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Report on the In-house Validation of a DNA Extraction Method from Soybean Seeds

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European Union Reference Laboratory for Genetically Modified Food and Feed

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Report on the In-house Validation of a DNA Extraction Method from Soybean Seeds

25 August 2023

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In accordance with relevant EU legislation [a], Perseus, on behalf of Beijing DaBeiNong Biotechnology Co., Ltd., provided to the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) a DNA extraction method for soybean and the relevant samples (ground soybean seeds).

In line with its mandate [b], the EURL GMFF conducted an in-house validation of the 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 EURL GMFF and the ENGL [c], and that the method satisfies the provisions of Annex III-3.C.2 to Regulation (EU) No 503/2013 [a].

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 503/2013 of 3 April 2013 "on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006".

[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/guidancedocs.htm

Quality assurance

The EURL GMFF is ISO 17025:2017 accredited [certificate number: Belac 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) in food and feed by DNA extraction, DNA identification and Real-time PCR and for determination of Genetically Modified content in % (cp/cp) in food and feed by DNA extraction and digital PCR)].

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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Content

1. Introduction

This report describes the validation of a large-scale genomic DNA extraction method derived from the publicly available "CTAB" method [1] followed by silica resin purification using commercially available columns (Wizard® DNA Clean-Up System, Promega) and size-exclusion spin columns with Sephacryl matrix (Mobi Spin S-300, MoBiTec). The report also describes the method's applicability on the samples of food and feed provided by the applicant. This protocol can be used for the extraction of DNA from ground soybean.

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

It is recommended that this method is carried out only by skilled laboratory personnel as the procedures comprise the use of hazardous chemicals and materials. It is 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 (Ultra Centrifugal Mill ZM 200)
2. Pipettes (Rainin)
3. Balances (Mettler Toledo XS2002S)
4. Centrifuges (Eppendorf 5810R and 5415D)
5. Vortex (MS1 Minishaker IKA)
6. Incubator (50°C and 65°C, with agitation; Eppendorf thermomixer comfort 5355)
7. Vacuum manifold and pump (Promega Vac-Man® Laboratory Vacuum Manifold)
8. Freezer -20°C and Fridge 4°C (any model appropriate)
9. Fume hood (any model appropriate)

2.2. Chemicals

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

1. CTAB (Sigma No. H6269)
2. Proteinase K (Sigma P2308)
3. RNase A (Sigma R6513)
4. Chloroform (Sigma C2432)
5. Isopropanol (Sigma I9516)
6. Ethanol (Fluka 02860)
7. EDTA solution (Sigma E7889)

8. Tris-EDTA Buffer Solution (Fluka 93283)
9. Nuclease free water (Promega P119C)
10. Wizard DNA Clean-UP System (Promega, Cat. no. A7280)
11. Mobi Spin S-300 Spin Columns (MoBiTec, SC0310)
12. Phenol:Chloroform:Isoamyl alcohol (25:24:1)(Roth, Cat. no. A156.2)
13. Isoamyl alcohol
14. Chloroform
15. Sodium acetate (Sigma 567422)
16. NaCl

2.3. Solutions

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

- **CTAB Lysis Buffer (2%)**
- 2 % w/v CTAB (H6269)
- 20 mM EDTA pH 8.0 (E7889)
- 1.4 M NaCl
- 100 mM Tris HCl pH 8.0
- Store at room temperature for up to 1 year

Commercial products are available, e.g. from PanReac AppliChem, Cat. no. A4150.

- **Tris-EDTA buffer (TE 0.1X)**
- 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}. Dilute 1mL of TE 1X with 9 mL of H_2O_{deion} to obtain TE 0.1X. Autoclave.
Store at room temperature for up to 2 years.
If a commercial product is used (e.g. TE 1X from Sigma, Cat. no. 93283), dilute 1mL of TE 1X with 9 mL of water (Ambion AM9937) to obtain TE 0.1X.

- **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.

- **RNase A (100 mg/mL)**
- Dissolve 500 mg RNase A in 5 mL of $H_2O_{deion.}$
- Aliquot and store at -20 °C for up to 2 years.

- **Chloroform:Isoamyl alcohol (24:1)**
- Isoamyl alcohol
- Chloroform

For 100 mL, mix 4 mL of isoamyl alcohol with 96 mL of chloroform.
Store at room temperature for up to 1 year.

- **Ethanol 70% (v/v)**

For 100 mL combine 70 mL 100% ethanol and fill up to 100 mL with H_2O_{deion}.
Store at room temperature for up to 1 year.

- **Isopropanol 80% (v/v)**

For 100 mL combine 80 mL 100% isopropanol and fill up to 100 mL with H_2O_{deion}.
Store at room temperature for up to 1 year.

- **3 M Sodium Acetate pH 5.2**

For 100 mL of 3 M sodium acetate, dissolve 24.6 g sodium acetate in 70 mL of H_2O_{deion}, adjust the pH to pH 5.2 with glacial acetic acid and fill to 100 mL with H_2O_{deion}. The solution must be autoclaved and can be stored at room temperature for up to 2 years.
Commercial product 3 M Sodium Acetate pH 5.2 is available, e.g. from Sigma 567422.

2.4. Plasticware

1. 50 mL conical tubes (Corning)
2. 15 mL conical tubes (Corning)
3. 1.5 mL microcentrifuge tubes (Eppendorf DNA LoBind® Tubes)
4. 2.0 mL microcentrifuge tubes (Eppendorf DNA LoBind® Tubes)
5. Filter tips for pipettes

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

2.5. Precautions

- The protocol is recommended for use by skilled personnel only because of the use of hazardous chemicals and materials
- Consideration of notice of products and operating instructions safety recommendations and guidelines is strongly recommended.
- Chloroform, isoamyl alcohol, and isopropanol are hazardous chemicals; therefore, all manipulations have to be performed according to safety guidelines, under a fume hood.
- Maintain strictly separate working areas for DNA extraction, PCR set-up and amplifications.
- All equipment and lab benches should be free of DNA residues.
- It is recommended to use clean containers with blenders grinding 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 large-scale high quality genomic DNA from soybean grains. Application of the method to other matrices may require adaptation and possible further validation.

3.2 Practicability

The primary downstream use of genomic DNA from seed material is for real-time PCR-based detection methods. Therefore, the preparation of DNA from seed material should take place in an area dedicated for this purpose. All equipment (e.g. pipettes, centrifuges), lab ware and reagents used in this process should be stored and used only in the dedicated area. The entire procedure takes about 6 hours of hands-on time.

3.3 Principle

The 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 of the ground soybean seeds in the presence of CTAB lysis buffer (containing Proteinase K), followed by isopropanol precipitation of DNA. During lysis, the cetyltrimethylammonium bromide (CTAB) binds polysaccharides, cell wall debris and denatured proteins. After lysis, precipitation and re-suspension, the DNA is further purified from lipids and proteins using chloroform-isoamyl alcohol, then precipitated again with isopropanol.

This crude precipitate is re-suspended and incubated in a digestion buffer with RNAse A. This enzymatic treatment digests any remaining RNA and strips the DNA from all bound proteins, facilitating their removal during the purification process; it is then followed by an additional purification step with chloroform-isoamyl alcohol and precipitation using sodium acetate and ethanol.

The final step removes remaining inhibitors by loading the resulting lysate on a silica-resin purification system (Wizard® DNA Clean-Up System, Promega) and Sephacryl size-exclusion spin columns (Mobi Spin S-300, MoBiTec).

3.4 Grinding

Grinding of soybean seeds not only facilitates the lysis by mechanically disrupting cellular structures and increasing the surface area, but is also indispensable for the generation of representative test portions by reducing the particle size.

Prior to extraction, the soybean seed samples should be grounded to a fine powder using either a cutting mill for 1 minute at 10,000 rpm (e.g. Ultra Centrifugal Mill ZM 200) or a mortar and pestle in liquid nitrogen. When using liquid nitrogen, the sample should be kept frozen until Lysis Buffer is added.

3.5 DNA extraction protocol

3.5.1 Lysis and isopropanol precipitation of DNA

1. In one 50 mL conical tube, mix the following: 2 g seed powder, 20 mL CTAB Lysis Buffer (pre-warmed to 65 °C) and 100 µL Proteinase K (20 mg/mL). Incubate for 1 hour at 65 °C with agitation.
2. Centrifuge at 12,000 x g for 10 minutes at 4 °C.
3. Transfer the supernatants into a new 50 mL conical tube.
4. Add equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and mix by inversion for 5 minutes.

Note: *Chloroform can dissolve many types of plastic, use appropriate containers and glass pipettes while working with chloroform.*

5. Centrifuge at 12,000 x g for 10 minutes at 4 °C.
6. Transfer the upper aqueous phase to a new 50 mL conical tube.
7. Repeat steps 4-6.
8. Add equal volume of isopropanol (pre-cooled to -20 °C). Mix gently by inversion.
9. Keep the tube at -20 °C for 1 hour to precipitate the DNA.
10. Centrifuge at 12,000 x g for 5 minutes at 4 °C to pellet DNA.
11. Discard supernatant and add 10 mL of 70 % ethanol (pre-cooled to 4 °C) to wash the DNA pellet.
12. Centrifuge at 12,000 x g for 5 minutes at 4 °C.
13. Repeat steps 11-12.
14. Remove all supernatant and air-dry the pellet at 37 °C until all ethanol is evaporated.
15. Add 4 mL of 1X TE and incubate at 65 °C with agitation until pellet is completely dissolved.

Note: *If there are problems dissolving the pellet, the pellet can be dissolved over night at 4 °C.*

3.5.2 Purification and ethanol / sodium acetate precipitation of DNA

16. Add 5 µL of RNase A (100 mg/mL), mix gently and incubate at 37 °C for 30 minutes.
17. Add 4 mL of chloroform:isoamyl alcohol (24:1). Mix by inversion for 5 minutes.
18. Centrifuge at 12,000 x g for 10 minutes at 4 °C.
19. Transfer the upper aqueous phase to a 15 mL conical tube.

20. Add 1/10 volume of 3 M sodium acetate (pre-cooled to 4 °C) and 2 volumes of absolute ethanol (pre-cooled to 4 °C). Mix gently by inversion. Place at -20 °C for at least 1 hour to precipitate DNA.
21. Centrifuge at 12,000 x g for 10 minutes at 4 °C to pellet the DNA.
22. Discard supernatant and add 10 mL of 70 % ethanol (pre-cooled to 4 °C) to wash the DNA pellet.
23. Centrifuge at 12,000 x g for 5 minutes at 4 °C.
24. Repeat steps 22-23.
25. Remove all supernatant and air-dry at 37 °C until all ethanol is evaporated.
26. Add 1 mL of 1X TE and incubate at 65 °C with agitation until pellet is completely dissolved.

Note: *If there are problems dissolving the pellet, the pellet can be dissolved over night at 4 °C.*

27. Centrifuge at 12,000 x g for 10 minutes at 4 °C.
28. Transfer the supernatant to a 1.5 mL microcentrifuge tube and discard any pellet.

3.5.3 Purification Using Wizard® DNA Clean-Up System, Promega
(Based on Manufacturer's Instructions)

29. Thoroughly mix the Wizard® DNA Clean-Up resin before removing an aliquot.

Note: *If crystals or aggregates are present, dissolve them by warming the resin to 37°C for 10 minutes. The resin itself is insoluble. Cool to 25–30 °C before use.*

30. Use one Wizard® Minicolumn for each sample. Attach the provided Syringe Barrel to the Luer-Lok® extension of each Minicolumn. Insert the tip of the Minicolumn/Syringe Barrel assembly into the vacuum manifold (Promega Vac-Man® Laboratory Vacuum Manifold).
31. Mix the resin before use. Add 1 mL of Wizard® DNA Clean-Up Resin to a 1.5 mL microcentrifuge tube. Add 100 µL of the DNA sample to the Clean-Up Resin and mix by inverting several times. Incubate for 5 minutes at room temperature.
32. Pipet the resin/DNA mix into the Syringe Barrel. Apply a vacuum to draw the solution through the Minicolumn. Break the vacuum to the Minicolumn.
33. To wash the column, add 2 mL of 80 % isopropanol to the Syringe Barrel, and re-apply a vacuum to draw the solution through the Minicolumn.
34. Dry the resin by continuing to draw a vacuum for 30 seconds after the solution has been pulled through the column. Do not dry the resin for more than 30 seconds. Remove the Syringe Barrel and transfer the Minicolumn to a 1.5 mL microcentrifuge tube. Centrifuge the Minicolumn at maximum speed (10,000 x g) in a microcentrifuge for 2 minutes to remove any residual isopropanol.
35. Transfer the Minicolumn to a new microcentrifuge tube. Apply 100 µL of pre-warmed (65 °C) 0.1X TE buffer to the Minicolumn and wait 1 minute. Centrifuge the Minicolumn for 1 min at maximum speed (10,000 x g) to elute the bound DNA. Remove and discard the Minicolumn.

3.5.4 Purification Using Mobi Spin S-300, MoBiTec, Promega
(Based on Manufacturer's Instructions)

36. Resuspend the resin in the column by vortexing.
37. Bend off the tip of the column and loose the cap one fourth turn.
38. Place the column in a 2 mL microcentrifuge tube.
39. Pre-spin the column 1 minute at 800 x g in a microcentrifuge with a fixed-angle rotor.
40. Use the column immediately after removing the equilibration buffer from the resin to avoid drying up.
41. Place the column in a new 1.5 mL tube and slowly apply the sample (100 µL) to the upper side of the slanted matrix surface, taking care of not to disturbing the bed.
42. Spin the column 2 minutes at 800 x g. The purified sample is collected in the bottom of the support tube.

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

The EURL GMFF tested the method proposed by the applicant and described above on soybean samples 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

Wild type soybean grains were received from the applicant, 100 grams were ground, and 36 g were used for DNA extraction.

4.2 DNA extraction

DNA was extracted from six test portions (replicates) following the method described in paragraph 3.5 "DNA extraction protocol". 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 (Invitrogen).

Each DNA extract was measured three times and the three 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 average DNA concentration and the yield are reported in Table 1 below.

[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)

Table 1. Average DNA concentration (ng/µL) and relative yield

Non-GM soybean seeds*	
Overall average (ng/µL)	75.43
Standard deviation of all samples	8.01
Coefficient of variation (%)	10.62
Yield (µg DNA/g sample intake)	3.77

* in-house validation based on eighteen extracted samples

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

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

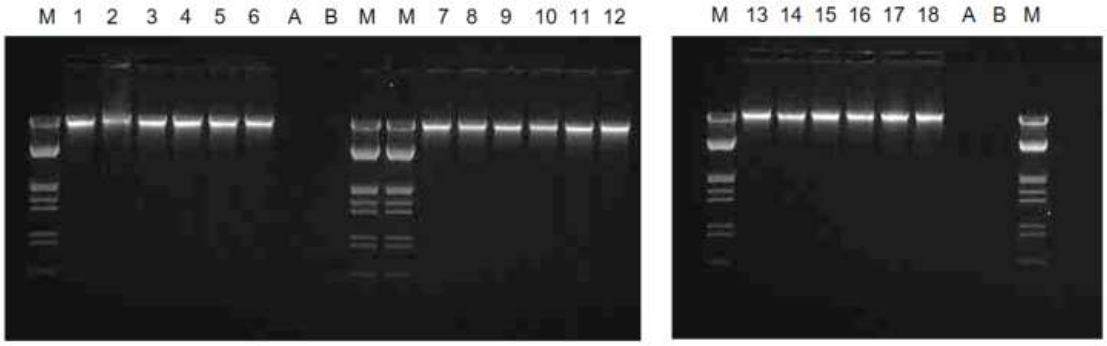
Sample	**Concentration (ng/µL)**	**Yield (µg/g)**
1	87.35	4.37
2	81.37	4.07
3	82.89	4.14
4	87.85	4.39
5	85.50	4.27
6	78.09	3.90
7	64.13	3.21
8	64.47	3.22
9	64.81	3.24
10	70.19	3.51
11	66.15	3.31
12	78.26	3.91
13	78.01	3.90
14	67.16	3.36
15	77.25	3.86
16	72.80	3.64
17	79.44	3.97
18	71.96	3.60

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 on a 1.0 % agarose gel electrophoresis, to verify that it is not excessively fragmented for subsequent analyses. One µL of the DNA solution was loaded per lane (Figure 1).

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



Lanes 1-6: samples extracted on day 1, lanes 7-12: samples extracted on day 2, lanes 13-18: samples extracted on day 3, A: Extraction negative control, B: Extraction environmental control, M: Lambda DNA/*Eco*RI+*Hind*III molecular weight markers.

The extracted genomic DNA samples appeared as distinct high molecular weight DNA bands on 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 25 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*. Five µL of the diluted extracts were used in each real-time PCR reaction. The Cq values obtained for "undiluted" and diluted DNA samples are reported in Table 3.

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

Cq values					
	Undiluted	Diluted extracts			
DNA extract	(25 ng / µL)	1:4	1:16	1:64	1:256
1	21.69	23.61	25.68	27.82	29.70
2	21.68	23.58	25.75	27.76	29.67
3	21.69	23.68	25.80	27.82	29.75
4	21.72	23.67	25.84	27.40	29.57
5	21.75	23.73	25.81	27.82	29.68
6	21.52	23.52	25.71	27.73	29.66
7	21.95	23.88	25.93	27.93	29.86
8	21.87	23.79	25.81	27.77	29.73
9	21.84	23.89	25.88	27.87	29.88
10	21.91	23.90	25.97	27.93	29.79
11	21.79	23.69	25.82	27.85	29.72
12	21.82	23.75	25.86	27.92	29.72
13	21.72	23.68	25.71	27.78	29.72
14	21.76	23.72	25.88	27.75	29.70
15	21.67	23.68	25.82	27.78	29.70
16	21.80	23.73	25.79	27.84	29.73
17	21.78	23.83	25.87	27.85	29.68
18	21.76	23.74	25.76	27.88	29.71

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 check for inhibition the Cq values of the four diluted samples were plotted against the logarithm of the dilution, while the Cq values for the "undiluted" samples (25 ng /µL) were extrapolated by linear regression.

Subsequently, the extrapolated Cq values for the "undiluted" samples were compared with the measured Cq data. It is assumed that PCR inhibitors are present if the measured Cq value for the "undiluted" sample is > 0.5 cycles from the calculated Cq value. Table 4 below reports the comparison of extrapolated Cq values versus measured Cq values for all samples and the values of linearity (R^2) and slope of all measurements.

Table 4. Comparison of extrapolated Cq values versus measured Cq values (amplification of soybean gene *lectin*).

DNA extraction	R^2	Slope	Cq extrapolated	mean Cq measured	ΔCq*
1	1.00	-3.39	21.60	21.69	0.10
2	1.00	-3.37	21.62	21.68	0.06
3	1.00	-3.36	21.71	21.69	0.01
4	1.00	-3.20	21.80	21.72	0.08
5	1.00	-3.30	21.79	21.75	0.05
6	1.00	-3.39	21.54	21.52	0.03
7	1.00	-3.31	21.92	21.95	0.03
8	1.00	-3.28	21.83	21.87	0.04
9	1.00	-3.32	21.89	21.84	0.05
10	1.00	-3.26	21.99	21.91	0.08
11	1.00	-3.34	21.74	21.79	0.05
12	1.00	-3.32	21.81	21.82	0.01
13	1.00	-3.35	21.68	21.72	0.05
14	1.00	-3.29	21.81	21.76	0.05
15	1.00	-3.33	21.74	21.67	0.07
16	1.00	-3.33	21.76	21.80	0.04
17	1.00	-3.24	21.92	21.78	0.14
18	1.00	-3.33	21.77	21.76	0.01

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 Cq = abs (Cq extrapolated - Cq measured)

According to the 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^2 [f] shall be ≥0.98.

The table indicates that all ΔCq values of extrapolated versus measured Cq are < 0.5. The R^2 coefficient of linear regression is > 0.99 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 DNA extraction method from ground soybean provided by the applicant produces DNA of suitable quantity and quality for subsequent PCR-based analyses.

When 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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