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Report on the In-house Validation of a DNA Extraction Method from Soybean Grains and Validated Method

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Report on the In-house Validation of a DNA Extraction Method from Soybean Grains and Validated Method

13 May 2014

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In accordance with relevant EU legislation[a], Dow AgroSciences LLC provided to the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF) a DNA extraction method for soybean grains and the relevant samples (ground soybean grains).

In line with its mandate[b], the EU-RL 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 EU-RL GMFF and the ENGL[c], and that it satisfies the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004.

The method is therefore fit for the purpose of producing soybean 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.

[a] Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003".

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

[c] Definition of minimum performance requirements for analytical methods of GMO testing. http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf

Quality assurance

The EU-RL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

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

The EU-RL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EU-RL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection IHCP provided by CERMET.

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Content

1. Introduction

This report describes the validation of a DNA extraction method derived from the publicly available "CTAB" method [(1)] followed by an anion exchange chromatography with commercially available columns "Genomic Tip 20/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 soybeans.

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

This protocol is recommended to be executed only by skilled laboratory personnel as the procedures comprise the use of hazardous chemicals and materials. It is strongly 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. Cutting mill (Retsch GRINDOMIX GM 200)
2. Pipettes (Gilson)
3. Incubator (Eppendorf Thermo-mixer Comfort 5355)
4. Balances (Mettler Toledo XS2002S)
5. Centrifuges (Eppendorf 5810 and 5415D)
6. Vortex (MS1 Minishaker IKA)

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 (Sigma S5150)
5. Proteinase K (Sigma P2308)
6. RNase A (Sigma R6513)
7. Sodium acetate (Sigma S7899)
8. 2-Mercaptoethanol (Sigma M3148)

9. Chloroform (Sigma C2432)
10. 1-Octanol (Sigma 293245)
11. Isopropanol (Sigma I9516)
12. Ethanol (Fluka 02860)
13. EDTA (Sigma E7889)
14. Polyvinylpyrrolidone 10, PVP10, (Sigma-Aldrich P2307)
15. Phenol/Chloroform/Isoamyl alcohol (25:24:1) (Sigma P2069)
16. Tris-EDTA Buffer Solution (Fluka 93283)

2.3. Solutions

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

1. **CTAB Lysis Buffer (2%)**

- 2% w/v CTAB
- 100 mM Tris HCl pH 8.0
- 20 mM EDTA pH 8.0
- 1.4 M NaCl
- 2% (w/v) PVP10
- Store at room temperature for up to 1 year

2. **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_2O_{deion}. Autoclave.
Store at room temperature for up to 2 years.

3. **Proteinase K (20 mg/mL)**

- For 10 mL proteinase K solution dissolve 200 mg proteinase K in 10 mL H_2O_{deion}.
- Aliquot and store at -20 °C for up to 2 years.

4. **RNase A (91 mg/mL)**

- Dissolve 250 mg RNase A in 2750 µL of $H_2O_{deion.}$
- Aliquot and store at -20 °C for up to 2 years.

5. **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.

6. **Ethanol 70% (v/v)**
For 200 mL combine 140 mL 100% ethanol with 60 mL H_2O_{deion}.
Store at room temperature for up to 5 years.

2.4. Plasticware

1. "Genomic Tip 20/G" columns (Qiagen 10223)
2. 50 mL conical tubes (BD 352070)
3. 15 mL conical tubes (BD 352097)
4. 2.0 microcentrifuge tubes (Eppendorf 0030 120.094)
5. filter tips for pipettes

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

2.5. Precautions

- Chloroform, octanol, and isopropanol are hazardous chemicals; therefore, all manipulations have to be performed according to safety guidelines, under a 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
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 high quality genomic DNA from soybean grains. Application of the method to other matrices may require adaptation and possible further specific validation.

3.2 Practicability

The DNA extraction method described below requires only standard molecular biology equipment, e.g. a centrifuge, an incubator and pipettes. The procedure takes about 4 hours of hands-on time.

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 method starts with a lysis step in the presence of CTAB lysis buffer containing and high concentrations of NaCl for keeping DNA released from the sample material in solution followed by extraction with phenol-chloroform-isoamyl alcohol and chloroform-octanol to remove contaminants such as lipophilic molecules, proteins and CTAB/polysaccharide complexes.
A crude DNA precipitate is then generated by using isopropanol. The pellet is dissolved in low salt buffer. Remaining inhibitors are removed by anion-exchange chromatography using the commercially available gravity-flow column "Genomic Tip 20/G" (Qiagen). After elution from the column, a final isopropanol precipitation step desalts and concentrates the DNA.

3.4 Grinding

Soybean grains (e.g. 100 gr) should be ground prior to extraction procedure for 1 minute at 10,000 rpm in a cutting mill.

3.5 Lysis and isopropanol precipitation of DNA

1. Transfer 25 mL CTAB lysis buffer, 125 µL Proteinase K, 500 µL 2-mercaptoethanol and 100 µL RNase A to a 50 mL conical tube.
2. Weigh out 1 g of ground soybeans and add to the tube containing CTAB lysis buffer, Proteinase K, 2-mercaptoethanol and RNase A, mix thoroughly.
3. Incubate for 2 hours at 65 °C with gentle shaking.
4. Centrifuge the sample tubes at 5000 x g for 20 minutes.
5. Pour the clear supernatants into clean 50 mL conical tubes and add equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to each sample and mix vigorously for 5 minutes.
6. Centrifuge the sample tubes at 5000 x g for 20 minutes.
7. Transfer the clear supernatants to clean 50mL conical tubes with plastic pipettes.
8. Repeat the phenol:chloroform:isoamyl alcohol (25:24:1) step and transfer the clear supernatants to clean 50mL conical tubes.
9. Add equal volume of chloroform:octanol (24:1) and mix vigorously for 5 minutes.
10. Centrifuge the sample tubes at 5000 x g for 20 minutes.
11. Transfer the clear supernatants to clean 50mL conical tubes with plastic pipettes.

12. Repeat the chloroforml:octanol (24:1) extraction/centrifugation step and transfer the clear supernatants to clean 50mL conical tubes.
13. Add equal volume (about 15 mL) of isopropanol to each sample and mix well by inverting the tubes several times (samples can be incubated at room temperature for 1 hour or at 4 °C overnight).
14. Centrifuge for 10 minutes at 5000 x g in a fixed angle rotor.
15. Decant and discard the supernatants.
16. Wash the DNA pellets with 10 mL of cold 70% ethanol and spin for 5 minutes at 3000 x g in a swinging bucket rotor or at 5000 x g in a fixed angle rotor.
17. Repeat the 70% ethanol wash step.
18. Discard the supernatants very carefully and dry the DNA pellets for 30 minutes at 37 °C in the Thermo-mixer.
19. Re-suspend the DNA pellets in 2 mL of 1x TE buffer (pH 8.0) pre-warmed to 65 °C.
20. Store the tubes at 4 °C over-night to dissolve the DNA pellets. The following day additional heating may be required to completely dissolve the pellets.
21. Centrifuge samples at 5000 x g for 10 minutes to remove all undissolved matter. Transfer the supernatants and discard any pellets.

3.6 DNA Purification using Genomic-tip 20/G

22. Set up a gDNA purification column (QIAGEN Genomic-tip 20/G) for each sample.
23. Equilibrate a QIAGEN Genomic-tip 20/G with 2 mL Buffer QBT. Let the liquid enter the column by gravity flow.
26. Mix the supernatant from Step 21 with an equal volume of Buffer QBT and then add the entire sample to the equilibrated QIAGEN Genomic-tip 20/G column. (Note: Slight positive pressure may be required to facilitate column flow. Do not exceed 4-10 drops/min).
27. Wash the QIAGEN Genomic-tip column three times with 1 mL Buffer QC.
28. Elute the genomic DNA with 1 mL QF buffer (pre-warmed at 50 °C) and collect the DNA in a 2 mL microcentrifuge tube.
29. Repeat the elution with 1 mL QF buffer and collect the DNA in a second 2 mL microcentrifuge tube.
30. Precipitate DNA by adding 700 µL cold isopropanol to each tube; mix carefully by inverting (about 10 times).
31. Centrifuge samples at 20000 x g on a tabletop centrifuge for 10 minutes.
32. Remove supernatants and wash pellets with 1 mL 70% ethanol.
33. Centrifuge samples at 20000 x g on a tabletop centrifuge for 10 minutes.
34. Remove supernatants very carefully and allow pellets to air-dry for 10 minutes at room temperature.
35. Re-suspend the pellets in 150 µL 1x TE buffer (pH 8.0) pre-warmed to 65 °C.
36. Store the tubes at 4 °C over-night to dissolve the DNA pellets. The following day additional heating may be required to dissolve the pellets completely.
37. Pool the solutions from the duplicate tubes into a single tube for a final volume of about 300 µL.

4. Testing of the DNA extraction method by the EU-RL GMFF

The EU-RL GMFF tested the method proposed by the applicant and described above on samples consisting of ground soybeans 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[d].

4.1 Preparation of samples

100 g of soybean grains received from the applicant were ground using a GRINDOMIX GM 200 (Retsch GmbH) mixer.

4.2 DNA extraction

DNA was extracted from six test portions (replicates) following the method described in paragraph 3.4 "Description of the methods". 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 (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.

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

The average DNA concentration and the yield are reported in Table 1 below.

	Concentration (ng/µL)	**Yield (µg)**
Overall average	75.2	22.6
Standard deviation of all samples	11.9	3.6
Coefficient of variation (%)	16	16

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

Table 2. DNA concentration (ng/µL) and yield of extracted samples

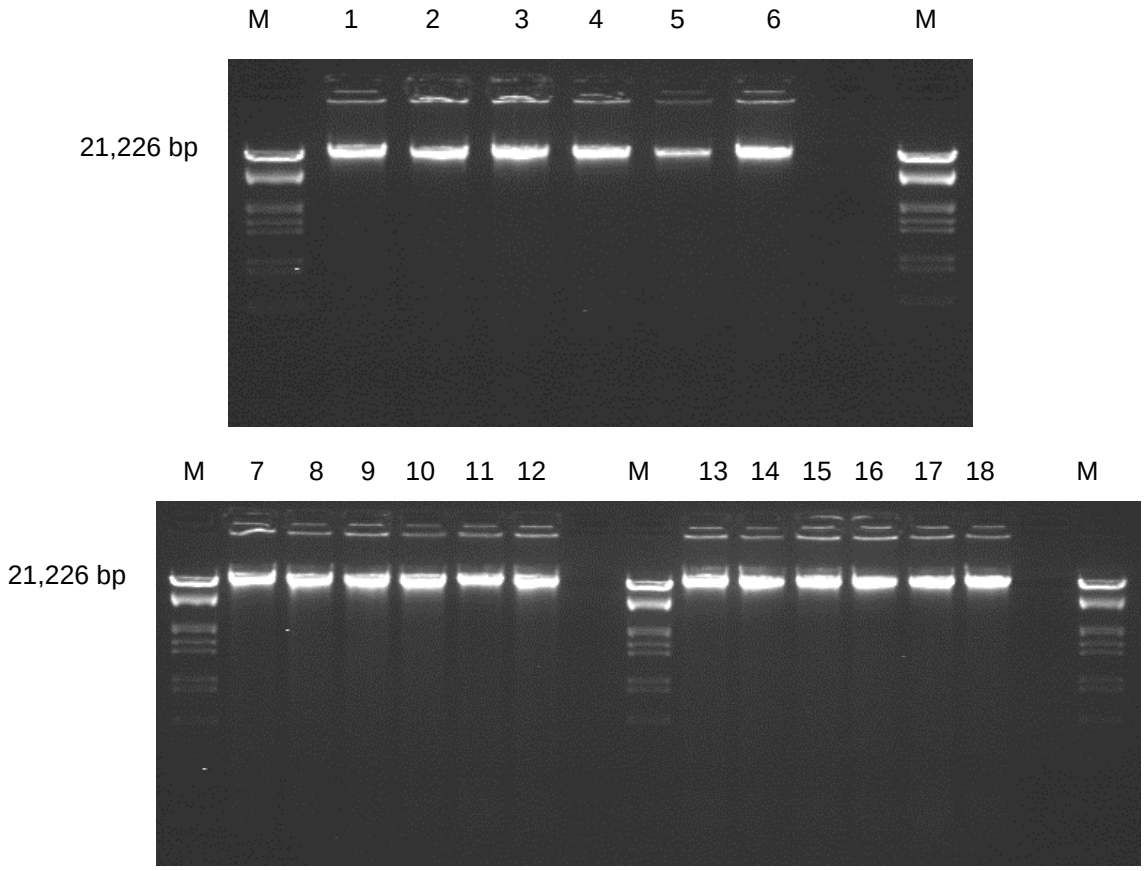
Sample	**Concentration (ng/µL)**	**Yield (µg)**
1	62.9	18.9
2	69.9	21.0
3	72.2	21.7
4	69.3	20.8
5	40.0	12.0
6	68.6	20.6
7	87.2	26.2
8	87.6	26.3
9	87.7	26.3
10	88.1	26.4
11	77.3	23.2
12	89.8	26.9
13	77.8	23.3
14	76.5	23.0
15	74.9	22.5
16	67.9	20.4
17	78.4	23.5
18	77.0	23.1

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 analysing it on a 1.0% agarose gel electrophoresis, to check that the DNA is not excessively fragmented for subsequent analyses. On the agarose gel, 4 µL of the DNA solutions are loaded (Figure 1).

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



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/EcoRI+HindIII molecular weight markers.

The extracted genomic DNA samples appeared as distinct high molecular weight DNA fluorescent banding patterns migrating through 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 30 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 *lectin Le1*). The Ct values obtained for "undiluted" and diluted DNA samples are reported in Table 3.

Table 3. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of soybean gene *Le1*.

Ct values					
	Undiluted	Diluted extracts			
DNA extract	(30 ng/µL)	1:4	1:16	1:64	1:256
1	21.32	23.36	25.52	27.58	29.65
2	21.46	23.40	25.36	27.38	29.58
3	21.30	23.24	25.37	27.40	29.43
4	21.30	23.16	25.12	27.39	29.34
5	21.09	22.96	25.07	27.17	29.16
6	21.34	23.47	25.43	27.55	29.78
7	21.82	23.86	25.93	27.99	30.05
8	21.90	23.87	26.01	27.93	29.96
9	21.94	23.86	25.89	27.98	29.96
10	21.86	23.72	25.83	27.94	29.85
11	21.83	23.89	25.98	27.97	29.95
12	21.87	23.84	25.95	27.96	30.02
13	21.55	23.63	25.66	27.55	29.70
14	21.50	23.51	25.52	27.68	29.72
15	21.60	23.61	25.60	27.80	29.79
16	21.47	23.46	25.55	27.56	29.62
17	21.59	23.55	25.51	27.61	29.78
18	21.43	23.41	25.56	27.53	29.62

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 measure inhibition, the Ct values of the four diluted samples were plotted against the logarithm of the dilution and the Ct values for the "undiluted" samples (30 ng/µL) were extrapolated from the equation calculated by linear regression.

Subsequently, the extrapolated Ct values for the "undiluted" samples were compared with the measured Ct data. It is assumed that PCR inhibitors are present if the measured Ct value for the "undiluted" sample is > 0.5 cycles from the calculated Ct value. Table 4 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 4. Comparison of extrapolated Ct values versus measured Ct values (amplification of soybean gene *Le1*)

DNA extraction	R^2	Slope	Ct extrapolated	mean Ct measured	ΔCt*
1	1.00	-3.48	21.29	21.32	0.02
2	1.00	-3.42	21.29	21.46	0.17
3	1.00	-3.42	21.21	21.30	0.09
4	1.00	-3.46	21.05	21.30	0.25
5	1.00	-3.44	20.91	21.09	0.18
6	1.00	-3.49	21.29	21.34	0.04
7	1.00	-3.42	21.81	21.82	0.01
8	1.00	-3.35	21.90	21.90	0.00
9	1.00	-3.39	21.82	21.94	0.11
10	1.00	-3.40	21.71	21.86	0.14
11	1.00	-3.35	21.91	21.83	0.08
12	1.00	-3.41	21.81	21.87	0.07
13	1.00	-3.34	21.61	21.55	0.06
14	1.00	-3.45	21.41	21.50	0.09
15	1.00	-3.44	21.52	21.60	0.08
16	1.00	-3.41	21.42	21.47	0.05
17	1.00	-3.45	21.42	21.59	0.18
18	1.00	-3.42	21.38	21.43	0.05

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 Ct = abs (Ct extrapolated - Ct measured)

According to ENGL definition of minimum performance requirements for analytical methods of GMO testing[e] 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^{2f} shall be ≥0.98.

The table indicates that all ΔCt values of extrapolated versus measured Ct are < 0.5. The R^2 coefficient of linear regression is > 0.98 for all DNA samples and the slopes of the curves are between -3.1 and -3.6 for all samples.

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

[f] R^2 is the correlation coefficient of a standard curve obtained by linear regression analysis.

5. Conclusions

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

The method is applicable to soybean samples provided by the applicant in accordance with the requirements of Annex I-2.C.2 to Regulation (EC) No 641/2004.
If applied to complex food or feed products containing soybean, 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.

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In line with its mandate , the EU RL 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 EU-RL GMFF and the ENGL , and that it satisfies the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004.

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