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Community Reference Laboratory
CRL
GM Food and Feed

Maize Seeds Sampling and DNA Extraction

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

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

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Content

1. Introduction

The purpose of the DNA extraction method described is to serve as a method to provide DNA 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 method and validation experiments, including results.

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. 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 Eppendorf tubes
6. Water bath adjustable to 65 ^{o}C ± 1 ^{o}C
7. 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 0.7M 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.

CTAB-based protocol

1. Transfer 2 grams of grounded seeds into a 50 ml polypropylene tube containing 10 ml of extraction buffer, pre-warmed to 65ºC. Invert the tube to mix the contents prior to incubating at 65ºC for 10 minutes. Mix gently by inversion 2 times during the incubation period.
2. Spin the 50 ml tubes at approximately 7200 x g for 10 minutes at room temperature, 22ºC.
3. Transfer the supernatant, ~7.5 ml, to a clean 15 ml polypropylene tube and extract with an equal volume of chloroform: isoamyl alcohol (24:1), by slowly inverting the tube 20 times.
4. Collect the upper aqueous phase after centrifugation at approximately 7200 x g for 10 minutes at room temperature (22ºC) and transfer it, ~ 7.0 ml, to a clean 15 ml polypropylene tube.
5. Add 10 µl RNase A at 10 mg/ml. Mix gently by inversion and incubate for 30 minutes at 37ºC.

6. Add 1/10th volume, ~ 700 µl of pre-warmed (55 ºC) 10% CTAB Buffer. Mix well by gentle inversion.
7. Extract with an equal volume of chloroform: isoamyl alcohol (24:1), by inverting the tube 20 times. Centrifuge at approximately 7200 x g for 10 minutes at room temperature.
8. Remove the upper aqueous layer into a fresh 50 ml polypropylene tube and add 3 volumes of precipitation buffer. Mix gently. Let rest at room temperature for 10 minutes.
9. Collect DNA by centrifugation at approximately 7200 x g for 15 minutes at room temperature.
10. Discard buffer solution and wash the pellet twice with 70% ethanol. The pellet is washed through the addition of 5 ml to the tube containing the DNA precipitate. The pellet is roused by agitation and gently swirled about in the ethanol for 10 seconds. Centrifuge at approximately 7200 x g for 5 minutes at room temperature. Carefully remove ethanol by decanting. The wash is then repeated once. To avoid buffer contamination rinse tube containing DNA precipitate well with 70% ethanol during the first alcohol wash.
11. Allow residual alcohol to evaporate then dissolve the DNA pellet in 200 µl TE.
12. Add 200 µl TE and allow DNA to dissolve overnight at 4 ºC.
13. Incubate sample at 65 ºC for 15 minutes and mix gently to ensure the pellet is completely dissolved.
14. Spin sample at maximum speed in bench top microfuge for 5 minutes to pellet any debris.

Micro and Ultra filtrations to eliminate contaminants and/or concentrate the DNA sample

Note: Carefully follow manufacturer's directions regarding the use of 0.2 µm pore size low DNA/protein binding membrane. Use the Pall Nanosep MF 0.2 µm Part No. ODM02C33:

15. Load the DNA sample into reservoir and spin at 14000 rpm until the sample has passed completely through the membrane.
16. Collect DNA sample from filtrate receiver and adjust concentration as needed.

Note: Further purification can be achieved using the Pall Nanosep 30K Omega Part No. OD030C33. Carefully follow the manufacturer's directions regarding the use of this ultrafiltration product. Use this to remove small molecular weight contaminants, remove salts, exchange buffers or concentrate samples.

17. Load the DNA sample into the reservoir and spin at 5000 x g for up to 20 minutes.
18. Following the spin, carefully remove the sample retained in the reservoir.
19. Adjust concentration of DNA solution as needed with TE.

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 a CTAB buffer (see extraction protocol in section 3 "Description of Methods"). The sample was further purified via two chloroform extractions and an RNase step. The gDNA was then precipitated with a Tris-HCl (pH=8.0), EDTA and CTAB buffer, washed in 70% ethanol and then suspended in TE. The DNA solution was then filtered through a Nanosep MF 0.2 µm column and further purified and concentrated using a Nanosep 30K Omega column.

4.1.2 Analysis:
PicoGreen® analysis determined the gDNA to be at a concentration of 284.6 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 284.6 ng/µl.

4.2. PicoGreen® quantification of gDNA extracted from maize grain

To demonstrate the repeatability of PicoGreen® quantification a standard solution of gDNA at 120 ng/µl was measured 10 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 #	Concentration (ng/µl)
1	120.3
2	117.0
3	115.4
4	121.4
5	119.6
6	120.5
7	116.9
8	117.2
9	118.9
10	115.9
Average Concentration (ng/µl)	118.3

Mean Concentration (ng/µl) (n=10)	Standard Deviation (n=10)	Actual Concentration (ng/µl)	% Difference between Actual and Measured Concentrations
118.3	2.1	120	1.4%

The gDNA isolated from maize grains 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	272.1
2	282.0
3	302.7
4	298.9
5	275.4
6	286.8
7	278.0
8	297.5
9	287.6
10	264.5
Average Concentration (ng/µl)	284.6

4.3 Spectrophotometer analysis of gDNA extracted from maize grains

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	0.073
260	0.149
280	0.081
260/280	2.0
260/230	2.2

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 maize grains, 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:

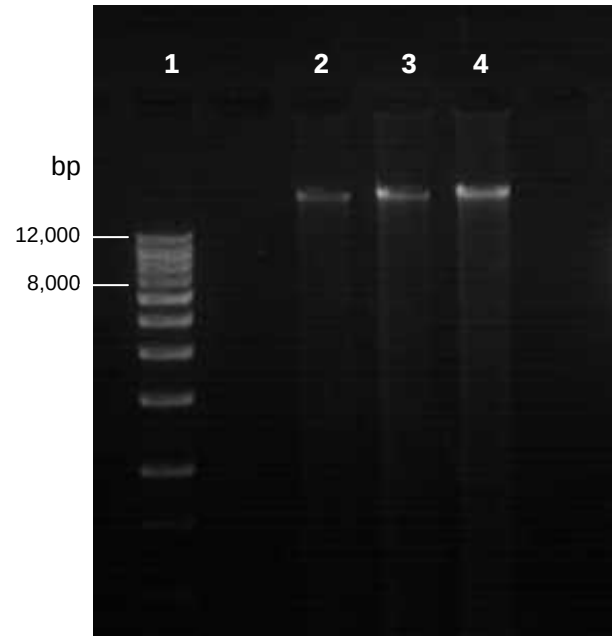
Lane 1 – 0.5 μg Kb DNA Ladder Stratagene (Cat.# 201115)

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 July 18, 2005



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

4.5 Inhibition Assay

In order to assess the quality of gDNA isolated from grain material intended for use with PCR applications, an inhibition study was conducted.

Extracted gDNA was serially diluted fourfold with 0.2X TE Buffer. The original undiluted sample (1:1) and the dilution series (1:4, 1:16, 1:64, 1:256) were analyzed using both the MIR604 event specific and the endogenous *Adh*1 assays.

To measure inhibition, the Ct values obtained using the four diluted samples were plotted against the natural logarithm of the dilution factor. The Ct Value of the undiluted sample was then extrapolated from this plot. The extrapolated and measured Ct values from the undiluted sample were compared.

With both MIR604 event specific and endogenous assays the differences between the extrapolated and measured values were <0.5 cycles, indicating no inhibitors were present in the gDNA preparation.

Data to support this conclusion are presented below in Table 4, Table 5, Figure 2 and Figure 3.

Table 4: Ct Values of undiluted and serial dilution of DNA extracts from maze grain.

Dilution Factor	Average VIC Ct Value (n=3)	Standard Deviation VIC Ct (n=3)	Average FAM Ct Value (n=3)	Standard Deviation FAM Ct (n=3)
1:1 MIR604 grain	24.00	0.03	21.31	0.01
1:4 MIR604 grain	25.85	0.01	23.26	0.13
1:16 MIR604 grain	28.04	0.06	25.33	0.26
1:64 MIR604 grain	29.94	0.04	27.50	0.04
1:256 MIR604 grain	31.98	0.03	29.31	0.05

Figure 2: Plot of Ct values vs. natural logarithm of dilution factor. Analysis performed with endogenous *Adh1* assay (top) and with MIR604 maize specific assay (bottom)

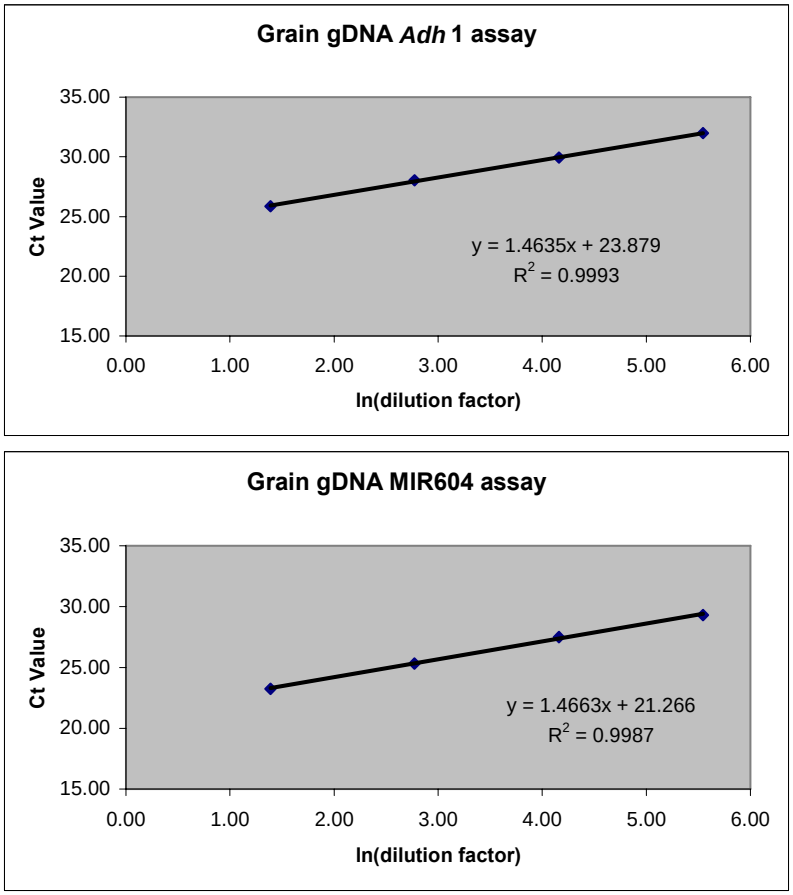


Table 5: Comparison of extrapolated Ct values vs. measured Ct values

DNA extract	Assay type	R^2	Ct Extrapolated	Ct Measured	ΔCt
MIR604 grain	*Adh* 1 endogenous	0.9993	23.88	24	0.12
MIR604 grain	MIR604 es	0.9987	21.26	21.31	0.05

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 "Extraction of gDNA from maize grain" method proposed by the applicant on samples of food and feed consisting of maize 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 300 g of maize seed material were ground using a GRINDOMIX GM 200 (Retsch GmbH) mixer.

5.1 DNA extraction

DNA was extracted following the "Extraction of gDNA from maize grain" method described above and in-house validated by the applicant.

The DNA extraction was carried out on 6 test portions (replicates) and repeated over three different days, giving a total of 18 DNA extracts.

5.2 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 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 Table 6 below:

Table 6. DNA concentration (ng/µl) of eighteen samples extracted from maize seeds 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/µl)
1	273.4
2	110.5
3	205.3
4	230.3
5	227.5
6	121.4
1	191.9
2	287.3
3	438.9
4	166.5
5	185.2
6	311.7
1	243.8
2	373.3
3	284.7
4	261.7
5	245.9
6	374.8

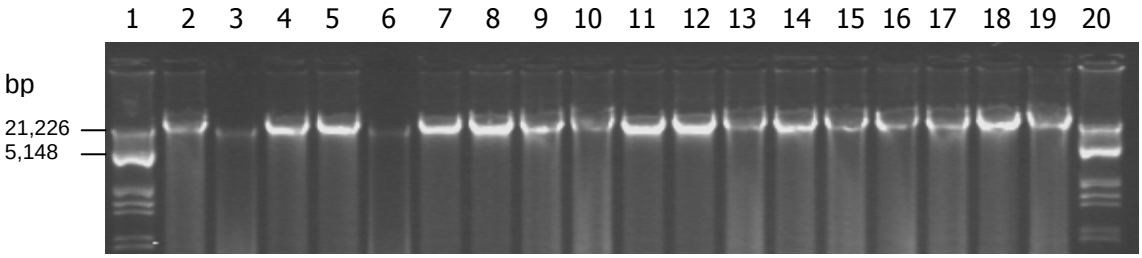
DNA concentration (ng/µl):

Overall average of all samples:	251.9 ng/µl
Standard deviation of all samples	86.4 ng/µl
Coefficient of variation	34.3 %

5.3 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 8 µl of the DNA solution were analysed on a 1.0% agarose gel (Figure 3).

Figure 3. Agarose gel electrophoresis of genomic DNA samples extracted from maize 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 Marker.



The 18 genomic DNA samples extracted as described above appeared as distinct fluorescent banding patterns migrating through the gel corresponding to high molecular weight DNA. DNA samples showed limited indications of degradation ('smearing').

5.4 Purity / Absence of PCR inhibitors

To assess the PCR quality of the DNA extracted, the experimental approach previously described (see paragraph 4.5) was followed.
The Ct values obtained for the "undiluted" and diluted samples are reported in Table 7 below:

Table 7. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of maize *Alcohol dehydrogenase* gene (*Adh1*)

	Undiluted (40 ng/µl)	**Diluted**			
DNA extract	**1:1**	**1:4**	**1:16**	**1:64**	**1:256**
1	22.36	24.20	25.88	27.90	29.80
2	21.92	23.77	25.71	27.71	29.85
3	22.60	24.36	26.40	28.43	30.35
4	22.65	24.65	26.61	28.82	30.45
5	22.60	24.68	26.74	28.82	30.67
6	22.75	24.94	26.74	28.79	30.70
1	21.21	23.70	25.43	27.24	29.53
2	21.28	23.57	25.57	27.51	29.22
3	21.77	23.61	25.82	27.69	29.66
4	21.31	23.07	25.16	27.42	29.26
5	22.42	24.47	26.34	27.99	30.31
6	21.72	23.81	25.67	27.60	29.39
1	21.96	23.78	25.78	27.95	29.58
2	21.95	24.18	25.98	27.76	29.85
3	22.03	24.02	26.26	28.33	29.88
4	22.11	24.06	26.03	28.26	29.94
5	22.45	24.32	26.09	28.03	30.28
6	22.55	24.42	26.25	28.29	30.10

The Table 8 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 8. 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	0.9984	-3.129	22.23	22.36	0.13
2	0.9993	-3.357	21.71	21.92	0.21
3	0.9965	-3.323	22.38	22.60	0.22
4	0.9945	-3.302	22.69	22.65	0.04
5	0.9984	-3.333	22.71	22.60	0.11
6	0.9988	-3.209	22.96	22.75	0.21
1	0.9929	-3.198	21.67	21.51	0.16
2	0.9959	-3.138	21.75	21.28	0.46
3	0.9970	-3.323	21.69	21.77	0.08
4	0.9978	-3.496	20.98	21.31	0.33
5	0.9917	-3.185	22.49	22.42	0.06
6	0.9978	-3.102	21.95	21.72	0.22
1	0.9963	-3.251	21.88	21.96	0.08
2	0.9976	-3.105	22.25	21.95	0.30
3	0.9934	-3.264	22.21	22.03	0.19
4	0.9859	-3.300	22.10	22.11	0.01
5	0.9964	-3.288	22.23	22.45	0.22
6	0.9993	-3.176	22.47	22.55	0.07

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)**

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.

All delta Ct values of extrapolated versus measured Ct are < 0.5.
R^2 of linear regression is > 0.99 for all DNA samples, except one (0.9859).

6. Conclusion

The data reported confirm that the extraction method, applied to maize grains/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 grains/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.

7. 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)]

8. References

1. Sambrook J. and D. W. Russell. Molecular Cloning. A laboratory manual. Third edition. 2001. Cold Spring Harbor Laboratory Press.