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JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Event-specific Method for the Quantification of Soybean Line 40-3-2 Using Real-time PCR

Validation Report and Protocol
Report on the Validation of a DNA Extraction Method for Soybean Seeds

Corrected version 1

European Union Reference Laboratory for
Genetically Modified Food and Feed

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Joint Research Centre

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The corrections made in the new version are:
In *Report on the Validation of a DNA Extraction Method for Soybean Seeds*

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"

Event-specific Method for the Quantification of Soybean Line 40-3-2 Using Real-time PCR

Validation Report

11 September 2007

Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit

Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the 40-3-2 transformation event in soybean DNA (unique identifier MON-∅4∅32-6). The collaborative trial was conducted according to internationally accepted guidelines (1, 2).

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and with Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto provided the detection method and the samples (soybean seeds containing the transformation event and conventional soybean seeds). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved fourteen laboratories from nine European countries.

The results of the international collaborative trial met the ENGL performance requirements and the scientific understanding about satisfactory method performance. Therefore, the CRL-GMFF considers the method validated as fit for the purpose of regulatory compliance.

The results of the collaborative study are made publicly available at http://gmo-crl.jrc.it/.

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Report on Steps 1-3 of the Validation Process

Monsanto submitted the detection method and control samples for soybean event 40-3-2 (unique identifier MON-∅4∅32-6) under Article 8 and 20 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Community Reference Laboratory for GM Food and Feed (CRL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the CRL-GMFF Validation Process", http://gmo-crl.jrc.it/guidancedocs.htm).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.it/guidancedocs.htm) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed and requests of complementary information addressed to the applicant. Upon reception of complementary information, the scientific evaluation of the detection method for event 40-3-2 was positively concluded in January 2006.

Between August 2006 and September 2006, the CRL-GMFF verified experimentally the method characteristics (step 3, experimental testing of samples and methods) by quantifying five blind GM-levels within the range 0.1%-8% on a copy number basis. The experiments were performed in repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision of the quantifications were within the limits established by the ENGL. The DNA extraction module of the method was tested on samples of food and feed.

A Technical Report summarising the results of tests carried out by the CRL-GMFF (step 3) is available on request.

Content

1. Introduction

Monsanto submitted the detection method and control samples for soybean event 40-3-2 (unique identifier MON-04032-6) under Article 8 and 20 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Directorate General-Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute for Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Regulation EC No 1829/2003) organised the international collaborative study for the event-specific method for the detection and quantification of 40-3-2 soybean. The study involved fourteen laboratories, all members of the European Network of GMO Laboratories (ENGL).

Upon reception of method, samples and related data (step 1), the JRC carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3) according to the requirements of Regulation (EC) No 641/2004 and following its operational procedures.

The internal in-house experimental evaluation of the method was carried out between August 2006 and September 2006.

Following the evaluation of the data and the results of the in-house laboratory tests, the international collaborative study was organised (step 4) and took place in October 2006.

A method for DNA extraction from soybean seeds, submitted by the applicant, was evaluated by the CRL-GMFF; laboratory testing of the method was carried out in August 2006 in order to confirm its performance characteristics. The protocol for DNA extraction and a report on method testing is available at http://gmo-crl.jrc.it/.

The operational procedure of the collaborative study included the following module:

- Quantitative real-time PCR (Polymerase Chain Reaction). The methodology is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event 40-3-2 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean *Lec* (*lectin*) endogenous assay (reference gene) and the target assay (40-3-2) are performed in separate wells.

The international collaborative study was carried out in accordance with the following internationally accepted guidelines:

- ISO 5725 (1994).
- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995).

2. List of participating laboratories

As part of the international collaborative study the method was tested in fourteen ENGL laboratories to determine its performance. Clear guidance was given to the laboratories with regards to the standard operational procedures to follow for the execution of the protocol. The participating laboratories are listed in alphabetical order in Table 1.

Table 1. Laboratories participating in the validation of the detection method for soybean line 40-3-2.

Laboratory	Country
BIOMI Ltd	Hungary
Bundesinstitut fuer Risikobewertung (BfR)	Germany
CRA-W, Dépt Qualité des productions agricoles	Belgium
Danish Institute for Food and Veterinary Research	Denmark
DEFRA - Central Science Laboratory (CSL)	UK
Ente Nazionale Sementi Elette (central office in Milano) / Laboratorio Analisi Sementi	Italy
INETI - LIA (Instituto Nacional de Engenharia Tecnologia e Inovação - Laboratório para a Insdústria Alimentar)	Portugal
Institute for Agricultural & Fisheries Research (ILVO)	Belgium
Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen Amtliche Lebensmittelüberwachung	Germany
LSGV Saarland (Landesamt für Soziales, Gesundheit und Verbraucherschutz)	Germany
LUFA Speyer	Germany
National Veterinary Institute	Norway
Scottish Agricultural Science Agency	UK
The Food and Consumer Product Safety Authority (VWA)	The Netherlands

3. Materials

For the validation of the quantitative event-specific method, genomic DNA was extracted from samples consisting of:

i) Seeds of soybean harbouring the 40-3-2 event (Line AG3701RR, Lot number GLP-0303-13709-S) and;

ii) Seeds of conventional soybean (Line A2553, lot number GLP-0212-13349-S)

Samples were provided by the applicant in accordance to the provisions of Regulation (EC) No 1829/2003, Art 2.11 [“control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)].

Samples containing mixtures of 100% 40-3-2 soybean and non-GM soybean genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the control samples provided, in a constant amount of total soybean DNA.

Participants received the following materials:

- ✓ Five calibration samples (80 µl of DNA solution each) for the preparation of the standard curve, labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (80 µl of DNA solution each), labelled from U1 to U20.
- ✓ Reaction reagents:

☐	Universal PCR Master Mix 2X, 3 vials:	5 ml each
☐	Sterile distilled water:	10 ml

- ✓ Primers and probes (1 tube each) as follows:

Lec reference system

☐	Lec primer forward	(10 µM):	195 µl
☐	Lec primer reverse	(10 µM):	195 µl
☐	Lec TaqMan® probe	(10 µM):	65 µl

40-3-2 system

☐	40-3-2 primer forward	(10 µM):	195 µl
☐	40-3-2 primer reverse	(10 µM):	195 µl
☐	40-3-2 TaqMan® probe	(10 µM):	130 µl

4. Experimental design

Twenty unknown samples (labelled from U1 to U20), representing five GM levels, were used in the validation study (Table 2). On each PCR plate, the samples were analyzed either for the 40-3-2 specific system or the *lec* specific system. In total, two plates were run per participating laboratory and four replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. Participating laboratories carried out the determination of the GM% according to the instructions provided in the protocol and using the electronic tool provided (Excel spreadsheet).

Table 2. 40-3-2 GM contents

40-3-2 GM % (GM copy number/soybean genome copy number *100)
0.1
0.4
0.9
4.0
8.0

5. Method

Description of operational steps followed

For the specific detection of event 40-3-2 DNA, an 84-bp fragment of the recombination region of parts of the construct inserted into the plant genome (5′ insert-to-plant junction) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM dye and a non fluorescent quencher MGBNFQ.

For the relative quantification of event 40-3-2 DNA, a soybean-specific reference system amplifies a 74-bp fragment of the soybean endogenous gene *lec* (*lectin,* accession number K00821), using a pair of *lec* gene-specific primers and a *lec* gene-specific probe labelled with FAM and TAMRA.

Standard curves are generated for both the 40-3-2 and the *lec* specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For relative quantification of event 40-3-2 DNA in a test sample, the 40-3-2 copy number is divided by the copy number of the soybean reference gene (*lec*) and multiplied by 100 to obtain the percentage value (GM% = 40-3-2 / *lec* * 100).

Calibration sample S1 was prepared by mixing the appropriate amount of 40-3-2 DNA in control non-GM soybean DNA to obtain a 10% GM 40-3-2 in a total of 200 ng soybean DNA. Samples S2 and S3 were prepared by 1:4 serial dilutions from the S1 sample and samples S4 and S5 were prepared by 1:3 serial dilutions from the S3 sample.

The absolute copy numbers in the calibration curve samples are determined by dividing the sample DNA weight (nanograms) by the published average 1C value for soybean genome (1.13) (3). The copy number values used in the quantification, the GM contents of the calibration samples and total DNA quantity used in PCR are provided in Table 3 (% GM calculated considering the 1C value for soybean genome as 1.13 pg) (3).

Table 3. % GM values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng/4 µl)	200	50	12.5	4.2	1.4
Soybean genome copies	176991	44248	11062	3687	1229
40-3-2 soybean copies	17699	4425	1106	369	123

6. Deviations reported

Nine laboratories reported no deviations from the protocol.

One laboratory performed two PCR runs for the 40-3-2 specific system because of a high variation between replicates in the first run. In the second run, the first replicate of sample U20 was eliminated. Concerning the *lec* system, the second replicate of S1 was eliminated.

One laboratory used the automatic Ct determination of the analysis software on ABI 7900HT.

One laboratory entered a reaction volume of 30 µl instead of 50 µl because the SDS 2.2.1 software did not allow the 50 µl volume set up.

One laboratory set up the PCR plates the day before, stored them refrigerated over night and run the experiment the day after.

7. Summary of results

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula ((10^(-1/slope))-1)*100] of the reference curve and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for the 40-3-2 system and the *lec* reference system are summarised in Table 4.

Table 4. Values of reference curve slope, PCR efficiency and linearity (R^2)

	40-3-2			***lec***		
LAB	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**
1	-3.30	99	0.98	-3.47	94	0.99
2	-3.17	93	1.00	-3.30	99	0.99
3	-3.26	97	0.99	-3.25	97	0.99
4	-3.54	92	0.99	-3.53	92	1.00
5	-3.54	92	0.99	-3.43	96	0.99
6	-2.83	75	0.96	-3.08	89	0.99
7	-3.07	88	0.98	-3.22	96	0.98
8	-3.34	99	0.99	-3.25	97	0.99
9	-3.33	100	0.99	-3.25	97	0.99
10	-3.47	94	0.99	-3.45	95	0.99
11	-3.14	92	0.96	-3.29	98	0.94
12	-3.56	91	0.99	-3.15	92	0.99
13	-3.67	87	0.99	-3.27	98	0.99
14	-3.02	86	0.93	-3.06	88	0.97
Mean	**-3.30**	**92**	**0.98**	**-3.28**	**95**	**0.98**

The mean PCR efficiency was 95% for the *lec* reference system and 92% for the 40-3-2 system. The linearity of the method was on average 0.98 for both systems. Data reported confirm the appropriate performance characteristics of the method tested in terms of efficiency and linearity.

GMO quantification

Table 5 shows the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

Table 5. GM% mean values determined by laboratories for unknown samples.

	Sample GMO content (GM% = GM copy number/soybean genome copy number *100)																			
Lab	**0.1**				**0.4**				**0.9**				**4.0**				**8.0**			
	Rep 1	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**
1	0.06	0.05	0.03	0.07	0.31	0.27	0.31	0.31	0.68	1.05	0.43	0.80	3.45	1.65	4.48	5.21	6.97	15.07	6.52	9.57
2	0.05	0.11	0.15	0.12	0.36	0.20	0.43	0.44	1.29	0.69	1.28	1.02	4.58	4.13	4.04	5.30	9.38	14.68	8.51	9.15
3	0.10	0.07	0.07	0.08	0.26	0.24	0.33	0.27	0.72	0.94	0.53	0.62	3.50	3.65	3.18	3.40	5.79	7.96	6.27	10.02
4	0.18	0.14	0.20	0.11	0.80	0.56	0.41	0.67	2.10	1.71	1.10	2.19	7.51	3.98	2.49	4.32	14.55	9.05	8.59	12.96
5	0.11	0.09	0.07	0.12	0.35	0.31	0.45	0.23	1.27	0.95	0.79	1.25	4.14	3.61	6.18	4.53	7.67	12.19	7.60	9.04
6	0.07	0.07	0.06	0.04	0.29	0.27	0.27	0.33	1.05	0.93	0.72	0.91	5.82	5.75	5.22	4.76	11.33	13.33	11.95	11.67
7	0.06	0.09	0.07	0.05	0.26	0.30	0.31	0.32	0.59	0.64	0.81	0.51	3.05	3.30	3.19	4.04	7.22	7.58	7.17	8.69
8	0.08	0.17	0.09	0.08	0.48	0.33	0.38	0.45	0.97	1.03	0.89	0.87	4.66	3.77	3.58	3.72	9.56	7.77	6.71	8.56
9	0.10	0.15	0.10	0.10	0.41	0.46	0.33	0.43	1.13	0.81	0.80	0.86	5.40	4.45	3.86	3.64	8.00	9.17	11.09	9.57
10	0.12	0.13	0.10	0.10	0.35	0.47	0.39	0.43	0.93	1.38	0.79	1.04	5.93	4.35	5.57	4.59	12.72	8.28	7.09	11.66
11	0.02	0.24	0.01	0.01	0.38	0.08	0.24	0.55	0.14	15.13	0.29	0.57	1.85	1.63	4.24	0.66	11.59	15.58	4.18	9.42
12	0.06	0.15	0.10	0.09	0.21	0.52	0.17	0.45	0.59	0.33	0.78	0.48	1.61	2.29	3.39	4.88	3.91	5.39	5.22	5.37
13	0.14	0.29	0.18	0.12	0.48	0.52	0.51	0.61	1.17	0.81	0.97	0.88	5.26	3.61	2.59	4.15	8.55	9.35	8.01	9.42
14	0.08	0.08	0.07	0.08	0.24	0.37	0.42	0.41	0.82	1.01	0.71	0.86	5.85	3.15	3.21	4.36	13.73	6.56	3.27	10.40

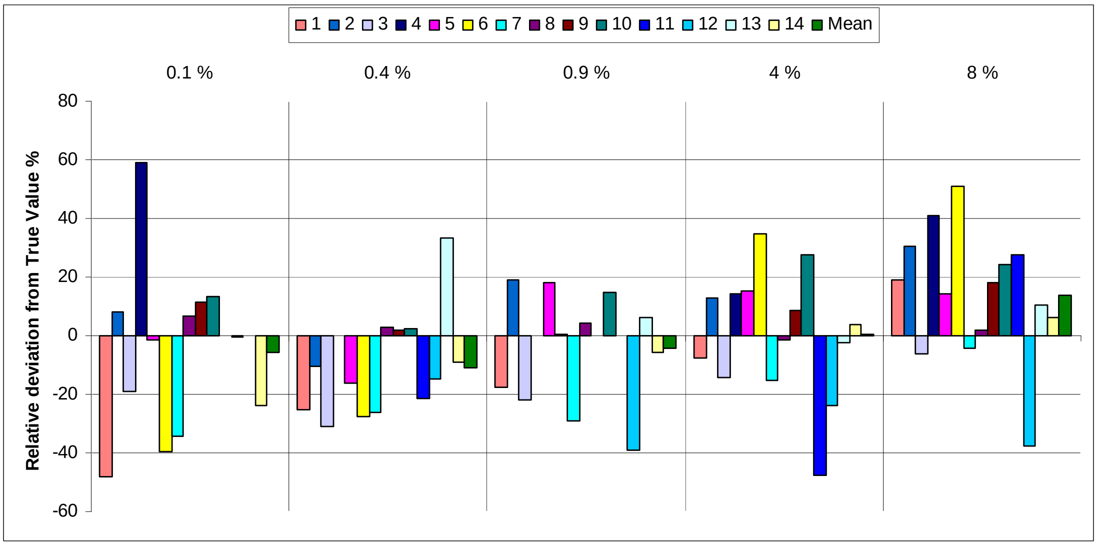
In Figure 1 the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the relative GM quantification obtained by the participating laboratories as well as the mean value (represented by the green bar).

As observed in Figure 1, relative deviations from the true values are mainly negative for GM levels of 0.1% and 0.4%, meaning that the GM content of unknown samples tends to be underestimated at these GM levels.

While at GM levels 0.9% and 4% the bias generated by all laboratories is randomly distributed, eleven out of fourteen laboratories overestimated the true value at 8%.

Overall, the average relative deviation was very limited at all GM levels tested, indicating a satisfactory accuracy of the method.

Figure 1. Relative deviation (%) from the true value of 40-3-2 for all laboratories



8. Method performance requirements

Among the performance criteria established by ENGL and adopted by the CRL-GMFF (http://gmo-crl.jrc.it/guidancedocs.htm, see also Annex 1), repeatability and reproducibility are assessed through an international collaborative trial, carried out with the support of ENGL laboratories (see Table 1). Table 6 illustrates the estimation of repeatability and reproducibility at various GM levels, according to the range of GM percentages tested during the collaborative trial.

The *relative reproducibility standard deviation* (RSD_R), that describes the inter-laboratory variation, should be below 33% at the target concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 6, the method fully satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R (%) is 40% at the 0.1% level and 32% at 4% and 8%, thus within the acceptance criterion.

Table 6. 40-3-2: summary of validation results.

Unknown sample GM%	Expected value (GMO %)				
	0.1	**0.4**	**0.9**	**4**	**8**
Laboratories having returned results	14	14	14	14	14
Samples per laboratory	4	4	4	4	4
Number of outliers	2	1	2	0	0
Reason for exclusion	2 C. test	1 DG test	1 C. test 1 G. test	-	-
Mean value	0.09	0.36	0.86	4.01	9.12
Relative repeatability standard deviation, RSD_r (%)	29	26	22	28	29
Repeatability standard deviation	0.03	0.09	0.19	1.12	2.62
Relative reproducibility standard deviation, RSD_R (%)	40	30	28	32	32
Reproducibility standard deviation	0.04	0.11	0.24	1.30	2.88
Bias (absolute value)	-0.01	-0.04	-0.04	0.01	1.12
Bias (%)	-6	-11	-4	0	14

C = Cochran's test; G= Grubbs' test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

Bias is estimated according to ISO 5725 data analysis protocol.

Table 6 further documents the *relative repeatability standard deviation (RSD_r)*, as estimated for each GM level. In order to accept methods for collaborative study evaluation, the CRL requires that RSD_r values be below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.it/guidancedocs.htm).

As it can be observed from the values reported in Table 6, the method demonstrates a relative repeatability standard deviation slightly higher than 25% over the dynamic range with a maximum of 29% at, except for GM level of 0.9% for which the RSD_r is 22%.

The *trueness* of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. In this case the method fully satisfies this requirement across the entire dynamic range tested; in fact, the highest value of bias (%) is 14% at the 8% level, well within the acceptance criterion.

9. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (as detailed under http://gmo-crl.jrc.it/guidancedocs.htm). The method acceptance criteria were reported by the applicant and used to evaluate the method prior to the international collaborative study (see Annex 1 for a summary of method acceptance criteria and method performance requirements).

The results obtained during the collaborative study indicate that the analytical module of the method submitted by the applicant complies with ENGL performance criteria with a minor exception of the RSD_r that is slightly higher than the acceptance criterion. The divergence is minor and since the reproducibility is within the range of acceptance, the method is considered applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. 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)]

11. References

1. Horwitz, W. (1995) Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67, 331-343.
2. International Standard (ISO) 5725. 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Swizerland.
3. Arumuganathan K, Earle ED. 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: The description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: The practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: The closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: The accuracy should be within ± 25% of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: The rate of amplification that leads to a theoretical slope of –3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $[10^{(-1/\text{slope})}] - 1$

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of ($-3.1 \geq \text{slope} \geq -3.6$)

R^2 Coefficient

Definition: The R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R^2 should be ≥ 0.98.

Repeatability Standard Deviation (RSD_r)

Definition: The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: The relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

Limit of Quantitation (LOQ)

Definition: The limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than 1/10th of the value of the target concentration with an $RSD_r \leq 25\%$. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

Acceptance Criterion: LOD should be less than 1/20th of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq$ 5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance Criterion: The response of an assay with respect to these small variations should not deviate more than ± 30%. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements

Dynamic Range

Definition: In the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD_R)

Definition: The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An RSD_R < 50 % is acceptable for concentrations below 0.2%.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within ± 25% of the accepted reference value over the whole dynamic range.

Event-specific Method for the Quantification of Soybean Line 40-3-2 Using Real-time PCR

Protocol

20 January 2009

Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit

Method development:

Monsanto Company

Method validated through collaborative trial by:

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

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Content

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of soybean event 40-3-2 DNA to total soybean DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in this method.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of soybean event 40-3-2 DNA, an 84-bp fragment of the recombination region between the insert and the plant genome (located at the 5' plant DNA region) is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM as a reporter at its 5′ end and with the non-fluorescent quencher MGBNFQ (minor groove binding non-fluorescent quencher [Applied Biosystems]) at its 3' end.

For the relative quantification of soybean event 40-3-2 DNA, a soybean-specific reference system amplifies a 74-bp fragment of the soybean endogenous *lectin* gene (*lec*), using a pair of specific primers and a *lec* gene-specific probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event Roundup Ready 40-3-2 DNA in a test sample, Roundup Ready 40-3-2 and *lec* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of soybean event 40-3-2 DNA to total soybean DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from soybean seeds and grains containing mixtures of genetically modified and conventional soybean.

The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GMO contents.

2.2 Collaborative trial

The method was validated in a collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with fourteen participating laboratories in October 2006.

Each participant received twenty blind samples containing Roundup Ready 40-3-2 genomic DNA at five GM contents, ranging from 0.10 % to 8.0 %.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM Roundup Ready 40-3-2 in four unknown samples. Four replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at http://gmo-crl.jrc.it/statusofdoss.htm

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.045% in 200 ng of total soybean DNA. The relative LOD was not assessed in a collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.09% in 200 ng of total soybean DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.10 %.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to soybean event 40-3-2 and thus imparts event-specificity to the method.

The specificity of event-specific assay was experimentally tested in real-time PCRs against DNA extracted from plant materials containing the specific targets of Roundup Ready® soybean 40-3-2, Roundup Ready® Canola (RT200), Roundup Ready® Canola (RT73), Conventional Canola, Roundup Ready® Corn (GA21), Roundup Ready® Corn (NK603), YieldGard® Corn Borer Corn (MON810), YieldGard® Rootworm/Roundup Ready® Corn (MON88017) , YieldGard® Rootworm corn (MON863), Lysine Maize (LY038), Conventional Corn, Roundup Ready® Cotton (MON1445), Bollgard® Cotton(MON531), Bollgard® Cotton (MON757), BollgardII® Cotton (MON15985), Conventional Cotton, Conventional Soybean, Roundup Ready® Wheat (MON71800), Conventional Wheat, Lentil, Quinoa, Sunflower, Buckwheat, Pinenuts, Rye berries, Millet, Barley, Basmati rice, Peanut.
None of the GM-lines tested, except the positive control soybean line 40-3-2, yielded detectable amplicons.

The specificity of the soybean reference assay *lec* was experimentally tested by the applicant against DNA extracted from plant materials containing Roundup Ready® soybean 40-3-2, Roundup Ready® Canola (RT200), Roundup Ready® Canola (RT73), Conventional Canola, Roundup Ready® Corn (GA21), Roundup Ready® Corn (NK603), YieldGard® Corn Borer Corn (MON810), YieldGard® Rootworm/Roundup Ready® Corn (MON88017) , YieldGard® Rootworm corn (MON863), Lysine Maize (LY038), Conventional Corn, Roundup Ready® Cotton (MON1445), Bollgard® Cotton (MON531), Bollgard® Cotton (MON757), BollgardII® Cotton (MON15985), Conventional Cotton, Conventional Soybean, Roundup Ready® Wheat (MON71800), Conventional Wheat, Lentil, Quinoa, Sunflower, Buckwheat, Pinenuts, Rye berries, Millet, Barley, Basmati rice, Peanut. None of the samples tested, except the positive control soybean line 40-3-2 and from conventional soybean, yielded detectable amplicons.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow the guidelines given by relevant authorities, e.g. ISO, CEN, Codex Alimentarius Commission.
- PCR-reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment used should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - should be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of soybean event 40-3-2

3.2.1 General

The PCR set-up for the taxon specific target sequence (*Lec*) and for the GMO (event 40-3-2) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended.
The method is developed for a total volume of 50 µl per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% 40-3-2 in non-GM soybean DNA for a total of 200 ng of DNA (corresponding to 176991 soybean genome copies with one genome assumed to correspond to 1.13 pg of haploid soybean genomic DNA) (1).

A calibration curve is produced by plotting the Ct-values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

The copy number measured for the unknown sample DNA is obtained by interpolation from the standard curves.

The ratio of transgene copy number and reference gene copy number multiplied by 100 gives the % GM contents of the samples.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. **Keep thawed reagents at 1-4°C on ice**.
2. In two reaction tubes (one for the 40-3-2 system and one for the *lec* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the 40-3-2 specific system.

Component	Final concentration	µl / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
40-3-2 AF (10 µM)	150 nM	0.75
40-3-2 AR (10 µM)	150 nM	0.75
40-3-2 AP (5 µM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the soybean *Lec* reference system.

Component	Final concentration	µl / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
lec F (10 µM)	150 nM	0.75
lec R (10 µM)	150 nM	0.75
lec P (10 µM)	50 nM	0.25
Nuclease free water	#	19.25
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the Roundup Ready 40-3-2 and one for the *Lec* master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of master mix (e.g. 46 x 3 = 138 µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 µl DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
6. Spin down the tubes in a microcentrifuge. Aliquot 50 µl in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 3 and Table 4:

Table 3. Cycling program for 40-3-2 system

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG		50 °C	120	No	1
2	Initial denaturation		95 °C	600	No	1
3	Amplification	Denaturation	95 °C	15	No	45
		Annealing & Extension	55 °C	60	Yes	

Table 4. Cycling program for soybean *Lec* system

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG		50 °C	120	No	1
2	Initial denaturation		95 °C	600	No	1
3	Amplification	Denaturation	95 °C	15	No	45
		Annealing & Extension	60 °C	60	Yes	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

a) Set the threshold: display the amplification curves of one system (e.g. Roundup Ready 40-3-2) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the “update” button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.

b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *Lec* system).

e) Save the settings and export all the data to a text file for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct-values for each reaction.

The standard curves are generated both for the *lec* and the 40-3-2 specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA.

For the determination of the amount of event 40-3-2 DNA in the unknown sample, the 40-3-2 copy number is divided by the copy number of the soybean reference gene (*lec*) and multiplied by 100 to obtain the percentage value (GM% = 40-3-2/*lec* * 100).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
40-3-2 target sequence	
40-3-2 AF	5' – TTC ATT CAA AAT AAG ATC ATA CAT ACA GGT T – 3'
40-3-2 AR	5' – GGC ATT TGT AGG AGC CAC CTT – 3'
40-3-2 AP (Probe)	6-FAM 5' – CCT TTT CCA TTT GGG – 3' MGBNFQ
Reference gene Lec target sequence	
lec F	5' – CCA GCT TCG CCG CTT CCT TC – 3'
lec R	5' – GAA GGC AAG CCC ATC TGC AAG CC – 3'
lec P (Probe)	6-FAM 5' – CTT CAC CTT CTA TGC CCC TGA CAC – 3' TAMRA

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

EURL
European Union Reference Laboratory
for GM Food & Feed

Report on the Validation of a DNA Extraction Method for Soybean Seeds

11 September 2007
Corrected version 1 – 25 July 2017

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

Method development and single laboratory validation:

Monsanto Company

Method testing and confirmation:

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 plant DNA extraction protocol derived from the publicly available "CTAB" method (1) 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 soybean seeds and grains.

The purpose of the DNA extraction method described is to provide DNA with purity suitable for real-time PCR based detection methods. The method should have a high yield and should be tailored for routine analysis in terms of ease of operations, sample throughput and costs.

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/Plasticware)

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. Plasticware

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 plasticware 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

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.

Scope and applicability:

The "Recommended Procedure for DNA Extraction from Plant Tissues" method for DNA extraction described below is suitable for the isolation of genomic DNA from a wide variety of soybean tissues and derived matrices. However, validation data presented here are restricted to ground soybean seeds. Application of the method to other matrices may require adaptation and possible further specific validation.

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).

Extraction of genomic DNA from soybean seed

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-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. Incubate for 60 minutes at 55 °C. Cool briefly on bench (10 minutes)
4. Add 20 mL of phenol:choloroform:isoamyl alcohol (PCI, 25:24:1). Cap the tube and mix vigorously by vortex or inversion.
5. Centrifuge for 10 minutes at 13000 x g and 20-25 °C to separate the aqueous and organic phases. Transfer upper aqueous phase to a clean 50 mL conical tube.
6. Repeat extraction two times for a total of three extractions (step 4-5).
7. Transfer upper aqueous phase to a new tube and add approximately 2/3 volume of -20 °C isopropanol and gently invert the tube several times to mix.

8. 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.
9. To pellet the DNA centrifuge the tubes at approximately 13000 x g for 20 minutes at 4 °C.
10. Re-dissolve the pellet in 4 mL of TE pH 8.0. Transfer to a 13-mL Sarstedt tube and add approximately 40 µL of 10 mg/mL RNase, then incubate at 37 °C for 30 minutes.
11. To extract the DNA add 4 mL of chloroform:isoamyl alcohol (CIA, 24:1). Centrifuge for 10 minutes at approximately 13000 x g at room temperature. Transfer the upper aqueous phase to a clean Sarstedt tube.
12. Repeat step 11 twice, then add half volume of 7.5 M ammonium acetate, gently mix by inversion/pipetting and add 2 volumes of 100% ethanol. Mix by inversion/pipetting and place at -20 °C for 30 minutes. DNA may be stored as ethanol precipitate at -20 °C for up to 1 year.
13. Centrifuge at 13000 x g for 20 minutes at 4 °C to pellet the DNA.
14. Rinse the DNA pellet twice with 70 % ethanol and remove residual ethanol by vacuum.
15. Re-suspend DNA in 1 mL TE, pH 8.0 and incubate at 65 °C for 1 hour with periodic gentle mixing.
16. 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.
17. Add equal volume of 20% PEG precipitation buffer to the extracted DNA solution. Mix well by pipetting or inversion.
18. Incubate the PEG/DNA mixture for 15 minutes at 37 °C.
19. Centrifuge the PEG/DNA mixture for 15 minutes at approximately 15000 x g at room temperature.
20. Pour off supernatant or remove by pipetting. Wash the walls of the tube and DNA pellet with 80% ethanol (1.25 times volume of the original PEG/DNA mixture). Pour off ethanol or remove by pipetting.
21. Repeat wash once for a total of two washes (step 20).
22. Completely dry any residual ethanol by vacufuge at low heat (4-6 minutes).
23. Re-suspend the pellet in TE or H_2O using approximately equal volume as original DNA solution.
24. Centrifuge the re-suspended DNA solution at 15000 x g for 15 minutes.
25. Transfer DNA solution to a clean tube without disturbing the pellet.

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 "Recommended Procedure for DNA Extraction from Plant Tissues" method proposed by the applicant on samples of food and feed consisting of ground soybean 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 soybean seed material were ground using a GRINDOMIX GM 200 (Retsch GmbH) mixer.

4.2 DNA extraction

DNA was extracted following the "Recommended Procedure for DNA Extraction from Plant Tissues" method described above; 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	111.3
2	216.5
3	134.5
4	198.0
5	203.8
6	222.1
1	196.0
2	219.9
3	198.2
4	172.5
5	181.0
6	215.4
1	99.8
2	111.0
3	101.5
4	141.8
5	187.8
6	127.7

- ✓ DNA concentration (ng/µL)

Overall average of all samples:	169 ng/µL
Standard deviation of all samples	44.5 ng/µL
Coefficient of variation	26.3 %

- ✓ Yield (total volume of DNA solution: 1000 µL)

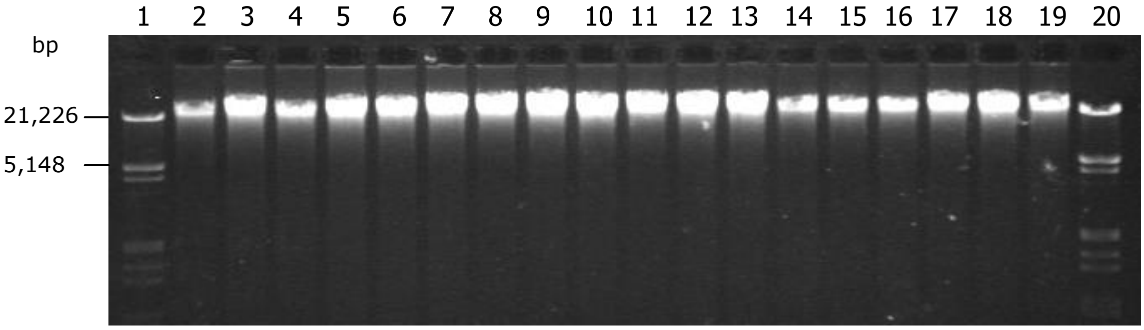
Overall average of all samples:	169 µg
Standard deviation	44.5 µg
Coefficient of variation	26.3 %

4.4 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 8 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 soybean 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.



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 endogenous control gene lectin, lec. 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 lectin gene, lec. 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	21.51	24.12	25.69	27.60	29.93
2	21.66	23.48	25.53	27.87	29.87
3	21.39	23.79	25.68	27.71	29.73
4	21.63	23.45	25.49	28.02	29.61
5	22.10	23.57	25.42	28.09	29.97
6	21.87	23.88	25.55	27.64	30.02
1	21.93	23.71	25.48	27.35	29.49
2	21.50	23.36	25.33	27.39	29.55
3	21.46	23.26	25.33	27.43	29.47
4	21.48	23.30	25.09	27.37	29.49
5	21.41	23.29	25.25	27.30	29.45
6	21.49	23.43	25.37	27.43	29.56
1	21.32	23.44	25.28	27.21	29.55
2	21.32	23.29	25.19	27.18	29.32
3	21.30	23.34	25.12	27.17	29.24
4	21.37	22.99	25.11	27.71	29.18
5	21.36	22.91	25.16	27.32	29.46
6	21.35	23.20	25.09	27.36	29.45

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 suppressed by > 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 lectin gene, *lec*)

DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**
1	0.991	-3.2	22.00	21.51	0.49
2	0.998	-3.6	21.31	21.66	0.35
3	0.998	-3.3	21.77	21.39	0.38
4	0.992	-3.5	21.39	21.63	0.25
5	0.994	-3.6	21.29	22.10	0.81
6	0.993	-3.4	21.64	21.87	0.23
1	0.997	-3.2	21.70	21.93	0.23
2	0.999	-3.4	21.25	21.50	0.25
3	0.999	-3.4	21.19	21.46	0.27
4	0.997	-3.5	21.10	21.48	0.38
5	0.999	-3.4	21.19	21.41	0.21
6	0.998	-3.4	21.34	21.49	0.15
1	0.995	-3.4	21.31	21.32	0.01
2	0.998	-3.3	21.22	21.32	0.10
3	0.997	-3.3	21.28	21.30	0.02
4	0.986	-3.5	20.95	21.37	0.42
5	0.997	-3.6	20.76	21.36	0.61
6	0.993	-3.5	21.02	21.35	0.34

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, with two exceptions: the sample number 5 extracted on day 1, with a value of 0.81 and sample number 5 extracted on day 3 with a value of 0.61.

R^2 of linear regression is > 0.99 for all DNA samples except one (0.986). The slope of the curve are all between -3.1 and -3.6, with no exceptions.

5 Conclusion

The data reported confirm that the extraction method, applied to soybean 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 soybean 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

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