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EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Community Reference Laboratory
GM Food and Feed

# Maize Seeds Sampling and DNA Extraction

# Report on the Validation of a DNA Extraction Method from Maize Seeds and Grains

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**Directorate General-Joint Research Centre**
**Institute for Health and Consumer Protection**
**Biotechnology & GMOs Unit**

**Method development and single laboratory validation:**

Syngenta Seeds S.A.S.

**Method testing and confirmation:**

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Biotechnology & GMOs Unit

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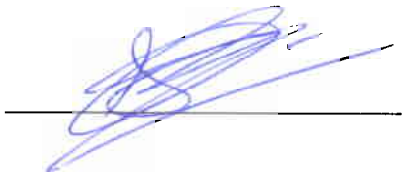

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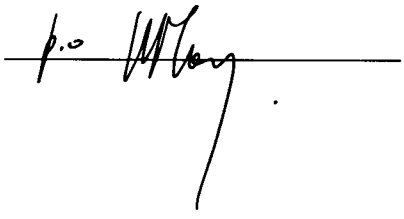

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# Content

# 1. Introduction

The purpose of the DNA extraction method described is to serve as a method to provide DNA from maize seeds or grains for subsequent PCR based detection methods. The method should yield DNA of sufficient quality and quantity and is required to be suitable for routine use in terms of ease of operations, sample throughput and costs. This report describes the results of the verification experiments conducted using a DNA extraction method previously validated on maize grains/seeds [1].

These protocols are recommended to be executed by skilled laboratory personnel as the procedures comprise working with hazardous chemicals and materials. 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. Grindomix GM 200 (Retsch GmbH) or equivalent
2. 200 ml mortar and pestle
3. Sorval RC-3B equipped with a H-6000A rotor for 5000 rpm that is equivalent to 7277g
4. Microfiltration Centrifugal Device: Pall Nanosep MF 0.2 µm (Pall Corporation P/N ODM02C33)
5. Ultrafiltration Centrifugal Device: Pall Nanosep 30K Omega (Pall Corporation P/N OD030C33)
6. Microcentrifuge with 18.000 x g for microcentrifuge tubes
7. Water bath adjustable to 65 $^{o}$C ± 1 $^{o}$C
8. UV spectrophotometer for DNA quantification

## 2.2. Chemicals

The following reagents 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**

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

**2. 10 % CTAB Solution**

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

**3. Precipitation Buffer**

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

**4. TE Buffer**

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

**5. RNase A (10 mg / ml)**

**6. Ethanol 70 %**

### 2.3. Plasticware

1. 50 ml conical tubes
2. 1.5 ml microcentrifuge tube
3. 2 ml microcentrifuge tube
4. 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 "CTAB-based protocol" method for DNA extraction described below is suitable for the isolation of genomic DNA from maize seed, grain and flour. Application of the method to other matrices may require adaptation and needs specific validation.

**Principle:**
The basic principle of DNA extraction consists of first releasing the DNA present in the matrix into aqueous solution and further purification of the DNA from PCR inhibitors. The "CTAB-based protocol" 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.

**Maize seed DNA extraction protocol and purification steps**

The protocol from Syngenta Seeds S.A.S. for DNA extraction of maize seeds was applied as described in [1].

# 4. 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/Nanosep” method proposed by the applicant [1] on samples of food and feed consisting of maize seeds provided by the applicant.

### 4.1 Preparation of samples

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

### 4.2 DNA extraction

DNA was extracted following the "CTAB/Nanosep" method described at http://gmo-crl.jrc.it/statusofdoss.htm [1]; the DNA extraction was carried out on 6 test portions.

### 4.3 DNA concentration 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 four point standard curve ranging from 1 to 500 ng/µl using a Biorad VersaFluor fluorometer.

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

Table 1. DNA concentration (ng/µl) of six samples extracted from maize seeds

| Sample | Concentration (ng/µl) |
|---|---|
| **1** | 687 |
| **2** | 270 |
| **3** | 262 |
| **4** | 300 |
| **5** | 248 |
| **6** | 348 |

DNA concentration (ng/µl):

| | |
|---|---|
| Overall average of all samples: | 353 ng/µl |
| Standard deviation of all samples | 168 ng/µl |
| Coefficient of variation | 48 % |

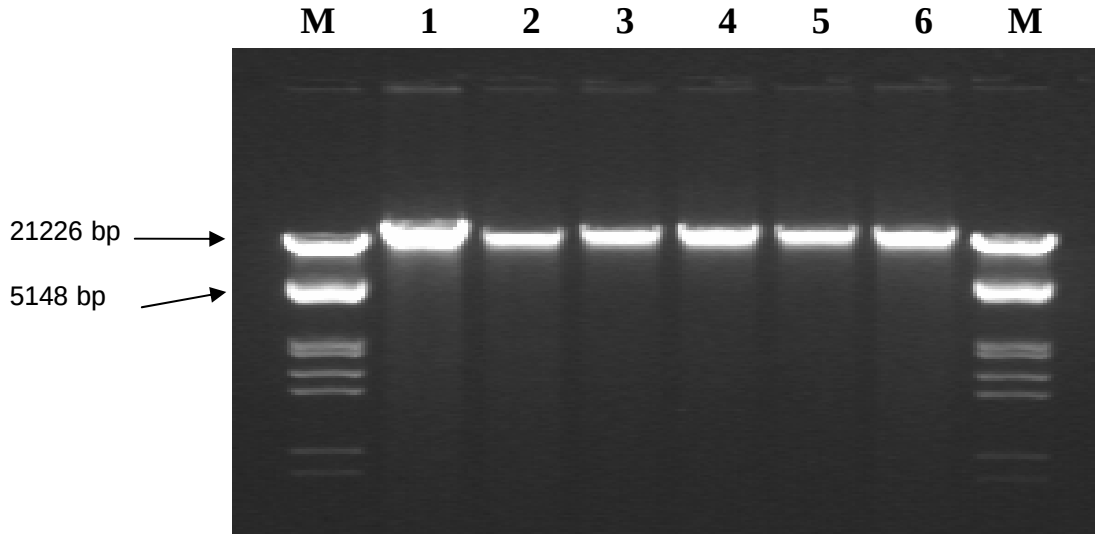
The high value of the coefficient of variation is due to the deviating high concentration of sample 1. Excluding this concentration, the overall average and standard deviation are 286 and 40 ng/µl respectively, and the coefficient of variation 14%.

With an average final volume per sample of 50 µl, the average yield is 9 µg of extracted DNA per gram of starting material.

### 4.4 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 0.8 µl of the DNA solution were analysed on a 1.0% agarose gel (Figure 1).

Figure 1. Agarose gel electrophoresis of six genomic DNA samples extracted from maize seeds (Lanes 1-6). M: Lambda DNA/EcoRI+HindIII Marker.




The 6 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 significant indication of degradation ('smearing').

### 4.5 Purity / Absence of PCR inhibitors

In order to assess their purity and to confirm the absence of PCR inhibitors, the extracted DNA solutions were adjusted to a concentration of 33 ng/µl (hereafter referred as "undiluted" samples). Subsequently fourfold serial dilutions of each extract were prepared with pure 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 2, while 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.

Table 2. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of maize *Alcohol dehydrogenase* gene (*Adh1*)

| | Undiluted (33 ng/µl) | Diluted | | | |
|---|---|---|---|---|---|
| DNA extract | 1:1 | 1:4 | 1:16 | 1:64 | 1:256 |
| 1 | 22.07 | 23.71 | 25.70 | 27.93 | 29.91 |
| 2 | 22.14 | 23.88 | 26.06 | 28.12 | 30.25 |
| 3 | 22.30 | 24.12 | 26.16 | 28.28 | 30.26 |
| 4 | 22.37 | 24.41 | 26.45 | 28.29 | 30.92 |
| 5 | 22.39 | 24.05 | 25.87 | 27.78 | 29.98 |
| 6 | 22.27 | 24.18 | 25.94 | 28.06 | 30.19 |

Table 3. Comparison of extrapolated Ct values versus measured Ct values (amplification of *Alcohol dehydrogenase* gene, *Adh1*)

| DNA extraction | $R^2$ | Slope* | Ct extrapolated | mean Ct measured | ΔCt** |
|---|---|---|---|---|---|
| 1 | 0.999 | -3.459 | 21.61 | 22.07 | 0.47 |
| 2 | 0.999 | -3.517 | 21.79 | 22.14 | 0.36 |
| 3 | 0.998 | -3.414 | 22.07 | 22.30 | 0.23 |
| 4 | 0.993 | -3.553 | 22.17 | 22.37 | 0.20 |
| 5 | 0.996 | -3.270 | 22.00 | 22.39 | 0.39 |
| 6 | 0.997 | -3.349 | 22.05 | 22.27 | 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 (33 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 more than 0.5 cycles from the calculated Ct value. In addition, the slope of the curve should be between -3.6 and -3.1.

All delta Ct values of extrapolated versus measured Ct are < 0.5; all values of the slopes are between -3.6 and -3.1, and $R^2$ of linear regression is > 0.99 for all samples.

Considering the results presented in Table 3, the six extracted samples did not indicate the presence of PCR inhibitors.

## 5. Conclusion

The data reported confirm that the extraction method, applied to maize seeds 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 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 its 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. Community Reference Laboratory for GM Food and Feed. “Maize Seeds Sampling and DNA Extraction - Report on the Validation of a DNA Extraction Method from Maize Seeds”. http://gmo-crl.jrc.it/statusofdoss.htm