EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
Food & Feed Compliance

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

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JOINT RESEARCH CENTRE
Health, Consumers and Reference Materials
Food & Feed Compliance

Method development:

Monsanto Company

Method testing and single laboratory validation:

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

Modification from the previous version:

At page 6:

5. PEG Precipitation Buffer (20% w/v) (store at room temperature)

Changed to

5. PEG Preciptation Buffer (20% w/v) (store at room temperature)

- 20% w/v PEG (MW 8000)

- 2.5 M NaCl

Note:

Since 01/12/2009 the term "Community Reference Laboratory (CRL)" is changed into "European Union Reference Laboratory (EURL)".

Since 01/03/2009 to 31/06/2016 the JRC-unit that hosts the EURL GMFF is named "Unit for Molecular Biology and Genomics" instead of "Biotechnology and GMO Unit".

Since 01/07/2016 the JRC-unit that hosts the EURL GMFF is named "Food and feed compliance"

Contents

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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1 Introduction

This report describes the validation of a DNA extraction protocol to extract high quality genomic DNA from processed plant tissue (e.g., leaf, grain, or seed) 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 maize seeds and grains. It is a modified extraction method from Rogers and Bendich (1985)[1].

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 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/Plastic ware)

2.1 Equipment

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

1. Centrifuge (Beckman Coulter Avanti J-251)
2. Shaker (LabLine Enviro 3527)
3. Thermometer (VWR Cat. No. 61222-504)
4. Vacufuge (Eppendorf 5301 22 82 010-9)
5. Water bath (Precision Cat. No. 51220046)
6. Microcentrifuge (Any appropriate model)

2.2 Chemicals

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

1. 24:1 chloroform:isoamyl alcohol (Sigma Cat. No. C-0549)
2. 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma Cat. No. P-3803)
3. Ammonium acetate 7.5 M (Sigma Cat. No. A-2706)
4. CTAB (Sigma Cat. No. H-6269)
5. 0.5 M EDTA, pH 8.0 (GibcoBRL Cat. no. 15575-038)
6. 100% ethanol (AAPER)
7. NaCl (Sigma Cat. No. S-5150)
8. 2-mercaptoethanol (Bio-Rad Cat. no. 161-0710)
9. RNase A (Roche Cat. No. 10 109 196 001)
10. Isopropanol (EM Science Cat. No. PX1835-9)
11. 1 M Tris HCl pH 8.0 (Sigma Cat. No. T-3038)
12. Proteinase K (Roche Cat. No. 03 115 836 001)
13. Polyethylene Glycol (MW 8000) (Sigma Cat. No. P2139)

2.3 Solutions

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

1. **CTAB Extraction Buffer (2%) (store at room temperature)**

- 2% w/v CTAB
- 100 mM Tris HCl pH 8.0
- 20 mM EDTA pH 8.0
- 1.4 M NaCl

2. **Tris-EDTA buffer (TE 1X) (store at room temperature)**

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

3. **Proteinase K (10 mg/mL) (store at -20 °C)**

4. **RNase A (10 mg/mL) (store at -20 °C)**

5. **PEG Precipitation Buffer (20% w/v) (store at room temperature)**

- 20% w/v PEG (MW 8000)
- 2.5 M NaCl

6. **Ethanol (70% v/v) (store at room temperature)**

7. **Ethanol (80% v/v) (store at room temperature)**

2.4 Plastic ware

1. 50 mL conical tubes (Corning Cat. No. 430290)
2. 13 mL Sarstedt tubes (Sarstedt Cat. No. 60.540)
3. 1.5 mL microcentrifuge tubes
4. filter tips

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

2.5 Precautions

- Phenol, chloroform, isoamyl alcohol, and isopropanol are hazardous chemicals; therefore, all manipulations have to be performed according to safety guidelines, under fume hood.
- 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

3 Description of the methods

3.1 Sampling

For sampling methods, it is referred 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) N. 1830/2003.

3.2 Scope and applicability

The method for DNA extraction described below is suitable for the isolation of genomic DNA from a wide variety of maize tissues and derived matrices. However, validation data presented here are restricted to ground maize seeds and grains. 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 Tris HCl, EDTA, CTAB and β-mercaptoethanol) followed by removal of contaminants such as lipophilic molecules and proteins by extraction with phenol and chloroform.

A DNA precipitate is then generated by using isopropanol. The pellet is dissolved in TE buffer. Remaining inhibitors are removed by PEG precipitation and re-suspension in TE buffer. Tissues crushing procedure

Tissues should be processed prior to extraction procedure. Possible methods of processing include a mortar and pestle with liquid nitrogen (leaf) or commercial blender (grain or seed).

3.4 Samples grinding procedure

Samples should be processed prior to extraction procedure. Possible methods of processing include a mortar and pestle with liquid nitrogen (leaf) or commercial blender (grain or seed).

3.5 Extraction of genomic DNA from maize seeds/grains

1. Weight out 6 g of processed tissue into a 50 mL conical tube appropriate for centrifugation. Note: For unprocessed tissue, weighing may occur prior to processing as long as entire processed sample is transferred to the conical tube.
2. For each 6 g sample add 25 mL of a solution consisting of 24.25 mL, pre-warmed CTAB extraction buffer, 0.5 mL 2-β-mercaptoethanol (2-ME), and 0.25 mL of 10 mg/mL proteinase K for a final concentration of 2% (2-ME) and 100 µg/mL (proteinase K).
3. Mix the tube vigorously by inversion for 45-60 seconds.
4. Incubate for 60 minutes at 55 °C and mix the tube vigorously for 40-60 seconds every 20 minutes. Cool the tube on bench for 10 minutes.
5. Add 20 mL of phenol:choloroform:isoamyl alcohol (PCI 25:24:1, pH 6.7). Cap the tube and mix vigorously by inversion at least for 1 minute.

6. Centrifuge for 10 minutes at 13000 x g at room temperature to separate the aqueous and organic phases. Transfer upper aqueous phase to a clean 50 mL conical tube.
7. Repeat extraction twice for a total of three extractions (step 5-6).
8. Transfer upper aqueous phase to a new tube, add 2/3 volume of -20 °C isopropanol and gently mix the tube by inversion.
9. To precipitate the DNA place the tube at -20 °C for 30 minutes. (DNA may be stored as isopropanol precipitate at -20 °C for up to 1 year).
10. To pellet the DNA, centrifuge the tubes at approximately 13000 x g for 20 minutes at 4 °C. Pour off isopropanol by pipette, and then perform a quick spin in the centrifuge to bring down the isopropanol from the side of the tube. Remove remaining isopropanol by pipette and ensure all residual isopropanol is removed before proceeding to the next step without over drying the pellet.
11. Re-dissolve the pellet in 4 mL of TE pH 8.0. Note: it may be necessary to incubate the tube at 60°C to resuspend the pellet.
12. Transfer the resuspended pellet to a 13-mL tube, add 40 µL of 10 mg/mL RNase and then incubate at 37°C for 30 minutes.
13. To extract the DNA, add 4 mL of chloroform:isoamyl alcohol (CIA 24:1), mix vigorously by inversion for 40-60 seconds and centrifuge for 10 minutes at 13000 x g at room temperature. Transfer the upper aqueous phase to a clean tube.
14. Repeat step 13 an additional time, then add half volume of 7.5 M ammonium acetate, gently mix by inversion and add 2 volumes of 100% ethanol. Mix by inversion and place at -20°C for 30 minutes. DNA may be stored as ethanol precipitate at -20 °C for up to 1 year.
15. Centrifuge at 13000 x g for 20 minutes at 4°C to pellet the DNA.
16. Rinse the DNA pellet twice with 10 mL of 70 % ethanol loosening the pellet from the side of the tube and remove residual ethanol by vacuum.
17. Re-suspend DNA in 1 mL TE, pH 8.0 and incubate at 65°C for at least 1 hour with periodic gentle mixing.
18. Centrifuge the DNA solution at 16000 x g for 10 minutes at 4°C. Transfer the aqueous portion to a clean tube without disturbing the pellet and store at 4°C.
19. Add equal volume of 20% PEG precipitation buffer (~1 mL) to the extracted DNA solution. Mix well by inversion.
20. Incubate the PEG/DNA mixture for 15 minutes at 37 °C.
21. Centrifuge the PEG/DNA mixture for 15 minutes at 15000 x g at room temperature.
22. Pour off supernatant. Wash the walls of the tube and DNA pellet with 1 mL of 80% ethanol loosening the pellet from the tube. Carefully pour off ethanol, centrifuge briefly and remove by pipetting any residual ethanol.
23. Repeat wash (step 22) for a total of two washes.
24. Completely dry any residual ethanol by vacufuge at low heat.
25. Re-suspend the pellet in 1 mL TE or H2O. If necessary, incubate the sample at 60°C to dissolve the pellet.
26. Centrifuge the re-suspended DNA solution at 15000 x g for 15 minutes.
27. Transfer DNA solution to a clean tube without disturbing the pellet and sore DNA at 4°C.

4 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 method proposed by the applicant on samples of food and feed consisting of ground 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.

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 method described above (see paragraph 3. "Description of the methods"); the DNA extraction was carried out on 6 test portions (replicates) and repeated over three different days, giving a total of 18 DNA extractions.

4.3 DNA concentration, yield 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 the Table 1 below.

Table 1. DNA concentration (ng/µL) of eighteen samples extracted 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	147.2
2	229.0
3	579.8
4	352.1
5	551.1
6	504.9
1	277.5
2	468.7
3	473.4
4	424.6
5	258.0
6	517.5
1	526.7
2	463.1
3	531.5
4	594.8
5	537.8
6	545.4

- ✓ DNA concentration (ng/µL)

Overall average	443.5 ng/µL
Standard deviation	133 ng/µL
Coefficient of variation	30 %

- ✓ Yield (total volume of DNA solution: 1 mL)

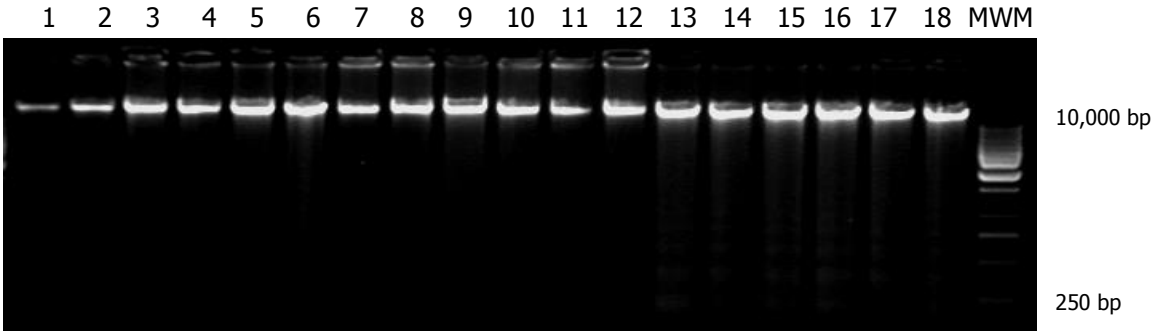
Overall average	443.5 µg
Standard deviation	133 µg
Coefficient of variation	30 %

4.4 Fragmentation state of DNA

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

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').

Figure 1. Agarose gel electrophoresis of eighteen genomic DNA samples extracted from maize seeds. Lanes 1-6: samples extracted on day 1; lanes 7-12 samples extracted on day 2; lanes 13-18 samples extracted on day 3; lane 19: 1 kb DNA molecular weight marker (MWM).



4.5 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 0.2x TE buffer (1:4, 1:16, 1:64, 1:256) and analysed using a real-time PCR system detecting the target sequence of the maize endogenous control gene, hmg (high mobility group). The Ct values obtained for "undiluted" and diluted DNA samples are reported in the Table 2.

Table 2. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of the maize endogenous gene, hmg. 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.

	Undiluted (50 ng/µL)	Diluted			
DNA extract		1:4	1:16	1:64	1:256
1	23.88	25.64	27.65	29.44	31.43
2	23.56	25.70	27.59	29.26	31.38
3	23.53	25.46	27.71	29.68	31.52
4	23.89	25.56	27.43	29.75	31.49
5	24.10	25.78	27.59	29.06	31.43
6	23.89	25.74	27.52	29.18	31.70
1	23.72	25.30	27.35	28.97	31.08
2	23.51	25.82	27.73	29.48	31.51
3	23.61	25.37	27.99	29.87	31.78
4	23.55	25.26	27.20	29.52	31.55
5	23.77	25.67	27.51	29.37	31.93
6	23.41	25.86	27.31	29.24	31.23
1	23.49	25.09	27.62	29.71	31.72
2	23.79	25.95	27.90	29.76	31.77
3	23.71	25.47	27.73	29.50	31.47
4	23.73	25.44	27.45	29.92	31.81
5	23.88	25.64	27.42	29.23	31.75
6	23.58	25.89	27.96	30.12	32.03

Table 3 below reports the comparison of extrapolated Ct values versus measured Ct values for all samples and the values of linearity (R2) 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 (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 > 0.5 cycles compared the calculated Ct value (ΔCt > 0.5) . In addition, the slope of the curve should be between -3.6 and -3.1.

Table 3. Comparison of extrapolated Ct values versus measured Ct values (amplification of maize *hmg* gene).

DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**
1	0.998	-3.18	23.75	23.88	0.13
2	0.995	-3.11	23.79	23.56	0.23
3	0.997	-3.35	23.55	23.53	0.03
4	0.996	-3.34	23.53	23.89	0.36
5	0.987	-3.10	23.76	24.10	0.33
6	0.989	-3.25	23.64	23.89	0.24
1	0.997	-3.149	23.43	23.72	0.28
2	0.999	-3.127	23.93	23.51	0.42
3	0.992	-3.507	23.47	23.61	0.13
4	0.997	-3.516	23.09	23.55	0.46
5	0.991	-3.427	23.46	23.77	0.31
6	0.995	-2.995	23.90	23.41	0.49
1	0.995	-3.598	23.15	23.49	0.35
2	0.999	-3.212	24.01	23.79	0.22
3	0.996	-3.278	23.61	23.71	0.10
4	0.996	-3.582	23.26	23.73	0.46
5	0.991	-3.345	23.47	23.88	0.41
6	0.998	-3.412	23.87	23.58	0.28

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)**

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

R2 of linear regression is > 0.99 for all DNA samples except two samples extracted on day 1: the sample 5 (0.987) and the sample number 6 (0.989). The slopes of the curve are all between -3.1 and -3.6, with one exception: the sample number 6 extracted on day 2, with a value of -2.995.

5 Conclusion

The data reported confirm that the extraction method, applied to samples of food and feed 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 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. Rogers S., Bendich A., 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Molecular Biology, 69-76.

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