Report on the Validation of a DNA Extraction Method for Ground Soybeans

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

1. Centrifuge (cooled, for 50 mL tubes, 10000 x g, e.g. Eppendorf 5810)
2. Centrifuge (cooled, for 2 mL tubes, 15000 x g, e.g. Beckman Coulter Allegra 25R)
3. Shaker/waterbath (LabLine Enviro 3527)
4. Thermomixer (Eppendorf)
5. Vacufuge (Eppendorf Concentrator 5301)

2.2. Chemicals

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

1. "Genomic DNA Buffers" Kit (Qiagen 19060)
2. CTAB (Sigma No. H6269)
3. Tris HCl (Sigma T3038)
4. Sodium chloride (Sigma S3014)
5. Proteinase K (Sigma P2308)
6. RNase A (Sigma R6513)
7. Sodium acetate (Sigma SA7899)
8. 2-Mercaptoethanol (Sigma M3148)
9. Chloroform (Sigma C2432)

10. 1-Octanol (Sigma-Aldrich 293245)
11. 2-Propanol (Isopropanol) (Sigma I9516)
12. Ethanol (Fluka 02860)

2.3. Solutions

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

1. **CTAB Lysis 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)** (e.g. from Fluka, 93283) (store at room temperature)
- 10 mM Tris HCl pH 8.0
- 1 mM EDTA pH 8.0

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

4. **RNase A (91 mg/ml)** (store at -20 ˚C)
- dissolve 250 mg RNase A in 2.5 mL Na Acetate (10 mM, pH 5.2)
- aliquot in 0.5 mL
- add 50 µl Tris-HCl (1 M, pH 7.4) to each aliquot

5. **Chloroform:octanol (24:1)** (store at room temperature, under fume hood for up to 6 months)

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

2.4. Plasticware

1. “Genomic Tip 20/G” columns (Qiagen 10223)
2. 50 mL conical tubes (e.g. Greiner 227270)
3. 15 mL conical tubes (e.g. Greiner 188261)
4. 2.0 and 1.5 mL microcentrifuge tubes (e.g. Eppendorf 0030 121.597; 0030 121.589)
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

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) No 1830/2003.

3.2 Scope and applicability

The method for DNA extraction described below is suitable for the isolation of genomic DNA from ground soybeans. 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 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 and CTAB/polysaccharide complexes by extraction with chloroform-octanol.
A crude DNA precipitate is then generated by using isopropanol. The pellet is dissolved in TE 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 procedure

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

3.5 Crude DNA extraction from soybeans

1. Transfer 15 mL CTAB lysis buffer, 60 µL Proteinase K, 200 µL 2-mercaptoethanol and 10 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; mix thoroughly.
3. Incubate for 1 hour at 65 °C with agitation, shaking every 15-20 minutes. Cool the tube on bench for 15 minutes.
4. Add 10 mL of choloroform:octanol (24:1) and mix vigorously by inversion or vortex.
5. Centrifuge for 10 minutes at 10000 x g at room temperature to separate the aqueous and organic phases.
6. Transfer upper aqueous phase to a clean 50 mL conical tube containing 10 mL isopropanol and invert the tube several times to mix.
7. To precipitate the DNA, centrifuge at 4 °C for 30 minutes at 10000 x g.
8. Discard supernatant and add 5 mL 70% ethanol and mix
9. Spin down at 4 °C for 20 minutes at 10000 x g.
10. Carefully discard ethanol.
11. Repeat the centrifugation step at 4 °C for 1 minute at 10000 x g and remove residual ethanol by pipetting.
12. Resuspend the pellet in 500 µL 1xTE buffer (pre-warmed to 50°C).

3.6 Purification using Genomic Tip 20/G

13. Add 5 mL G2 buffer (containing 10 µL RNase and 25 µL Proteinase K) and mix thoroughly.
14. Incubate 1 hour at 50°C with agitation.
15. Centrifuge at room temperature for 5 minutes at 10000 x g.
16. Transfer the supernatant to a 15 mL conical tube. (Note: pipetting debris should be avoided)
17. Equilibrate a column (“Genomic-Tip 20/G”) with 2 mL QBT buffer.
18. Apply the sample to the equilibrated column.
19. Wash the column with 1.5 mL buffer QC. Then wash it a second time, again with 1.5 mL QC.

20. Elute the genomic DNA with 1 mL QF buffer (warmed to 50°C) and collect the DNA in a 2 mL microcentrifuge tube.
21. Repeat the elution with 1 mL QF buffer and collect the DNA in a second microcentrifuge tube.
22. Add 700 µL isopropanol to each tube, mix carefully by inverting (about 10 times).
23. Centrifuge (30 minutes, 4°C, 10000 x g).
24. Discard the supernatant and wash the DNA pellets with 1 mL 70% ethanol each.
25. Centrifuge (10 minutes, 4°C, 15000 x g).
26. Discard the supernatant and let the ethanol evaporate without over-drying the pellet.
27. Dissolve the DNA pellets in 100 µL 1x TE buffer (pre-warmed to 50°C).
28. Incubate at 50°C for 30 minutes, with agitation.
29. Allow the DNA to homogenize over night at 4°C, with gentle agitation.
30. Centrifuge (15 minutes, room temperature, 15000 x g).
31. Combine the first and the second elutions which were obtained from the same column in a single microcentrifuge tube for a final volume of about 200 µL.

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

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 EURL-GMFF tested the method proposed by the applicant on samples of food and feed consisting of ground soybeans 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

50 g of soybeans 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 days, for 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	179.6
2	178.6
3	170.9
4	154.0
5	175.3
6	184.4
7	259.0
8	218.5
9	229.4
10	173.0
11	188.6
12	181.9
13	205.6
14	195.3
15	188.3
16	172.5
17	124.5
18	193.5

- ✓ DNA concentration (ng/μL)

Overall average	187.4 ng/μL
Standard deviation of all samples	29.1 ng/μL
Coefficient of variation	15.5 %

- ✓ Yield (total volume of DNA solution: 3600 μL)

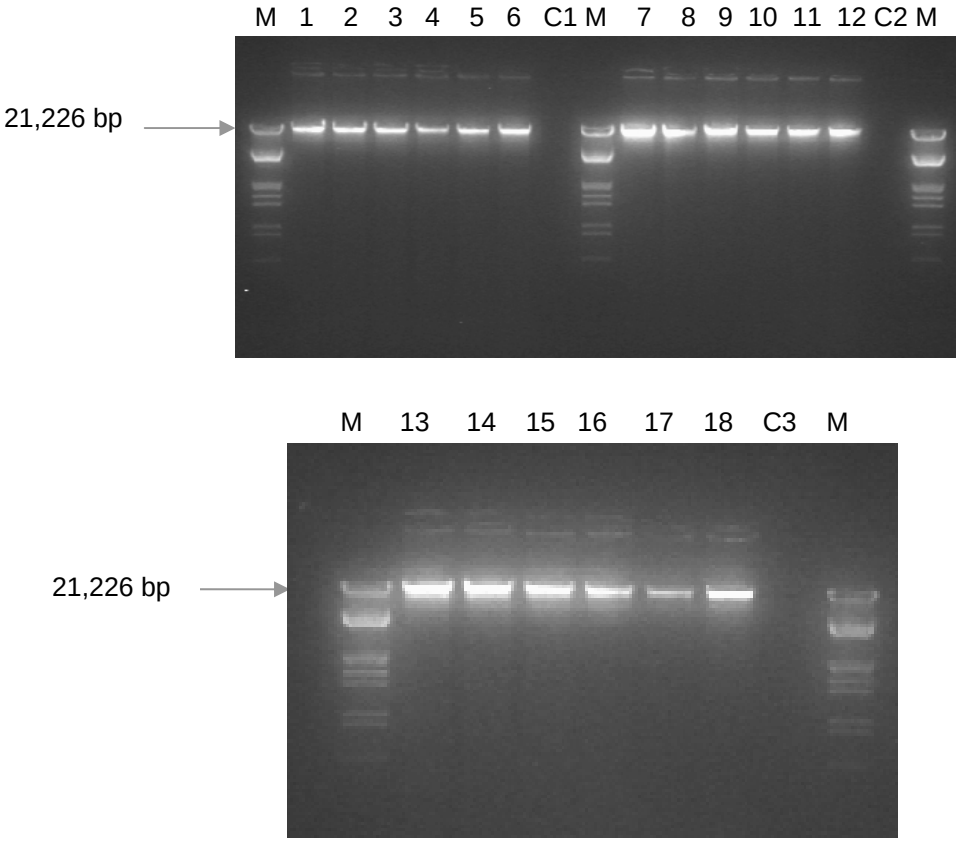
Overall average	37.48 μg
Standard deviation	5.82 μg
Coefficient of variation	15.5 %

4.4 DNA Fragmentation

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 1.5 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 high molecular weight DNA fluorescent banding patterns migrating through the gel. None of the DNA samples showed indication of significant degradation ('smearing').

Figure 1. Agarose gel electrophoresis of eighteen genomic DNA samples extracted from soybeans. Lanes labelled 1-6: samples extracted on day 1; lanes labelled 7-12 samples extracted on day 2; lanes labelled 12-18 samples extracted on day 3; lanes labelled M: Lambda DNA/EcoRI+HindIII molecular weight marker; lanes labelled C1-3: extraction controls of days 1-3



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 20 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 (1mM Tris, 10µM EDTA, pH 8.0) and analysed using a real-time PCR system detecting the target sequence of the endogenous gene *lectin 1* (*le1*). 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 soybean gene *le1*. 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.

Ct values					
	Undiluted	Diluted extracts			
DNA extract	(20 ng/µL)	1:4	1:16	1:64	1:256
1	21.72	23.72	25.83	27.95	29.89
2	21.74	23.76	25.98	28.03	29.97
3	21.79	23.74	25.89	27.90	29.90
4	21.69	23.69	25.80	27.96	30.00
5	21.83	23.81	25.98	28.07	30.01
6	21.80	23.88	26.00	28.04	30.10
7	21.81	23.82	25.89	28.01	29.80
8	21.82	23.80	25.99	28.07	29.99
9	21.81	23.88	25.94	27.99	29.90
10	21.73	23.75	25.96	28.01	29.98
11	21.51	23.55	25.67	27.78	29.96
12	21.65	23.70	25.81	27.97	30.11
13	21.07	23.13	25.23	27.33	29.23
14	21.17	23.17	25.30	27.49	29.42
15	21.14	23.15	25.23	27.35	29.28
16	21.06	23.06	25.15	27.26	29.35
17	21.14	23.17	25.19	27.29	29.41
18	21.09	23.10	25.14	27.29	29.29

Table 3 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 (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 from the calculated Ct value. 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 soybean gene *le1*)

DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**
1	0.9994	-3.427	21.69	21.72	0.03
2	0.9988	-3.435	21.76	21.74	0.03
3	0.9987	-3.402	21.74	21.79	0.05
4	0.9997	-3.506	21.58	21.69	0.11
5	0.9989	-3.437	21.79	21.83	0.04
6	0.9997	-3.438	21.83	21.80	0.03
7	0.9985	-3.332	21.86	21.81	0.05
8	0.9989	-3.429	21.80	21.82	0.02
9	0.9989	-3.340	21.90	21.81	0.09
10	0.9987	-3.446	21.74	21.73	0.01
11	0.9993	-3.547	21.40	21.51	0.11
12	0.9997	-3.552	21.55	21.65	0.10
13	0.9994	-3.391	21.12	21.07	0.05
14	0.9991	-3.476	21.11	21.17	0.06
15	0.9993	-3.404	21.13	21.14	0.01
16	0.9994	-3.485	20.96	21.06	0.10
17	0.9994	-3.456	21.06	21.14	0.07
18	0.9995	-3.444	21.02	21.09	0.07

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.

***The expected slope for a PCR with 100% efficiency is -3.322**

****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. The slope of the curve are between -3.1 and -3.6.

5. Conclusion

The results obtained confirm that the extraction method, applied to ground soybeans 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 soybean 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 EURL-GMFF operates according to ISO 9001:2008 (certificate number: CH-32232) and technical activities under ISO 17025:2005 [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative /quantitative PCR) - Accredited tests available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

7. References

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