

# JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

## **Report on the In-house Validation of a DNA Extraction Method from Ground Maize Seeds and Validated DNA Extraction Method**

European Union Reference Laboratory for  
Genetically Modified Food and Feed

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# **Report on the In-house Validation of a DNA Extraction Method from Ground Maize Seeds and Validated DNA Extraction Method**

**10 April 2018**

**European Union Reference Laboratory for GM Food and Feed**

## **Executive Summary**

In accordance with relevant EU legislation<sup>a</sup>, Pioneer Overseas Corporation, on behalf of Pioneer Hi-Bred International, provided to the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) a DNA extraction method for maize and the relevant samples (ground maize seeds).

In line with its mandate<sup>b</sup>, the EURL GMFF has conducted an in-house validation of this DNA extraction method. To this end it tested the DNA extraction method on the samples provided and evaluated its performance in terms of DNA yield, integrity and quality.

The in-house validation study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL<sup>c</sup>, and that it satisfies the provisions of Annex III-3.C.2 to Regulation (EU) No 503/2013.

The method is therefore fit for the purpose of producing maize DNA of suitable quantity and quality for subsequent PCR-based analysis.

This report is published at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.htm>.

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<sup>a</sup> Regulation (EC) No 503/2013 of 3 April 2013 "on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006".

<sup>b</sup> Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed".

<sup>c</sup> Definition of minimum performance requirements for analytical methods of GMO testing. <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>

## Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: Belac 268 TEST (Flexible Scope for DNA extraction, DNA identification and real-time PCR) and ISO 17043:2010 accredited (certificate number: Belac 268 PT, proficiency test provider).

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

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# Content

<b>1. INTRODUCTION.....</b>	<b>4</b>
<b>2. MATERIALS (EQUIPMENT/CHEMICALS/PLASTIC WARE).....</b>	<b>4</b>
2.1. EQUIPMENT .....	4
2.2. CHEMICALS.....	4
2.3. SOLUTIONS.....	5
2.4. PLASTICWARE.....	6
2.5. PRECAUTIONS .....	6
2.6 ABBREVIATIONS.....	7
<b>3. DESCRIPTION OF THE METHOD .....</b>	<b>7</b>
3.1 SCOPE AND APPLICABILITY .....	7
3.2 PRACTICABILITY.....	7
3.3 PRINCIPLE.....	7
3.4 GRINDING .....	8
3.5 DNA EXTRACTION PROTOCOL .....	8
<b>4. TESTING OF THE DNA EXTRACTION METHOD BY THE EURL GMFF.....</b>	<b>10</b>
4.1 PREPARATION OF SAMPLES.....	10
4.2 DNA EXTRACTION .....	10
4.3 DNA CONCENTRATION, YIELD AND REPEATABILITY .....	10
4.4 DNA FRAGMENTATION .....	11
4.5 PURITY / ABSENCE OF PCR INHIBITORS .....	12
<b>5. CONCLUSIONS.....</b>	<b>15</b>
<b>6. REFERENCES.....</b>	<b>15</b>

## 1. Introduction

This report describes the validation of a large-scale genomic DNA extraction method derived from the publicly available "CTAB" method <sup>(1)</sup> followed by an anion exchange chromatography with commercially available columns "Genomic Tip 500/G" (Qiagen) 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 ground maize.

The purpose of the DNA extraction method described is to provide DNA with purity and quantity suitable for real-time PCR based detection methods.

It is recommended that this method is carried out only by skilled laboratory personnel as the procedures comprise the use of hazardous chemicals and materials. It is advised to take particular notice of products safety recommendations and guidelines.

## 2. Materials (Equipment/Chemicals/Plastic ware)

### 2.1. Equipment

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

1. Pipettes (Gilson)
2. Balances (Mettler Toledo XS2002S)
3. Centrifuges (Eppendorf 5810R and 5415D)
4. Vortex (MS1 Minishaker IKA)
5. Incubator (for 50 mL tubes, 50°C and 65°C, with agitation) (Amersham Hybridization Oven / Shaker)
6. Freezer -20°C and Fridge 4°C (any model appropriate)
7. Fume hood (any model appropriate)

### 2.2. Chemicals

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

1. "Genomic DNA Buffer Set" Kit (Qiagen 19060)
2. CTAB (Sigma No. H6269)
3. Tris-HCl (Sigma T3038)
4. Sodium chloride solution 5M (Sigma S5150)
5. Proteinase K (Sigma P2308)
6. RNase A (Sigma R6513)

7. 2-Mercaptoethanol (Sigma M3148)
8. Chloroform (Sigma C2432)
9. 1-Octanol (Sigma 293245)
10. Isopropanol (Sigma I9516)
11. Ethanol (Fluka 02860)
12. EDTA solution (Sigma E7889)
13. Tris-EDTA Buffer Solution (Fluka 93283)
14. Nuclease free water (Promega P119C)

### 2.3. Solutions

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

#### 1. **CTAB Extraction Buffer (1%)**

- 1 % w/v CTAB (H6269)
- 10 mM EDTA pH 8.0 (E7889)
- 0.7 M NaCl
- 100 mM Tris HCl pH 8.0
- For 2 litres CTAB extraction buffer, measure 20 g CTAB, 40 mL 0.5 M EDTA, 280 ml of 5M NaCl and 200 mL 1M Tris HCl; add about 1600 mL H<sub>2</sub>O<sub>deion</sub> and stir (overnight) until all the CTAB and NaCl are dissolved. Adjust to 2 L with H<sub>2</sub>O<sub>deion</sub>. Filter-sterilize.
- Store at room temperature for up to 1 year

#### 2. **CTAB Precipitation Buffer (1%)**

- 1% w/v CTAB
- 10 mM EDTA pH 8.0
- 50 mM Tris HCl pH 8.0
- For 2 litres CTAB precipitation buffer, measure 20 CTAB, 40 mL 0.5M EDTA and 100 mL 1M Tris HCl; add about 1600 mL H<sub>2</sub>O<sub>deion</sub> and stir (overnight) until all the CTAB is dissolved. Adjust to 2 L with H<sub>2</sub>O<sub>deion</sub>. Autoclave or sterile filter.
- Store at room temperature for up to 1 year

#### 3. **Tris-EDTA buffer (TE 1X)** (e.g. from Fluka, 93283)

- 10 mM Tris HCl pH 8.0
- 1 mM EDTA pH 8.0

To prepare 100 mL 1x TE buffer combine 1 mL 1 M Tris (pH 7.5 to 8.0) and 200 µL 0.5 M EDTA (pH 8.0) and adjust the volume to 100 mL with H<sub>2</sub>O<sub>deion</sub>. Autoclave.

Store at room temperature for up to 2 years.

#### 4. **Proteinase K (20 mg/mL)**

- For 10 mL proteinase K solution dissolve 200 mg proteinase K in 10 mL H<sub>2</sub>O<sub>deion</sub>.
- Aliquot and store at -20 °C for up to 2 years.

**5. RNase A (100 mg/mL)**

- Dissolve 250 mg RNase A in 5 mL of H<sub>2</sub>O<sub>deion.</sub>
- Aliquot and store at -20 °C for up to 2 years.

**6. Chloroform:octanol (24:1)**

- 1-Octanol
- Chloroform

For 1 litre, mix 40 mL of 1-octanol with 960 mL of chloroform.

Store at room temperature for up to 1 year.

**7. Ethanol 70% (v/v)**

For 200 mL combine 140 mL 100% ethanol and fill up to 200 mL with H<sub>2</sub>O<sub>deion.</sub>

Store at room temperature for up to 1 year.

**2.4. Plasticware**

1. "Genomic Tip 500/G" columns (Qiagen 10262)
2. 50 mL conical tubes (BD 352098)
3. 2.0 microcentrifuge tubes (Eppendorf 0030 129.597)
4. sterile filters (Nalgene 514-0026)
5. filter tips for pipettes

*Note: all plastic ware should be sterile and free of DNases, RNases and nucleic acids.*

**2.5. Precautions**

- The protocol is recommended for use by skilled personnel only because of the use of hazardous chemicals and materials
- Consideration of notice of products and operating instructions safety recommendations and guidelines is strongly recommended.
- Chloroform, octanol, and isopropanol are hazardous chemicals; therefore, all manipulations have to be performed according to safety guidelines, under a fume hood.
- Maintain strictly separate working areas for DNA extraction, PCR set-up and amplifications.
- All equipment and lab benches should be free of DNA residues.
- 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
CTAB	cetyltrimethylammonium bromide

## 3. Description of the method

### 3.1 Scope and applicability

The method for DNA extraction described below is suitable for the isolation of large-scale high quality genomic DNA from ground maize. Application of the method to other matrices may require adaptation and possible further validation.

### 3.2 Practicability

The primary downstream use of genomic DNA from seed material is for real-time PCR-based detection methods. Therefore, the preparation of DNA from seed material should take place in an area dedicated for this purpose. All equipment (e.g. pipettes, centrifuges), lab ware and reagents used in this process should be stored and used only in the dedicated area. The entire procedure takes about 30 minutes for the first day, and about 6 hours for the second day.

### 3.3 Principle

The 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 method starts with an overnight thermal lysis step of the ground maize seed in the presence of CTAB extraction buffer (containing RNase A and Proteinase K). During lysis, the cetyltrimethylammonium bromide (CTAB) binds polysaccharides, cell wall debris and denatured proteins. After lysis and a chloroform extraction, a crude DNA extract is generated by precipitation with CTAB precipitation buffer.

This crude precipitate is re-suspended and incubated in a digestion buffer with RNase A and proteinase K. This incubation digests any remaining RNA and strips the DNA from all bound proteins, which facilitates the removal of the protein during the purification process.

The final step involves loading the resulting lysate on an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins, and low-molecular-weight impurities are

removed by a medium-salt wash. Genomic DNA is eluted in a high-salt buffer, concentrated and desalted by isopropanol precipitation (Qiagen, 2001).

### 3.4 Grinding

Grinding of maize 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.

Depending on the seed material to be extracted (water content, particle size, etc.), it might be advisable to reduce the amount of starting material used per QIAGEN Genomic-tip 500/G in the standard protocol. If the DNA shows signs of impurities, the protocol should be repeated using two tubes of 4 g starting ground seed material (Step 1), and transferring the entire content of the supernatant (Step 14) into one Genomic-tip 500/G (Step 17). However, high yields do not necessarily mean that there will be problems with impurities in the DNA preparation.

Certain uses of the large-scale CTAB extraction method may involve scaling down for certain steps; these modifications have not been tested. With the scaled-down method (using only one tube of 4 g tissue powder at Step 1), the supernatant is transferred to a 50 mL conical tube at Step 14, and all subsequent steps will be followed to use the volumes and tips shown in brackets (Steps 15-25). Further scaling down of the method for less than 1 g starting material is possible with use of QIAGEN Genomic-tip 20/G.

### 3.5 DNA extraction protocol

1. In each of two 50 mL conical tubes, mix the following: 4 g seed powder, 18 mL CTAB Extraction Buffer, 17.3  $\mu$ L RNase A (100 mg/mL) and 60  $\mu$ L Proteinase K (20 mg/mL).
2. Add 200  $\mu$ L 2-Mercaptoethanol to each tube.
3. Securely cap the tubes and lay the tubes horizontally in a shallow container and incubate overnight at  $\sim 65^{\circ}\text{C}$  with agitation.
4. Allow samples to cool down to room temperature for at least 15 minutes.
5. Add 10 mL Chloroform/1-Octanol (24:1) per tube and mix the tubes  $\sim 5$  minutes in an overhead shaker.

**Note:** Chloroform can dissolve many types of plastic, use appropriate containers and glass pipettes while working with chloroform.

6. Centrifuge at room temperature for 10 minutes at  $\sim 7000\ g$  to separate phases.
7. Carefully transfer supernatant to a fresh 50 mL conical tube containing 22 mL CTAB Precipitation Buffer.

**Note:** starch and proteins will form a thick layer separating the aqueous layer from the Chloroform/1-Octanol. Some of the Chloroform/1-Octanol may seep through the layer (appear slightly yellow in contrast to the clear, DNA-rich aqueous layer), and should be avoided when pipetting off the aqueous layer.

8. Gently mix by inversion. Incubate  $\sim 30$  minutes at room temperature.
9. Centrifuge at room temperature for 20 minutes at  $\sim 9200\ g$  to pellet.

10. Carefully pour off and discard supernatant. Dissolve pellet in 2 mL pre-warmed ( $\sim 50^{\circ}\text{C}$ ) 1X TE Buffer. Pipette carefully up and down or tap gently until the pellet is detached from the wall of the tube.
11. Add 9.5 mL QIAGEN Buffer G2, 17.3  $\mu\text{L}$  RNase A and 50  $\mu\text{L}$  Proteinase K to each tube.
12. Securely cap the tubes, lay the tubes horizontally in a shallow container and incubate at least 1 hour at  $\sim 50^{\circ}\text{C}$  with gentle agitation.
13. Centrifuge at room temperature for 5 minutes at  $\sim 9200\text{ g}$  to remove any particulate matter in order to prevent clogging of the QIAGEN Genomic-tip.
14. Pool supernatant of the two tubes of the replicate samples into one 50 mL tube.
15. Saturate one QIAGEN Genomic-tip 500/G [100/G] with 10 mL [4 mL] Buffer QBT. Allow the buffer to run through the column before proceeding.
16. Vortex the sample for 10 seconds at maximum speed.
17. Apply combined sample [or single sample] to the saturated QIAGEN Genomic-tip. Allow it to enter the resin by gravity flow.
18. Rinse the tip 2 times with 15 mL [7.5 mL] Buffer QC. Discard the flow through.
19. Elute DNA into a fresh 50 mL tube with 15 mL [5 mL] Buffer QF (pre-warmed to  $\sim 50^{\circ}\text{C}$ ).
20. Precipitate DNA by adding 0.7 volumes room temperature isopropanol. Invert 10 to 20 times.
21. Centrifuge to pellet at  $\sim 4^{\circ}\text{C}$  for  $\sim 30$  minutes at  $\geq 9200\text{ g}$  using a fixed angle rotor.
22. Discard supernatant. Wash the pellet with 4 mL 70% Ethanol. Vortex briefly to break up pellet.
23. Centrifuge to pellet at  $\sim 4^{\circ}\text{C}$  for  $\sim 10$  minutes at  $\geq 9200\text{ g}$ .
24. Remove as much supernatant as possible and air dry for 5 to 10 minutes.
25. Add 2 mL [500  $\mu\text{L}$ ] 1X TE (pre-warmed to  $\sim 50^{\circ}\text{C}$ ) to resuspend the pellet.
26. Resuspend the DNA  $\sim 30$  minutes at  $\sim 50^{\circ}\text{C}$  in an incubator or water bath.
27. Transfer the DNA into a 2 mL microcentrifuge tube. Incubate overnight at  $\sim 4^{\circ}\text{C}$  before proceeding to further resuspend the DNA and increase yield.
28. Spin samples in a microfuge for  $\sim 2$  minutes at max speed and transfer supernatant to a fresh 2 mL tube.
29. Store samples at  $\sim 4^{\circ}\text{C}$  until ready to use.

## 4. Testing of the DNA extraction method by the EURL GMFF

The EURL GMFF tested the method proposed by the applicant and described above on samples consisting of ground maize provided by the applicant. DNA extraction procedures should result in repeatable recovery, fragmentation profile, concentration and PCR quality of DNA extracts. The extracted DNA should be of suitable quantity and quality for the intended purpose<sup>d</sup>.

### 4.1 Preparation of samples

Ground maize was received from the applicant and used for DNA extraction. Eight grams divided in two falcon tubes and pooled together at step 14 were used per sample.

### 4.2 DNA extraction

DNA was extracted from six test portions (replicates) following the method described in paragraph 3.5 "DNA extraction protocol". The procedure was repeated over three days, for a total of 18 DNA extractions.

### 4.3 DNA concentration, yield and repeatability

The concentration of the extracted DNA solutions was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Invitrogen).

Each DNA extract was measured three times and the three 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 average DNA concentration and the yield are reported in Table 1 below.

	<b>Concentration (ng/μL)</b>	<b>Yield (μg)</b>
Overall average	353.20	88.30
Standard deviation of all samples	39.13	9.78
Coefficient of variation (%)	11.08	11.08

<sup>d</sup> EURL/ENGL guidance document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>)

Table 2 reports the data of DNA concentration and yield for the 18 extracted samples.

Table 2. DNA concentration (ng/ $\mu$ L) and yield of extracted samples

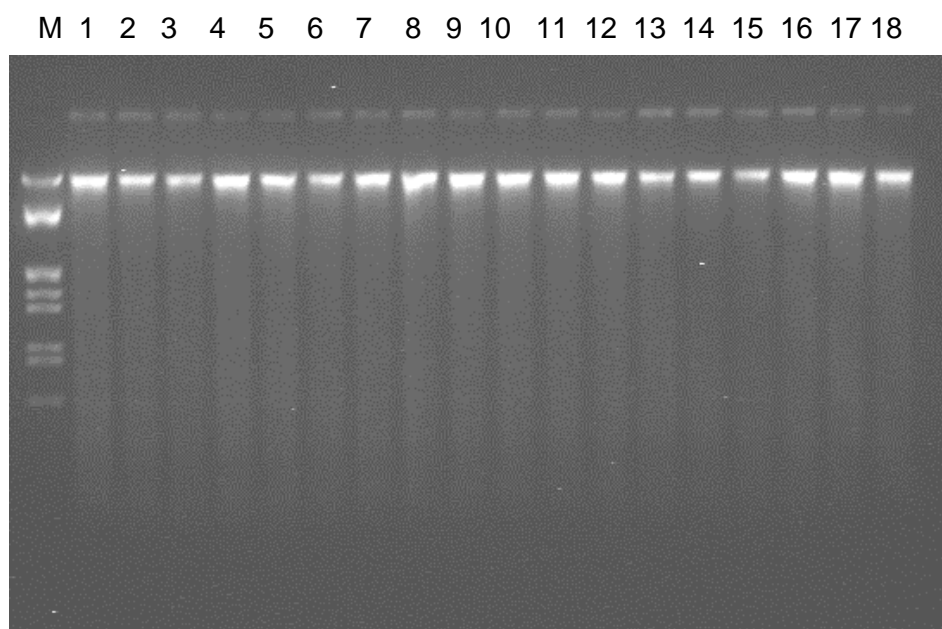
Sample	Concentration (ng/ $\mu$ L)	Yield ( $\mu$ g)
1	367.3	91.82
2	331.3	82.82
3	284.9	71.22
4	378.0	94.51
5	382.9	95.73
6	323.8	80.95
7	411.8	102.95
8	402.3	100.58
9	387.3	96.81
10	366.8	91.70
11	383.3	95.82
12	373.2	93.30
13	346.9	86.71
14	265.8	66.46
15	348.0	87.01
16	343.0	85.75
17	352.7	88.17
18	308.3	77.09

Note: In yellow boxes samples extracted on day 1; in green boxes samples extracted on day 2; in blue boxes samples extracted on day 3.

#### 4.4 DNA Fragmentation

The size of the extracted DNA was evaluated by analysis on a 1.0% agarose gel electrophoresis, to check that the DNA is not excessively fragmented for subsequent analyses. One  $\mu$ L of the DNA solution was loaded per lane (Figure 1).

Figure 1. Agarose gel electrophoresis of eighteen genomic DNA samples extracted from ground maize.



Lanes 1-6: samples extracted on day 1, lanes 7-12: samples extracted on day 2, lanes 13-18: samples extracted on day 3; M: Lambda DNA/*Eco*RI+*Hind*III molecular weight markers.

The extracted genomic DNA samples appeared as distinct high molecular weight DNA bands on the gel. None of the DNA samples showed indication of significant degradation.

#### 4.5 Purity / Absence of PCR inhibitors

In order to assess the purity and to conduct a test for the presence of PCR inhibitors, the extracted DNA solutions were adjusted to a concentration of 40 ng/μL (hereafter referred as “undiluted” samples).

Subsequently, fourfold serial dilutions (1:4, 1:16, 1:64, 1:256) of each extract were prepared with TE low buffer (1 mM Tris, 10 μM EDTA, pH 8.0) and analysed in triplicate using a real-time PCR system detecting the target sequence of the endogenous gene *hmg*. Five μL were used in each real-time PCR reaction. The Cq values obtained for “undiluted” and diluted DNA samples are reported in Table 3.

Table 3. Cq values of undiluted and fourfold serially diluted DNA extracts after amplification of maize gene *hmg*.

Cq values					
DNA extract	Undiluted (40 ng / $\mu$ L)	Diluted extracts			
		1:4	1:16	1:64	1:256
1	22.78	24.66	26.67	28.77	30.71
2	22.78	24.72	26.68	28.90	30.74
3	22.74	24.73	26.63	28.71	30.67
4	22.77	24.51	26.59	28.78	30.76
5	22.70	24.61	26.82	28.61	30.85
6	22.64	24.61	26.71	28.77	30.74
7	22.45	24.33	26.15	28.31	30.32
8	22.40	24.32	26.26	28.28	30.36
9	22.27	24.27	26.29	28.40	30.49
10	22.41	24.24	26.45	28.44	30.35
11	22.38	24.39	26.47	28.50	30.58
12	22.37	24.21	26.23	28.13	30.29
13	23.23	25.11	27.22	29.28	31.45
14	23.17	25.01	27.03	29.24	31.01
15	23.16	25.10	27.22	29.15	31.10
16	23.33	25.18	27.24	29.39	31.30
17	22.99	24.92	26.89	28.95	31.06
18	23.11	25.01	27.05	29.00	31.13

Note: In yellow boxes samples extracted on day 1; in green boxes samples extracted on day 2; in blue boxes samples extracted on day 3.

To check for inhibition the Cq values of the four diluted samples were plotted against the logarithm of the dilution and the Cq values for the “undiluted” samples (40 ng /  $\mu$ L) were extrapolated from the equation calculated by linear regression.

Subsequently, the extrapolated Cq values for the “undiluted” samples were compared with the measured Cq data. It is assumed that PCR inhibitors are present if the measured Cq value for the “undiluted” sample is > 0.5 cycles from the calculated Cq value. Table 4 below reports the comparison of extrapolated Cq values versus measured Cq values for all samples and the values of linearity ( $R^2$ ) and slope of all measurements.

Table 4. Comparison of extrapolated Cq values versus measured Cq values (amplification of maize gene *hmg*)

DNA extraction	R <sup>2</sup>	Slope	Cq extrapolated	mean Cq measured	ΔCq*
<b>1</b>	0.9997	-3.37	22.63	22.78	0.14
<b>2</b>	0.9973	-3.37	22.69	22.78	0.09
<b>3</b>	0.9992	-3.31	22.71	22.74	0.03
<b>4</b>	0.9968	-3.48	22.43	22.77	0.35
<b>5</b>	0.9967	-3.40	22.60	22.70	0.10
<b>6</b>	0.9984	-3.40	22.59	22.64	0.05
<b>7</b>	0.9967	-3.34	22.25	22.45	0.21
<b>8</b>	0.9979	-3.34	22.28	22.40	0.13
<b>9</b>	0.9993	-3.45	22.17	22.27	0.10
<b>10</b>	0.9981	-3.37	22.30	22.41	0.11
<b>11</b>	0.9993	-3.42	22.33	22.38	0.05
<b>12</b>	0.9989	-3.35	22.18	22.37	0.20
<b>13</b>	0.9992	-3.50	23.00	23.23	0.23
<b>14</b>	0.9981	-3.36	23.02	23.17	0.15
<b>15</b>	0.9994	-3.31	23.16	23.16	0.002
<b>16</b>	0.9980	-3.41	23.15	23.33	0.17
<b>17</b>	0.9992	-3.40	22.84	22.99	0.16
<b>18</b>	0.9995	-3.38	22.96	23.11	0.15

Note: In yellow boxes samples extracted on day 1; in green boxes samples extracted on day 2; in blue boxes samples extracted on day 3.

\*delta Cq = abs (Cq extrapolated - Cq measured)

According to ENGL definition of minimum performance requirements for analytical methods of GMO testing<sup>e</sup> the expected slope for a PCR with 100% efficiency is -3.3; the accepted average value should be in the range of -3.6 and -3.1. In addition the average value of R<sup>2f</sup> shall be ≥0.98.

The table indicates that all ΔCq values of extrapolated versus measured Cq are < 0.5. The R<sup>2</sup> coefficient of linear regression is > 0.99 for all DNA samples and the slopes of the curves are between -3.1 and -3.6 for all samples.

<sup>e</sup> EURL/ENGL guidance document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>)

<sup>f</sup> R<sup>2</sup> is the correlation coefficient of a standard curve obtained by linear regression analysis.



## 5. Conclusions

The results confirm that the DNA extraction method from ground maize provided by the applicant produces DNA of suitable quantity and quality for subsequent PCR-based analyses.

When applied to complex food or feed products containing maize, because of the known difficulties in extracting high quality and quantity of DNA from such materials, particular care must be taken with regard to verifying the suitability of the extracted DNA for subsequent analyses.

## 6. References

1. Murray M.G. and Thompson W.F., 1980. Rapid Isolation of High Molecular Weight Plant DNA. *Nucleic Acids Res.* 8, 4321-4325.

## JRC Mission

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