Report on the Validation of a "Dellaporta-Derived" Method for DNA Extraction from Ground Maize Grains and Seeds

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Content

1. Introduction

This report describes the validation of the performance of a DNA extraction protocol derived from the publicly available Dellaporta method (Dellaporta [1983] Plant Molecular Biology Reporter 1, nr 4, pp. 19-21) and its applicability on the samples of food and feed provided by the applicant.

The modified protocol can be used for extraction of DNA from seeds and grains ground to powder using a Warring blender or any other appropriate seed crushing device.

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

This protocol is recommended to be executed by skilled laboratory personnel as the procedures comprise working with hazardous chemicals and materials. It is strongly advised to take notice of the 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. Warring blender, model 7010S/7010G/7010HS/7010HG
2. CAC32 Pulveriser for Warring blender
3. Sorvall RC-5B Superspeed Centrifuge with SLA-1500 rotor for 250 ml centrifuge buckets
4. Miracloth filtration membrane (Calbiochem Cat. No. 475855)
5. Micro centrifuge with 18,000 x g for Eppendorf tubes
6. Table centrifuge (swinging buckets) with 3000 x g for Falcon tubes
7. Water bath adjustable to 65°C +/- 1°C
8. 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 (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. KAc; potassium acetate (Merck Cat. No. 1.04820.1000)
5. NaAc; sodium acetate (Merck Cat. No. 1.06268.1000)

6. SDS; sodium dodecyl sulphate (BDH Cat. No. 442444H)
7. β-mercaptoethanol (Sigma Cat. No. M6250)
8. RNase A (Roche Cat. No. 0109-142)
9. Ethanol p.a. (Merck Cat. No. 1.00983.1000)
10. Isopropanol p.a. (Merck Cat. No. 1.09634.2500)
11. Phenol: chloroform: isoamylalchol (25:24:1) (Sigma Cat.No. P-3803 equilibrated at pH 8.0: Cat. No. P-2069)
12. Chloroform p.a. (Merck Cat. No. 1.02445.2500)
13. DNA Clean&Concentrator TM 25 kit (Zymo Research Cat. No. D4005)

2.3 Solutions

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

1. **Extraction buffer**

- 100 mM Tris HCl pH 8.0
- 50 mM EDTA pH 8.0
- 500 mM NaCl
- 10 mM β-mercaptoethanol

2. **Tris-EDTA buffer (TE 1X);**

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

3. **Tris-EDTA buffer (TE 0.1X);**

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

4. **RNase A (10 mg/ml) (store at -20˚C)**

5. **SDS 20%**

6. **Ethanol 70%**

7. **5 M KAc**

8. **3 M NaAc**

Note: all the reagents with the exception of RNAse (-20ºC) can be stored at room temperature.

2.4 Plasticware

1. 250 mL Buckets

2. 50 mL Falcon tubes
3. 2.0 mL microcentrifuge tubes
4. 1.5 mL microcentrifuge tubes
5. filter tips

Note: all plasticware should be sterile, DNase and RNase free.

2.5 Abbreviations

EDTA	Ethylendiamintetracetic acid
PCR	polymerase chain reaction
RNase A	Ribonuclease A
TE	Tris EDTA
Tris	Tris (hydroxymethyl) aminomethane
SDS	Sodium dodecyl sulphate
KAc	Potassium acetate
NaAc	Sodium acetate

3. Description of the methods

3.1 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/.

3.2 Scope and applicability

The method for DNA extraction described below is suitable for the isolation of genomic DNA from maize grains and seeds. Application of the method to other matrices may require adaptation and possible further specific validation.

3.3 Principle

The basic 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 present method starts

with a lysis step (thermal lysis in the presence of 2-βmercaptoethanol and EDTA) followed by two precipitations with potassium acetate and isopropanol. After removal of RNA by digestion with RNAse A, contaminants, such as lipophilic molecules and proteins, are removed by two extractions: the first one performed with phenol:chloroform:isoamylalcohol (25:24:1) equilibrated at pH 8.0 and the second one with chloroform. This first crude extract is subsequently re-suspended, precipitated with isopropanol and washed with 70% ethanol. The DNA is purified using the "DNA Clean & Concentrator™ - 25 (ZYMO RESEARCH CORP.).

3.4 Precautions and recommendations

- Phenol, chloroform, isoamyalcohol and isopropanol are hazardous chemicals; therefore, any manipulations have to be performed under fume hood according to safety guidelines.
- Containers for warring blenders and centrifuge buckets have to be decontaminated thoroughly by immersing for an hour in a 10% bleach solution, rinsing with de-mineralised water, washing and autoclaving.
- All tubes and pipette tips have to be discarded as biological hazardous material.

3.5. Seed crushing procedure

The seeds are crushed using a Warring 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. All seeds should be crushed until a fine powder is obtained. Thorough grinding will also produce a homogenous powder. Prevent cross-contamination between the samples by dust particles.

3.6 Extraction of genomic DNA from maize grains/seeds

1. Transfer 10 gr powder into a 250 mL centrifuge bucket
2. Add 150 mL Extraction Buffer
3. Add 10.5 mL 20% SDS, mix well by inversion
4. Incubate at 65°C for 40 minutes; Note: mix samples every 10 minutes by inversion
5. Centrifuge 10 minutes at 10,000 x g
6. Filter the supernatant over a Miracloth filtration membrane
7. Transfer 30 mL filtered supernatant to a new 50 mL tube
8. Add 9 mL 5M KAc, shake vigorously for 1 minute
9. Incubate on ice for 30 minutes; Note: Mix samples every 10 minutes by inversion
10. Centrifuge for 20 minutes at 3,000 x g
11. Transfer 25 mL supernatant to a new 50 mL tube using a 25 mL pipette
12. Add 20 mL isopropanol, mix gently for 1 minute
13. Incubate on ice for 5 minutes
14. Centrifuge for 20 minutes at 3,000 x g
15. Remove supernatant and air-dry the pellet at 37°C until all isopropanol residue is evaporated
16. Dissolve the pellet in 1 mL TE

17. Transfer the DNA solution to a new 2.0 mL tube
18. Add 10 µL RNAse A (10 mg/ml), mix gently and incubate for 20 minutes at 37°C
19. Add 800 µL phenol: chloroform: isoamylalcohol (25:24:1)
20. Mix well for 1 minute
21. Centrifuge for 10 minutes in a microcentrifuge at maximum speed
22. Transfer the upper aqueous phase (900 µL) to a new 2 mL tube. Do Not disturb the interphase
23. Add 800 µL of chloroform
24. Mix well for 1 minute
25. Centrifuge for 10 minutes in a micro centrifuge at maximum speed
26. Transfer the upper aqueous phase (800 µL) to a new 2 mL tube containing 90 µL 3M NaAc. Do not disturb the interphase
27. Add 600 µL isopropanol
28. Mix gently by inversion for 1 minute
29. Centrifuge for 1 minute in a microcentrifuge at maximum speed to pellet the DNA
30. Remove all supernatant
31. Add 1 mL 70% ethanol to wash the DNA pellet. Make sure the DNA pellet is not stuck on the bottom. Shake the samples for 1 hour
32. Centrifuge for 5 minutes in a microcentrifuge at maximum speed
33. Remove supernatant and air-dry the pellet at 37°C until all ethanol residue is evaporated
34. Add 200 µL TE 0.1x to the DNA pellet
35. Store samples over night at 4°C
36. Shake gently samples for 1 hour
37. Centrifuge for 1 minute in a micro centrifuge at maximum speed
38. Purify the DNA samples using the DNA Clean & Concentrator TM 25 kit according to the manufacturer's instructions
39. Elute the DNA from the column twice using two times 100 µL TE 0.1X

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

Experimental data is provided from an in-house validation in which the method module has been successfully applied to the relevant matrix in the context of the application for authorization. Four samples of about 1000 corn seeds were ground to fine powders, and 10 gr subsamples flour were taken from each ground sample for DNA extractions. The procedure was performed three times under repeatability conditions (within short intervals of time (i.e. days) by the same operator, using the same equipment) resulting in 12 DNA samples.

4.1 DNA concentration, yield and repeatability

The concentration of the extracted DNA was determined by spectrophotometry (Ultrospec 2000, Pharmacia Biotech). 20 µL of the extracted DNA was diluted 1:5 in water. Absorption was measured for both blank (TE 0.1X diluted in water) and diluted DNA solutions at 260 nm. DNA

concentration was calculated based on the assumption that an OD of 1 corresponds to 50 µg/mL DNA.

Results are shown in Table 1; the average DNA efficiency was 26.1 ± 4.2 µg DNA/10 gr meal.

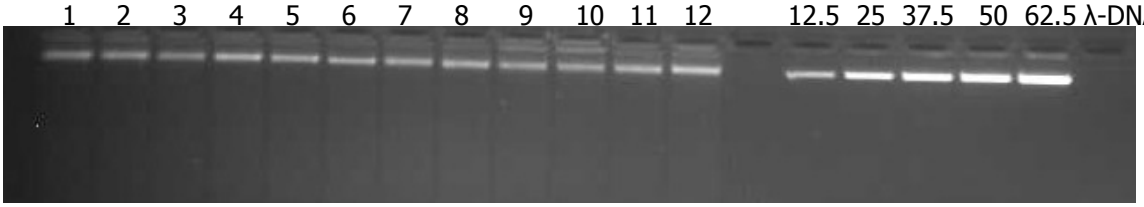
Table 1. Results of the 6 DNA extractions: DNA concentration (ng/µL) and DNA extraction efficiency

DNA extraction	Meal mass (gram)	[DNA] (ng / µl)	DNA extraction efficiency (µg DNA / 10 gram meal)
A1	10.0	145	29.0
A2	10.0	140	28.0
A3	10.0	100	20.0
A4	10.0	105	21.0
B1	10.0	125	25.0
B2	10.0	110	22.0
B3	10.0	170	34.0
B4	10.0	140	28.0
C1	10.0	150	30.0
C2	10.0	115	23.0
C3	10.0	145	29.0
C4	10.0	120	24.0

Therefore, the maize DNA extraction procedure is sufficiently efficient and yields adequate amounts of genomic DNA.

4.2 Analysis of DNA fragmentation

The analysis of DNA fragmentation was performed by ethidium bromide-stained 1% agarose gel electrophoresis compared to a series of standard (non restriction enzyme digested) lambda DNA references of different molecular weight. The 12 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 12 genomic DNA samples showed indications of degradation (smearing).



Therefore, it was concluded that the procedure yields DNA of high structural integrity.

4.3 Evidence of absence of PCR inhibitory compounds

The absence of PCR inhibitory compounds in the DNA preparations were demonstrated by teal-time PCR using the oligonucleotides directed to the endogenous control gene alcohol dehydrogenase, *adh1* gene on serial dilutions of the DNA preparations.
The difference in threshold cycle (Ct) values of a Real-time PCR analysis between the Ct values corresponding to the dilutions should match the dilution factor applied, e.g. if DNA is diluted 10X then the ∆Ct should be approximately 3.32, while if the DNA is diluted 2X, the ΔCt should be 1, etc. Deviations from this relationship may indicate that the extracted DNA contains PCR inhibitors, or that the DNA solution is not homogenous.

This relationship was used to analyze the serial dilutions of the DNA preparations for the presence of PCR inhibitory compounds by plotting the mean Ct values against the logarithm of the DNA mass, and determining the slope (PCR efficiency) and the linearity of the correlation.

A two-fold serial dilution of the extracted DNA was prepared yielding eight different amounts of DNA (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128) of which the Ct values were determined in triplicate in a Real-time PCR run with the oligonucleotide primers and probe. In total, the analysis was executed three times with DNA samples extracted on different days.

To analyse the data, the mean Ct values (y axis) were plotted against the logarithm of the DNA mass (x axis), and by linear regression a trend line (y = ax + b) was calculated, as well as a correlation coefficient, R^2, as a measure of linearity.

The ideal slope value, a (optimal PCR efficiency) then becomes a = -3.32 (typically a 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 results of the three real-time PCR runs are shown in the Table 2 below and show no evidence for PCR inhibitory compounds. The PCR efficiency has been calculated by the following equation: Efficiency (%) = 100 x $[10^{(-1/\text{slope})}]$ -1.

Table 2. Results of the analysis for PCR inhibitory compounds; comparison of extrapolated Ct values versus measured Ct values

DNA	Slope, a	Efficiency (%)	Intercept, b	Linearity, r^2
1A	-3.4327	95.58	29.787	0.9984
1B	-3.2919	101.27	29.819	0.9968
1C	-3.3718	97.96	29.547	0.9982

The results reported in Table 2 demonstrate that the DNA extraction procedure is fit-for-purpose.

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 "Dellaporta-derivated" 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 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 "Dellaporta-derivated" method previously described; the DNA extraction was carried out on 6 test portions (replicates) for three times (days) reaching 18 DNA extractions.

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 Bio-Rad VersaFluor fluorometer. The DNA concentration for all samples is reported in Table 3.

Table 3. DNA concentration (ng/μL) of eighteen samples extracted in three days: yellow cells for samples extracted on day 1, green cells for samples extracted on day 2 and blue boxes for samples extracted on day 3.

Sample	Concentration (ng/μL)
1	170.7
2	137.1
3	141.0
4	97.5
5	133.1
6	88.8
7	129.9
8	90.0
9	88.3
10	88.7
11	143.4
12	99.9
13	272.9
14	127.4
15	116.8
16	174.6
17	110.0
18	212.2

✓ DNA concentration (ng/μL)

Overall average:	134.6 ng/μL
Standard deviation of all samples:	48.6 ng/μL
Coefficient of variation	36.1%

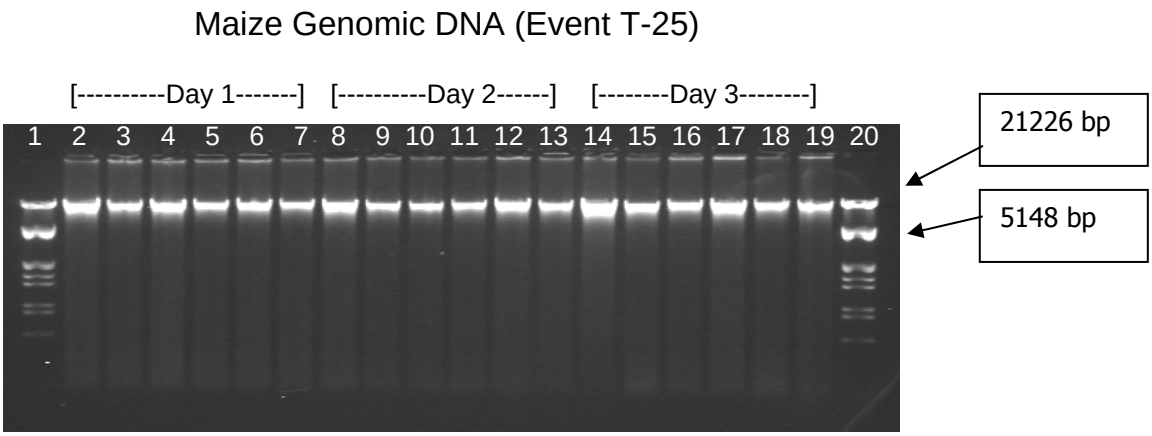
✓ Yield (total volume of DNA solution: 200 μL)

Overall average:	26.9 μg
Standard deviation of all samples:	9.7 μg
Coefficient of variation:	36.1%

5.4 Fragmentation of DNA

The size of the eighteen extracted DNA was evaluated by agarose gel electrophoresis; 2 μL of the DNA solution were analysed on a 0.8% agarose gel (Figure 2). A Lambda DNA/EcoRI+HindIII marker was used.

Figure 2. Agarose gel electrophoresis of eighteen 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 eighteen 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 40 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 Table 4.

Table 4. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of maize gene *adh1*. Yellow cells for samples extracted on day 1, green cells for samples extracted on day 2 and blue boxes for samples extracted on day 3.

	Undiluted (40 ng/µL)	Diluted			
DNA extract	1:1	1:4	1:16	1:64	1:256
1	22.59	24.19	25.92	27.94	29.76
2	22.11	24.20	26.04	28.18	30.01
3	22.07	23.76	26.09	27.98	30.24
4	22.55	24.55	26.35	28.58	30.49
5	22.24	23.91	26.12	28.19	30.43
6	22.36	24.69	26.51	28.54	30.33
7	22.13	24.33	26.36	28.00	30.01
8	22.01	24.07	26.36	28.64	30.53
9	22.33	24.36	26.32	28.92	30.67
10	22.27	24.01	25.82	27.57	30.14
11	21.95	24.13	26.04	28.06	29.92
12	21.39	23.63	26.17	28.18	30.04
13	22.53	24.54	26.61	28.63	31.08
14	22.07	23.87	26.08	28.02	30.50
15	22.36	23.92	26.04	27.87	29.98
16	22.39	23.99	25.98	27.96	30.04
17	21.64	24.25	26.17	28.27	30.73
18	21.81	29.98	26.41	28.16	30.17

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.

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 (ΔCt) cycles from the calculated Ct value. In addition, the slope of the curve should be between -3.6 and -3.1.

Table 5. Comparison of extrapolated Ct values versus measured Ct values (amplification of maize *alcohol dehydrogenase* gene, *adh1*). yellow cells for samples extracted on day 1, green cells for samples extracted on day 2 and blue boxes for samples extracted on day 3.

DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**
1	0.9927	-3.109	22.27	22.59	0.32
2	0.9888	-3.254	22.21	22.11	0.10
3	0.9916	-3.545	21.68	22.07	0.39
4	0.9967	-3.326	22.49	22.55	0.06
5	0.9978	-3.592	21.76	22.24	0.48
6	0.9993	-3.148	22.78	22.36	0.42
7	0.9968	-3.1113	22.47	22.13	0.34
8	0.9978	-3.597	21.99	22.01	0.03
9	0.9932	-3.574	22.19	22.33	0.15
10	0.9804	-3.342	21.85	22.27	0.42
11	0.9984	-3.223	22.19	21.95	0.24
12	0.9934	-3.456	21.84	21.39	0.45
13	0.9954	-3.593	22.30	22.53	0.22
14	0.9959	-3.587	21.70	22.07	0.38
15	0.9942	-3.328	21.94	22.36	0.41
16	0.9895	-3.344	21.96	22.39	0.43
17	0.9943	-3.524	22.02	21.64	0.39
18	0.9891	-3.372	22.10	21.81	0.29

***The expected slope for a PCR with 100% efficiency is -3.32**

****delta Ct = abs (Ct extrapolated - Ct measured)**

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 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.

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. Dellaporta *et al.*, 1983. Plant Molecular Biology Reporter 1, nr. 4, pp. 19-21

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