The purpose of the DNA extraction method described is to provide DNA with purity suitable for real-time PCR based detection methods. The method should have a high yield and should be tailored for routine analysis in terms of ease of operations, sample throughput and costs.

This protocol is recommended to be executed by skilled laboratory personnel since hazardous chemicals and materials are exploited at some steps. It is strongly advised to take particular notice of all product 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. 200 ml mortar and pestle
2. Sorval RC-3B equipped with a H-6000A rotor for 5000 rpm that is equivalent to 7277g
3. Microfiltration Centrifugal Device: Pall Nanosep MF 0.2 µm (Pall Corporation P/N ODM02C33)
4. Ultrafiltration Centrifugal Device: Pall Nanosep 30K Omega (Pall Corporation P/N OD030C33)
5. Microcentrifuge with 18.000 x g for microcentrifuge tubes (or 1.5-2.0 ml reaction tubes)
6. Water bath adjustable to 65 °C ± 1 °C
7. UV spectrophotometer for DNA quantification

2.2. Chemicals

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

1. Na_2-EDTA; Titriplex III (Sigma Cat. No. E-7889)
2. Tris-HCl; Tris(hydroxymethyl)aminomethane hydrochloride (Sigma Cat. No. T-3038)
3. NaCl; sodium chloride (Sigma Cat. No. S-7653)
4. CTAB; hexadecyltrimethylammonium bromide (Sigma Cat. No. H-6269)

5. PVP 40000; polyvinylpyrrolidone (Sigma Cat. No. PVP-40)
6. RNase A (Roche Cat. No. 0109-142)
7. Chloroform:Isoamyl alcohol (24:1); (Sigma Cat. No. C-0549-1PT)
8. Ethanol p.a. (Merck Cat. No. 1.00983.1000)

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

1. Extraction buffer (store at room temperature)

- 1.4 M NaCl
- 2% (w/v) CTAB
- 0.1 M Tris-HCl pH 8.0
- 0.02 M EDTA pH 8.0
- 1% (w/v) PVP 40000

2. 10% CTAB Solution (store at room temperature)

- 10% (w/v) CTAB in 0.7 M NaCl

3. Precipitation Buffer (store at room temperature)

- 1% (w/v) CTAB
- 0.05 M Tris-HCl pH 8.0
- 0.01 M EDTA pH 8.0

4. TE Buffer (store at room temperature)

- 0.01 M Tris-HCl pH 8.0
- 0.001 M EDTA pH 8.0

5. RNase A (10 mg/ml) (store at -20 °C)

6. Ethanol 70% (store at room temperature)

2.3. Plasticware

1. 50 ml conical tubes
2. 15 ml conical tubes
3. 1.5 ml microcentrifuge tube
4. 2 ml microcentrifuge tube
5. filter tips

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

2.4. 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:
For sampling of seeds and grains of maize, 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 method for DNA extraction described below is suitable for the isolation of genomic DNA from maize grain. Application of the method to other matrices may require adaptation and needs specific validation.

Principle:
The basic principle of the DNA extraction consists of first releasing the DNA present in the matrix under study into aqueous solution and further purification of the DNA from PCR inhibitors. The present method starts with a lysis step (thermal lysis in the presence of CTAB and EDTA) followed by removal of RNA by digestion with RNase A and removal of contaminants such as lipophilic molecules and proteins by two extractions with chloroform. Afterwards a crude DNA-extract is generated using CTAB precipitation buffer (under low salt conditions DNA precipitates in the presence of CTAB) and washed in 70% ethanol. The pellet is dissolved in TE-buffer.
Further concentration and purification of genomic DNA is achieved using the Pall Nanosep MF 0.2m and Pall Nanosep 30K Omega.

Extraction of gDNA from maize grain

The "CTAB-based" protocol from Syngenta Seeds S.A.S. (http://gmo-crl.jrc.it/statusofdoss.htm; MIR 604, DNA extraction, CRLVL-04/05XP) for DNA extraction of maize grain was applied.

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

4.1 Summary

4.1.1 Extraction:
Genomic DNA was extracted from ground maize grain using the "CTAB-based" method (see section 3 "Description of Methods").

4.1.2 Analysis:
PicoGreen® analysis determined the gDNA to be at a concentration of 69.50 ng/µl.
Spectrophotometric analysis determined that the gDNA was pure, having no significant protein or polysaccharide contamination.
Gel electrophoresis determined that the gDNA extracted was of a high molecular weight with no evidence of degradation.
Eight extractions (each using two grams of maize grain as starting material) were pooled to yield 400 µl of gDNA at 69.50 ng/µl.

4.2 PicoGreen® quantification of gDNA extracted from maize event GA21 grain

To demonstrate the repeatability of PicoGreen® quantification a standard solution of gDNA at 100 ng/µl was measured four times. The values were averaged and compared to the expected value. Results are reported in Table 1:

Table 1. Repeatability of measurement of DNA concentration using PicoGreen® quantification

Replicate #	Measured Concentration ng/µl
1	97.36
2	101.20
3	101.10
4	98.83

Mean Concentration (n=4)	Standard Deviation (n=4)	Actual Concentration ng/µl	% Difference between Actual and Measured Concentrations
99.62	1.86	100	0.38%

The gDNA isolated from event GA21 grain was diluted 1:1000 and quantified using PicoGreen®. The concentration of the pooled extracts was measured and the average concentration calculated. This estimation of concentration only includes double stranded DNA, and does not include all free nucleic acids that may be present in the sample. Results are reported in Table 2.

Table 2: DNA concentration of individual extracted samples and average value as determined by PicoGreen® quantification

Replicate #	Concentration ng/µl
1	79.7
2	94.5
3	51.9
4	49.8
5	27.8
6	153.3
7	66.6
8	32.4
Average concentration	**69.50 ng/µl**

4.3 Spectrophotometer analysis of gDNA extracted from Maize Event GA21 grain

The DNA was diluted 1:100 and a 250 µl aliquot was analysed using a Thermospectronic Genesys 6 spectrophotometer. The absorbance was measured from 200 nm to 400 nm.
The absorbance values at 230, 260 and 280 nm are shown in Table 3 with 260/230, 260/280 ratios. Ratios determined from specific absorbance provide indications about the purity of the genomic DNA preparation. A ratio of 260/230 that is greater than 1.7 indicates that the sample is free from polysaccharide contamination. A ratio of 260/280 that is greater than 1.7 indicates that the sample is free from protein contamination.

Table 3. Specrophotometric analysis of DNA extracted from maize grains

Wavelength nm	Absorbance
230	0.238
260	0.482
280	0.268
260/280	1.8
260/230	2.0

These ratios indicate that the gDNA extracted from maize grain is free from polysaccharide and protein contamination.

4.4 Gel analysis of gDNA quality: fragmentation state of DNA

To determine the quality of the gDNA isolated from Event GA21 grain, 100, 200 and 300 ng of DNA were loaded into lanes 2, 3 and 4, respectively, of a 100 ml 0.8% TAE Agarose gel. The gel was stained by adding 1.5 µg of ethidium bromide directly to the gel which was subjected to electrophoresis at 50 volts for 4 hours. The molecular weight of the gDNA bands are determined by comparison to Lane 1 which contains 0.5 µg of Kb DNA Ladder.

Figure 1. Agarose gel electrophoresis of genomic DNA samples extracted from maize grains (lanes 2-3-4); lane 1: Kb DNA Ladder.

Gel Layout:

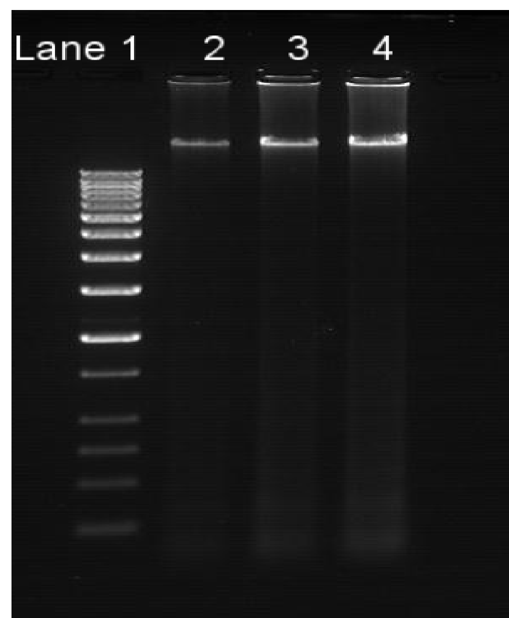
Lane 1 – 0.5 µg Kb DNA Ladder Stratagene (Cat.# 201115)

Lane 2 – 100 ng of Maize gDNA extracted from grain

Lane 3 – 200 ng of Maize gDNA extracted from grain

Lane 4 – 300 ng of Maize gDNA extracted from grain

gDNA extractions performed on April 18, 2005



Gel electrophoresis showed that the DNA extracted was of a high molecular weight with no evidence of degradation.

5. 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-based" method proposed by the applicant on samples of food and feed consisting of ground maize grain material 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 maize seed material were ground using a GRINDOMIX GM 200 (Retsch GmbH) mixer.

5.2 DNA extraction

DNA was extracted following the "CTAB-based" 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 4 below.

Table 4. DNA concentration (ng/µl) of six DNA extractions from samples of maize grain

Sample	Concentration (ng/µl)
1	346
2	510
3	500
4	301
5	225
6	524

DNA concentration (ng/µl)

Overall average:	401 ng/µl
Standard deviation:	127 ng/µl
Coefficient of variation	31.7%

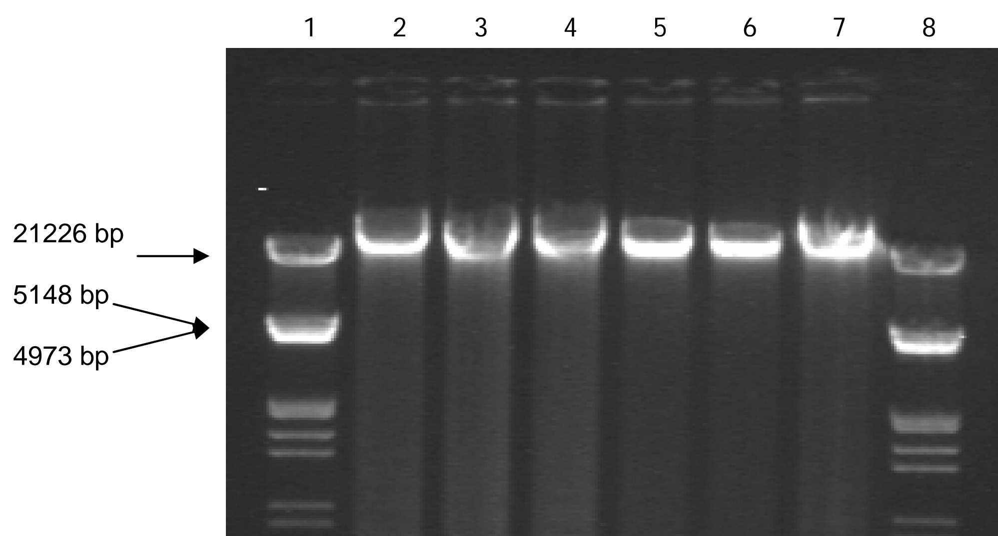
Yield (total volume of DNA solution: 20 µl)

Overall average:	8.02 µg
Standard deviation:	2.54 µg
Coefficient of variation:	31.7%

5.4 Fragmentation state of DNA

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

Figure 2. Agarose gel electrophoresis of six genomic DNA samples extracted from maize grain (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').

5.5 Purity / Absence of PCR inhibitors

In order to assess the purity and to confirm the absence of PCR inhibitors, the extracted DNA solutions were adjusted to a concentration of 50 ng/µl (hereafter referred as "undiluted" samples). Subsequently fourfold serial dilutions of each extract were prepared with water (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*, *Adh1*. The Ct values obtained for "undiluted" and diluted DNA samples are reported in the Table 4 below:

Table 5. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of maize *Alcohol dehydrogenase* gene, *Adh1*.

	Undiluted (50 ng/µl)	**Diluted**			
DNA extract	**1:1**	**1:4**	**1:16**	**1:64**	**1:256**
1	22.61	24.26	26.42	28.08	30.51
2	21.78	23.91	25.37	27.15	29.73
3	22.10	24.07	25.74	27.58	29.85
4	22.00	24.09	25.53	27.59	29.86
5	21.99	23.94	25.85	27.75	29.70
6	21.97	24.20	25.86	27.77	29.86

Table 6 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 6. Comparison of extrapolated Ct values versus measured Ct values (amplification of maize *Alcohol dehydrogenase* gene, *Adh1*)

DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**
1	0.9949	-3.390	22.21	22.61	0.40
2	0.9804	-3.194	21.73	21.78	0.05
3	0.9922	-3.183	22.02	22.10	0.08
4	0.9885	-3.220	21.92	22.00	0.08
5	0.9958	-3.185	22.02	21.99	0.03
6	0.9952	-3.139	22.20	21.97	0.22

***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 (50 ng/µl) was extrapolated from the equation calculated by linear regression.

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

All ΔCt values of extrapolated versus measured Ct are < 0.5.

R^2 of linear regression is > 0.98 for all DNA samples.

6. Conclusion

The data reported confirm that the extraction method, applied to maize grain material provided by the applicant, produces DNA of suitable quantity and quality for subsequent PCR based detection applications. The method is consequently applicable to samples of maize grain 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.

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.