EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Community Reference Laboratory
CRL
GM Food and Feed

Event-specific Method for the Quantification of Soybean Line A2704-12 Using Real-time PCR

Validation Report and Protocol
Soybean Seeds Sampling and DNA Extraction

M. Mazzara, C. Delobel, E. Grazioli, S. Larcher, C. Savini and G. Van den Eede

14 May 2007

Method development:
Bayer CropScience GmbH

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

Institute for Health and Consumer Protection
2007

EUR 22910 EN

The mission of the Institute for Health and Consumer Protection is to provide scientific support to the development and implementation of EU policies related to health and consumer protection.

The Institute for Health and Consumer Protection carries out research to improve the understanding of potential health risks posed by chemical, physical and biological agents from various sources to which consumers are exposed.

European Commission
Directorate-General Joint Research Centre
Institute for Health and Consumer Protection

Contact information
Address: Biotechnology and GMOs Unit
E-mail: JRC-BGMO@ec.europa.eu
Tel.: +39 0332 786706
Fax: +39 0332 786159

http://ihcp.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

EUR 22910 EN
ISBN 978-92-79-06928-4
ISSN 1018-5593
Luxembourg: Office for Official Publications of the European Communities

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Community Reference Laboratory
CRL
GM Food and Feed

Event-specific Method for the Quantification of Soybean Line A2704-12 Using Real-time PCR

Validation Report

14 May 2007

Directorate General-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 A2704-12 transformation event in soybean DNA (unique identifier ACS-GMØØ5-3). The collaborative trial was conducted according to internationally accepted guidelines [1, 2].

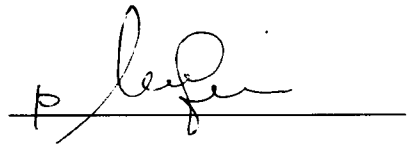
In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Bayer CropScience provided the detection method and the samples (genomic DNA extracted from wild-type and 100% soybean A2704-12 event). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The international collaborative trial involved thirteen laboratories from ten 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.

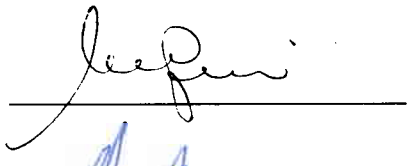
The results of the collaborative study are made publicly available at http://gmo-crl.jrc.it/.

Drafted by:
C. Delobel

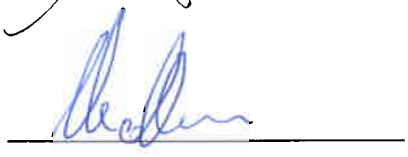
Report Verification Team:
1) W. Moens



2) M. Querci



Scientific and technical approval:
M. Mazzara



Compliance with CRL Quality System:
S. Cordeil

Authorisation to publish:
G. Van den Eede

Address of contact laboratory:

European Commission, Directorate General-Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 1, 21020 Ispra (VA) - Italy

Report on Steps 1-3 of the Validation Process

Bayer CropScience submitted the detection method and control samples for soybean event A2704-12 (unique identifier ACS-GMØØ5-3) under Article 5 and 17 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 to 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 evaluated 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 and step 3 (scientific assessment of documentation and data and experimental testing of the samples and methods, respectively) four 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 A2704-12 was positively concluded in May 2006.

In May 2006, the CRL-GMFF verified experimentally the method characteristics (step 3, experimental testing of the samples and methods) by quantifying five blind GM levels within the range 0.1%-3.3% 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 5

2. LIST OF PARTICIPATING LABORATORIES 6

3. MATERIALS 7

4. EXPERIMENTAL DESIGN 8

5. METHOD 8

DESCRIPTION OF OPERATIONAL STEPS FOLLOWED 8

6. DEVIATIONS REPORTED 9

7. SUMMARY OF RESULTS 9

PCR EFFICIENCY AND LINEARITY 9
GMO QUANTIFICATION 10

8. METHOD PERFORMANCE REQUIREMENTS 12

9. CONCLUSIONS 13

10. QUALITY ASSURANCE 13

11. REFERENCES 13

12. ANNEX 1: METHOD ACCEPTANCE CRITERIA AND METHOD PERFORMANCE REQUIREMENTS AS SET BY THE EUROPEAN NETWORK OF GMO LABORATORIES (ENGL) 14

1. Introduction

Bayer CropScience submitted the detection method and control samples for soybean event A2704-12 (unique identifier ACS-GMØØ5-3) under Article 5 and 17 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 A2704-12. The study involved thirteen 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 in May 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 June-July 2006.

A method for DNA extraction from A2704-12 seeds, submitted by the applicant, was evaluated by the CRL-GMFF; laboratory testing of the method was carried out between April 2006 and February 2007 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 A2704-12 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean lectin (*Le1*) endogenous assay (reference gene) and the target assay (A2704-12) 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 thirteen 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 A2704-12.

Laboratory	Country
Agricultural Biotechnology Center	Hungary
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (Bavarian Health and Food Safety Authority)	Germany
Centro Nacional de Alimentación - Agencia Española de Seguridad Alimentaria	Spain
CRA-W, Dépt Qualité des productions agricoles	Belgium
Ente Nazionale Sementi Elette (central office in Milano) / Laboratorio Analisi Sementi	Italy
Finnish Customs Laboratory	Finland
General Chemical State Laboratory, Food Division	Greece
Istituto Superiore di Sanità, ISS, National Center for Food Quality and Safety - GMO and Mycotoxins Unit	Italy
Laboratoire national de Santé	Luxembourg
Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen Amtliche Lebensmittelüberwachung	Germany
LSGV Saarland (Landesamt für Soziales, Gesundheit und Verbraucherschutz)	Germany
National Veterinary Institute	Norway
The Food and Consumer Product Safety Authority (VWA)	Netherlands

3. Materials

For the validation of the quantitative event-specific method, control samples consisting of:

i) a DNA stock solution homozygous for the GM-event A2704-12 (Lot Number 32RRMM0020)

and

ii) non-GM DNA stock solution (Lot Number 32RRMM0029) extracted from a genetically similar wild-type line

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% A2704-12 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 (200 µl of DNA solution each) for the preparation of the standard curve, labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (100 µl of DNA solution each), labelled from U1 to U20.
- ✓ Amplification reagent control for use on each PCR plate.
- ✓ Reaction reagents:

  - ❑ Universal PCR Master Mix 2X, 2 vials: 5 ml each
  - ❑ Sterile distilled water: 4 ml

- ✓ Primers and probes (1 tube each) for the *Le1* reference gene and for the A2704-12 specific systems as follows:

  Le1 system
  - ❑ KVM164 primer (10 µM): 160 µl
  - ❑ KVM165 primer (10 µM): 160 µl
  - ❑ TM021 TaqMan® probe (10 µM): 160 µl

  A2704-12 soybean system
  - ❑ KVM175 primer (10 µM): 320 µl
  - ❑ SMO001 primer (10 µM): 320 µl
  - ❑ TM031 TaqMan® probe (10 µM): 160 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, samples were analysed in parallel with both the A2704-12 and *Le1* specific systems. In total, two plates were run per participating laboratory, with two replicates for each GM level analysed on each run. In total, 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. A2704-12 GM contents

A2704-12 GM % (GM copy number/soybean genome copy number *100)
0.10
0.40
0.90
2.00
3.30

5. Method

Description of operational steps followed

For specific detection of event A2704-12 genomic DNA, two specific primers amplify a 64-bp fragment of an internal sequence of the A2704-12 event that consists of the unique junction of rearranged sequences of the original transforming plasmid. The amplicon is located on a junction fragment containing one copy of the 3' *bla* sequence and one copy of the 5' *bla* sequence, integrated in an inverted orientation, as compared to the transforming plasmid configuration.

PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For the relative quantification of event A2704-12 DNA, a soybean-specific reference system amplifies a 105-bp fragment of the soybean endogenous gene *Le1* (*lectin,* accession number K00821), using two *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with FAM and TAMRA.

For relative quantification of event A2704-12 DNA in a test sample, the normalised ΔCt values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCt values against the logarithm of the amount of event A2704-12 DNA). The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of event A2704-12 DNA is estimated.

Calibration samples denominated from S1 to S5 were prepared by mixing the appropriate amount of A2704-12 DNA from the stock solution with non-GM cotton DNA to obtain the following relative contents of A2704-12: 3.6%, 1.8%, 0.9%, 0.45% and 0.09%. Total DNA amount per reaction was 200 ng, when 5 µl of a DNA solution at the concentration of 40 ng/µl were loaded.

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/5 µl)	200	200	200	200	200
% GM (DNA/DNA)	3.60	1.80	0.90	0.45	0.09

6. Deviations reported

No deviations from the protocol were reported.

7. Summary of results

PCR efficiency and linearity

The values of the slopes of the standard curve [from which the PCR efficiency is calculated using the formula ((10^(-1/slope))-1)*100] and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for both runs (plates A and B) are summarised in Table 4.

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

LAB	PLATE	Slope	PCR Efficiency (%)	Linearity (R^2)
1	A	-3.23	95.85	1.00
	B	-3.32	99.73	1.00
2	A	-3.31	99.58	1.00
	B	-3.41	96.27	1.00
3	A	-3.39	97.37	1.00
	B	-3.36	98.37	1.00
4	A	-3.54	91.60	1.00
	B	-3.67	87.12	0.99
5	A	-3.49	93.53	1.00
	B	-3.49	93.57	1.00
6	A	-3.55	91.22	1.00
	B	-3.41	96.37	1.00
7	A	-3.60	89.58	1.00
	B	-3.41	96.60	1.00
8	A	-3.45	94.90	1.00
	B	-3.49	93.47	1.00
9	A	-4.23	72.37	0.97
	B	-3.55	91.29	0.98
10	A	-3.50	93.25	1.00
	B	-3.44	95.29	1.00
11	A	-3.59	89.95	1.00
	B	-3.49	93.57	1.00
12	A	-3.52	92.18	1.00
	B	-3.29	98.46	1.00
13	A	-3.60	89.59	1.00
	B	-3.57	90.75	1.00
	Mean	**-3.50**	**93.15**	**1.00**

The mean PCR efficiency was above 93%. The linearity of the method was on average above 0.99. Therefore, the data reported confirm the appropriate performance characteristics of the method tested.

GMO quantification

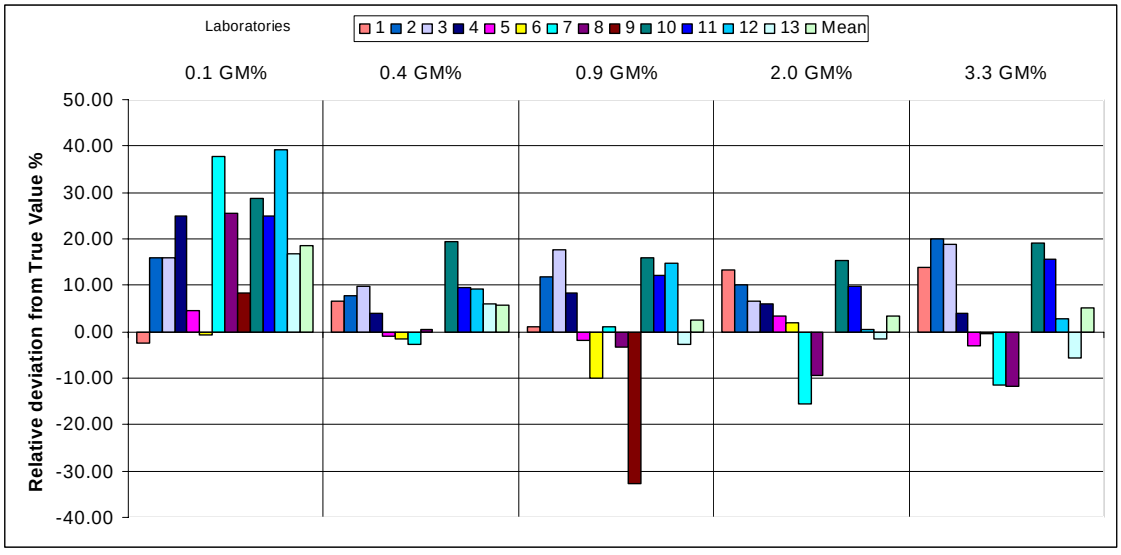
Table 5 shows the mean values of the four replicates for each GM level as calculated and provided by all laboratories. Each mean value is the average of three PCR repetitions.

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				2.0				3.3			
	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	0.09	0.09	0.11	0.10	0.41	0.39	0.42	0.48	0.89	0.81	1.03	0.91	2.21	2.13	2.40	2.32	3.78	4.07	3.51	3.66
2	0.10	0.13	0.11	0.13	0.42	0.40	0.46	0.44	0.87	0.90	1.04	1.21	2.23	2.42	2.18	1.98	4.33	4.09	3.45	3.96
3	0.11	0.12	0.12	0.11	0.42	0.42	0.43	0.48	0.92	1.09	1.06	1.17	2.03	1.99	2.16	2.34	3.77	4.00	4.06	3.85
4	0.11	0.11	0.14	0.14	0.41	0.37	0.47	0.41	0.85	0.83	1.09	1.12	2.01	2.33	2.09	2.06	3.31	3.69	3.20	3.52
5	0.10	0.11	0.11	0.10	0.40	0.37	0.41	0.41	0.89	0.94	0.86	0.84	2.08	2.02	2.21	1.96	3.27	2.99	3.35	3.21
6	0.10	0.10	0.10	0.09	0.43	0.39	0.39	0.38	0.85	0.78	0.78	0.82	2.13	1.96	2.13	1.93	3.04	3.18	3.51	3.41
7	0.15	0.17	0.12	0.11	0.41	0.40	0.44	0.30	0.92	0.89	0.82	1.01	1.76	1.88	1.57	1.56	2.78	3.55	2.34	3.02
8	0.13	0.13	0.12	0.12	0.41	0.42	0.44	0.34	0.88	0.92	0.86	0.82	1.90	1.84	1.82	1.68	3.01	3.03	2.81	2.80
9	0.07	0.11	0.12	0.13	0.23	0.23	0.59	0.49	0.37	0.61	0.69	0.76	1.02	1.01	2.02	2.97	1.21	1.63	4.75	3.92
10	0.12	0.11	0.15	0.13	0.47	0.43	0.53	0.47	0.92	0.95	1.21	1.08	2.21	2.32	2.41	2.28	3.46	3.91	4.07	4.29
11	0.14	0.14	0.11	0.11	0.44	0.47	0.45	0.40	1.07	0.99	0.91	1.06	2.16	2.34	2.20	2.08	4.09	4.00	3.46	3.72
12	0.14	0.17	0.13	0.13	0.46	0.40	0.45	0.43	0.96	1.12	1.06	0.99	1.74	2.10	2.09	2.11	3.35	4.11	3.10	3.01
13	0.11	0.12	0.12	0.12	0.44	0.43	0.42	0.41	0.88	0.86	0.85	0.91	2.03	1.90	2.01	1.93	2.96	3.25	3.30	2.94

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.

Figure 1. Relative deviation (%) from the true value of A2704-12 for all laboratories



As observed in Figure 1, a general trend of overestimation is visible for most of the laboratories at the lowest GM levels 0.1% and 0.4%. The highest relative deviation from the true value of A2704-12 is observed at 0.1% GM level, although the mean value is well within the limit of the trueness acceptance level (bias < 20%). The mean overestimation of higher GM levels 0.9%,

2.0% and 3.3% is very limited. One laboratory underestimated the GM level 0.9% by more than 30%. Overall, the average relative deviation from the true value was acceptable at all GM levels tested, indicating a satisfactory accuracy of the method.

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 16% at the 0.1% and 0.9% levels, thus within the acceptance criterion.

Table 6. A2704-12: summary of validation results.

Unknown sample GM%	Expected value (GMO %)				
	0.1	0.4	0.9	2.0	3.3
Laboratories having returned results	13	13	13	13	13
Samples per laboratory	4	4	4	4	4
Number of outliers	0	1	0	1	1
Reason for exclusion	-	1 C. test	-	1 C. test	1 C. test
Mean value	0.12	0.42	0.92	2.07	3.47
Relative repeatability standard deviation, RSD_r (%)	13	8	11	6	9
Repeatability standard deviation	0.02	0.03	0.10	0.13	0.30
Relative reproducibility standard deviation, RSD_R (%)	16	9	16	10	14
Reproducibility standard deviation	0.02	0.04	0.15	0.21	0.47
Bias (absolute value)	0.02	0.02	0.02	0.07	0.17
Bias (%)	18	6	2	3	5

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 satisfies this requirement throughout the whole dynamic range tested.

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 satisfies this requirement across the entire dynamic range tested; in fact, the highest value of bias is 18% at the 0.1% 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 at 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. The method is therefore 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 = $[10^{(-1/slope)}] - 1$

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of (-3.1 ≥ slope ≥ - 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 $1/10^{th}$ 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 $1/20^{th}$ 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 $\pm$ 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 $\pm$ 25% of the accepted reference value over the whole dynamic range.

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Community Reference Laboratory
CRL
GM Food and Feed

Event-specific Method for the Quantification of Soybean Line A2704-12 Using Real-time PCR

Protocol

14 May 2007

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

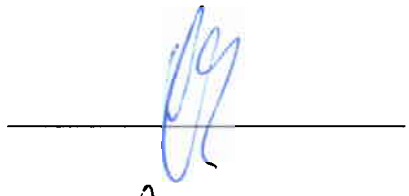
Method development:

Bayer CropScience GmbH

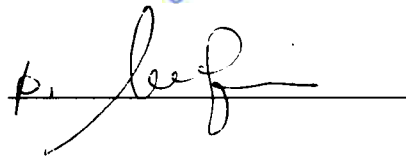
Collaborative trial:

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

Drafted by
C. Delobel



Report Verification Team
1) W. Moens

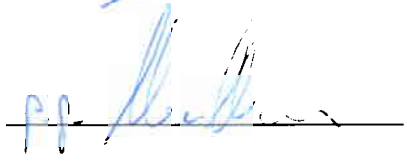


2) M. Querci

Scientific and technical approval
M. Mazzara

Compliance to CRL Quality System
S. Cordeil

Authorisation to publish
G. Van den Eede



Address of contact laboratory:
European Commission, Directorate General Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 1, 21020 Ispra (VA) - Italy

Content

1. GENERAL INFORMATION AND SUMMARY OF THE METHODOLOGY 4

2. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS 4

2.1 General 4
2.2 Collaborative trial 5
2.3 Limit of detection (LOD) 5
2.4 Limit of quantification (LOQ) 5
2.5 Molecular specificity 5

3. PROCEDURE 6

3.1 General instructions and precautions 6
3.2 Real-time PCR for quantitative analysis of A2704-12 6
3.2.1 General 6
3.2.2 Calibration 7
3.2.3 Real-time PCR set-up 7
3.3 Data analysis 9
3.4 Calculation of results 9

4. MATERIALS 9

4.1 Equipment 9
4.2 Reagents 10
4.3 Primers and Probes 10

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 event A2704-12 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 specific detection of event A2704-12 genomic DNA, a 64-bp fragment of the unique junction of rearranged sequences of the original transforming plasmid 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 two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantification of event A2704-12 DNA, a soybean-specific reference system amplifies a 105-bp fragment of the lectin gene (*Le1*), a soybean endogenous gene, using two specific primers and a *Le1* 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 A2704-12 DNA in a test sample, the normalised ΔCt values of the calibration samples are used to calculate by linear regression a reference curve ΔCt-formula. The normalised ΔCt values of the unknown samples are measured and, by means of the regression, the relative amount of A2704-12 event DNA is estimated.

2. Validation status and performance characteristics

2.1 General

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

The reproducibility and trueness of the method was tested through collaborative trial using 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 thirteen participating laboratories in June-July 2006.

Each participant received twenty blind samples containing A2704-12 soybean genomic DNA at five GM contents, ranging from 0.1 % to 3.3 %.

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 A2704-12 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found under 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 0.023% 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 0.045% in 200 ng of total soybean DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.1 %.

2.5 Molecular specificity

The method exploits a unique DNA sequence of the junction of rearranged sequences of the original transforming plasmid. The sequence is specific to A2704-12 event and thus imparts event-specificity to the method.

The specificity of the event-specific assay was experimentally tested against DNA extracted from plant materials containing the specific targets of maize T25, MON 810, Bt11, Bt176, GA21, NK603, CBH351, Roundup Ready Soybean, OSR Ms1, Ms8, Rf1, Rf2, Rf3, Topas19/2, T45, LLRICE62 and LLCotton25. None of the materials yielded detectable amplification in replicate experiments.

The specificity of the soybean endogenous reference system *Le1* was experimentally tested by the applicant on soybean related species (50 ng of genomic DNA in the PCR reaction and PCR reaction of 40 thermo cycles). The *Le1* endogenous reference system produced amplification signals for *glycine max* and *glycine soj* DNA samples. Out of the eight soybean related species, two did not react with the soybean endogenous system (i.e. lentil *Castelluccio lentil* and *Vicia faba Boccli*) while weak amplification signals (i.e. above 37 cycles for a maximum of 7 replicate

out of 12 replicates) were obtained for six species (Lentil lentil 1, *Vicia faba* WJK-PRC-47, *Vicia faba Madfredini, Arachis hypogaea* US1175, *Arachis rasteiro* and Peas).

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 A2704-12

3.2.1 General

The PCR set-up for the taxon specific target sequence (*Le1*) and for the GMO (A2704-12) 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 25 µl per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curve consists of five samples containing different amounts of A2704-12 DNA in a total amount of 200 ng soybean DNA. The GM content of the standard samples ranges from 3.6% to 0.09%.

A calibration curve is produced by plotting the ∆Ct-values of calibration samples against the logarithm of the respective GM % contents; the slope (a) and the intercept (b) of the standard curve (y = ax + b) are then used to calculate the mean % GM content of the blind samples based on their normalised ΔCt values.

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 on ice**.
2. In two reaction tubes (one for A2704-12 system and one for the *Le1* 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 soybean *Le1* reference system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM164 primer (10 µM)	200 nM	0.5
KVM165 primer (10 µM)	200 nM	0.5
TM021 probe (10 µM)	200 nM	0.5
Nuclease free water	#	6
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the A2704-12 specific system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM175 primer forward (10 µM)	400 nM	1
SMO001 primer reverse (10 µM)	400 nM	1
TM031 Probe (10 µM)	200 nM	0.5
Nuclease free water	#	5
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the A2704-12 and one for the *Le1* 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. 20 x 3 = 60 µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 x 3 = 15 µl DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
6. Spin down the tubes in a microcentrifuge. Aliquot 25 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:

Table 3. Cycling program for A2704-12/*Le1* systems

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. A2704-12) 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. *Le1* system).

e) Save the settings and export all the data into an Excel 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 reference ΔCt-curve is generated by plotting the ΔCt-values measured for the calibration points against the logarithm of the GM% content, and by fitting a linear regression line into these data.

Thereafter, the reference curve ΔCt-curve formula is used to estimate the relative amount (%) of A2704-12 event in the unknown samples of DNA.

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')
A2704-12 target sequence	
KVM175	5'- GCA AAA AAG CGG TTA GCT CCT -3'
SMO001	5'- ATT CAG GCT GCG CAA CTG TT -3'
TM031 (Probe)	FAM-5'- CGG TCC TCC GAT CGC CCT TCC -3'-TAMRA
Reference gene Le1 target sequence	
KVM164	5'- CAC CTT TCT CGC ACC AAT TGA CA -3'
KVM165	5'- TCA AAC TCA ACA GCG ACG AC -3'
TM021 (Probe)	FAM-5'- CCA CAA ACA CAT GCA GGT TAT CTT GG - 3'-TAMRA

CRLVL13/05XP

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Community Reference Laboratory
CRL
GM Food and Feed

Soybean Seeds Sampling and DNA Extraction

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

14 May 2007

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

Method development and single laboratory validation:

Bayer CropScience GmbH

Method testing and confirmation:

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

Drafted by:
E. Grazioli

Report Verification Team:
1) W. Moens

2) M. Querci

Scientific and technical approval:
M. Mazzara

Compliance with CRL Quality System:
S. Cordeil

Authorisation to publish:
G. Van den Eede

Address of contact laboratory:
European Commission, Directorate General-Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 1, 21020 Ispra (VA) - Italy

Content

1. INTRODUCTION..... 4
2. MATERIALS (EQUIPMENT/CHEMICALS/PLASTICWARE)..... 4
3. DESCRIPTION OF THE METHODS..... 6
4. TESTING OF THE DNA EXTRACTION METHOD BY THE METHOD DEVELOPER..... 8
5. EXPERIMENTAL TESTING OF THE DNA EXTRACTION METHOD BY THE COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED..... 12
6. CONCLUSION..... 17
7. QUALITY ASSURANCE..... 17
8. REFERENCES..... 17

1. Introduction

This report describes a plant DNA extraction protocol derived from the publicly available "Dellaporta" method [1]. This protocol can be used for the extraction of DNA from soybean seeds and grains ground to powder with a Waring™ blender or with any other appropriate seeds/grains crushing device.

These protocols are 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/Plasticware)

2.1. Equipment

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

1. Waring blender, model 7010S/7010G/7010HS/7010HG or equivalent
2. 70 mm Blender Base (Eberbach Corp. – Cat.No. 8495) for Waring blender or equivalent
3. Micro centrifuge with 18,000 x g for microcentrifuge tubes
4. Table centrifuge (swinging buckets) with 3,000 x g for Falcon tubes
5. Water bath adjustable to 65°C ± 1°C
6. Fluostar Galaxy type 0403 from BMG LabTechnologies
7. PC with Fluostar software (Fluo32)

2.2. Chemicals

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

1. Na_2-EDTA; Titriplex III (Merck Cat. No. 1.08418.1000)
2. Tris-HCl; Tris(hydroxymethyl)aminomethane hydrochloride (USB Cat. No. 22676)
3. NaCl; sodium chloride (Duchefa Cat No. S0520)
4. KAc; potassium acetate (Merck Cat. No. 1.04820.1000)
5. NaAc; sodium acetate (Merck Cat. No. 1.06268.1000)
6. SDS; sodium dodecyl sulphate (BDH Cat. No. 442444H)
7. 2-mercaptoethanol (Sigma Cat. No. M6250)
8. RNase A (Roche Cat. No. 0109-142)
9. Ethanol p.a. (Merck Cat. No. 1.00983.1000)
10. Isopropanol p.a. (Merck Cat. No. 1.09634.2500)
11. Phenol:chloroform:isoamylalcohol (25:24:1) equilibrated at pH 8.0 (Sigma Cat. No. P-2069)

12. Chloroform p.a. (Merck Cat. No. 1.02445.2500)
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. **Extraction Buffer pH 8.0**

- 100 mM Tris HCl pH 8.0
- 50 mM EDTA
- 500 mM NaCl
- 10 mM 2-mercaptoethanol

2. **Tris-EDTA buffer (TE) pH 8.0**

- 10 mM Tris HCl pH 8.0
- 1 mM EDTA

3. **Tris-EDTA buffer (TE0.1) pH 8.0**

- 10 mM Tris HCl pH 8.0
- 0.1 mM EDTA

4. **RNase A (10 mg/ml)**

5. **SDS 20%**

6. **Ethanol 70%**

7. **5M KAc**

8. **3M NaAc**

2.4. Plasticware

1. 50 ml conical tubes
2. 15 ml conical tubes
3. 2 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 following safety guidelines and 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:
Sampling approaches for seeds and grains are referred to in technical guidance documents and protocols described in:
- International Organization for Standardizations, Switzerland: ISO standard 6644, ISO standard 13690, ISO standard 5725;
- International Rules for Seed Testing (2004) International Seed Testing Association (ISTA), Switzerland. ISBN 3-906549-38-0;
- USDA-GIPSA (2001) Sampling grains for the detection of Biotech grains http://www.usda.gov/gipsa/.

Scope and applicability:
The "Dellaporta-derived" method for DNA extraction described below is suitable for the isolation of genomic DNA from ground soybean seeds and grains. 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 "Dellaporta-derived" method starts with a lysis step (thermal lysis in the presence of Tris HCl, EDTA, NaCl and 2-mercaptoethanol) followed by isopropanol precipitation and removal of contaminants such as lipophilic molecules and proteins by extraction with phenol:chloroform:isoamylalcohol.
A further DNA precipitate is then generated by using isopropanol, followed by RNAse treatment, phenol:chloroform:isoamylalcohol purification and final isopropanol precipitation. The resulting pellet is dissolved in TE buffer.

Seed crushing procedure:

- The seeds/grains are crushed using a Waring blender
- Grind in intervals of 10 seconds for 4 times at maximum speed. Shake between intervals until all powder is loose to improve crushing-procedure
- All seeds/grains should be crushed until a fine powder is obtained. Thorough grinding will also produce a homogenous powder
- Prevent cross-contamination by dust particles between the samples

Soybean seed DNA extraction protocol

1. Transfer 1 g powder into a 50 ml Falcon tube
2. Add 30 ml Extraction Buffer
3. Add 2.1 ml 20% SDS, mix well by inversion
4. Incubate at 65°C for 30 minutes
   Note: Mix samples every 10 minutes by inversion
5. Centrifuge for 20 minutes at 3,000 x g
6. Transfer 20 ml supernatant to a new 50 ml Falcon tube using a 25 ml pipette
7. Add 6 ml 5M KAc, shake vigorously for 1 minute
8. Incubate on ice for 30 minutes
   Note: Mix samples every 10 minutes by inversion
9. Centrifuge for 20 minutes at 3,000 x g
10. Transfer 20 ml supernatant to a new 50 ml Falcon tube using a 25 ml pipette
11. Add equal volume of isopropanol, mix gently for 1 minute
12. Incubate on ice for 5 minutes
13. Centrifuge for 20 minutes at 3,000 x g
14. Remove supernatant and air-dry the pellet at 37°C until all isopropanol residue is evaporated
15. Dissolve the pellet in 10 ml TE
16. Shake the samples for 1 hour. Make sure the pellet is completely dissolved
17. Add 12 ml phenol:chloroform:isoamylalcohol (25:24:1)
18. Mix well for 1 minute
19. Centrifuge for 20 minutes at 3,000 x g
20. Transfer the upper aqueous phase (8 ml) to a new 50 ml Falcon tube using a 10 ml pipette. Do not disturb the interphase
21. Add 900 µl 3M NaAc
22. Add 6 ml isopropanol
23. Mix gently by inversion for 1 minute
24. Place on ice for 5 minutes
25. Centrifuge for 20 minutes at 3,000 x g to pellet the DNA
26. Remove supernatant and air-dry the pellet at 37°C until all isopropanol residue is evaporated
27. Dissolve the pellet in 1 ml TE

28. Shake the samples for 1 hour. Make sure the pellet is completely dissolved
29. Transfer the DNA solution to a new 2.0 ml microcentrifuge tube
30. Add 10 µl RNase A (10 mg/ml), mix gently and incubate for 20 min at 37°C
31. Add 800 µl phenol:chloroform:isoamylalcohol (25:24:1)
32. Mix well for 1 minute
33. Centrifuge for 10 minutes in a microcentrifuge at maximum speed
34. Transfer the upper aqueous phase (about 900 µl) to a new 2 ml microcentrifuge tube
35. Add 800 µl chloroform
36. Mix well for 1 minute
37. Centrifuge for 10 minutes in a micro centrifuge at maximum speed
38. Transfer the upper aqueous phase (about 800 µl) to a new 2 ml microcentrifuge tube containing 90 µl 3M NaAc
39. Add 600 µl isopropanol
40. Mix gently by inversion for 1 minute
41. Place on ice for 5 minutes
42. Centrifuge for 1 minute in a micro centrifuge at maximum speed to pellet the DNA
43. Remove all supernatant
44. Add 1 ml 70% ethanol to wash the DNA pellet. Make sure the DNA pellet is not stuck to the bottom. Shake the samples for 1 hour
45. Centrifuge for 5 minutes in a micro centrifuge at maximum speed
46. Remove supernatant and air-dry the pellet at 37°C until all ethanol residue is evaporated
47. Add 200 µl TE 0.1 to the DNA pellet
48. Allow pellet to dissolve for about 10 hours at 4°C
49. Shake samples for minimal 3 hours at 4°C
50. Centrifuge for 1 minute in a micro centrifuge at maximum speed
51. Transfer the supernatant to a new microcentrifuge tube

4. Testing of the DNA extraction method by the method developer

4.1 Performance characteristics

A soybean seed bulk sample was ground to fine powder. Six replicated independent DNA extractions were performed on 1 gram flour sub-samples. This experiment was repeated twice under repeatability conditions (within short intervals of time [i.e. days] by the same operator, using the same equipment) resulting in 18 DNA samples.

4.2 DNA concentration, yield and repeatability standard deviation (RSDr)

The concentration of the extracted DNA was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit. 1:40 dilutions of each DNA sample were prepared in duplicate and mixed with the PicoGreen reagent. The DNA concentration was determined on

the basis of a nine-point standard curve ranging from 10 ng/ml to 200 ng/ml, using a Fluostar for fluorescence detection. Each concentration was obtained as an average of two readings per sample (Table 1).

Table 1. Results of the 18 (6 X 3) DNA extractions: DNA concentration (ng/µl) and DNA extraction efficiency

DNA extraction	Sample mass (gram)	[DNA] (ng / µl)	Yield (µg DNA / gram sample)
A1	1.0	423.6	84.7
A2	1.0	480.5	96.1
A3	1.0	484.8	97.0
A4	1.0	450.8	90.2
A5	1.0	455.3	91.1
A6	1.0	506.1	101.2
B1	1.0	399.3	79.9
B2	1.0	426.6	85.3
B3	1.0	418.5	83.7
B4	1.0	484.2	96.8
B5	1.0	442.4	88.5
B6	1.0	435.5	87.1
C1	1.0	436.0	87.2
C2	1.0	502.5	100.5
C3	1.0	450.5	90.1
C4	1.0	463.0	92.6
C5	1.0	482.7	96.5
C6	1.0	483.8	96.8

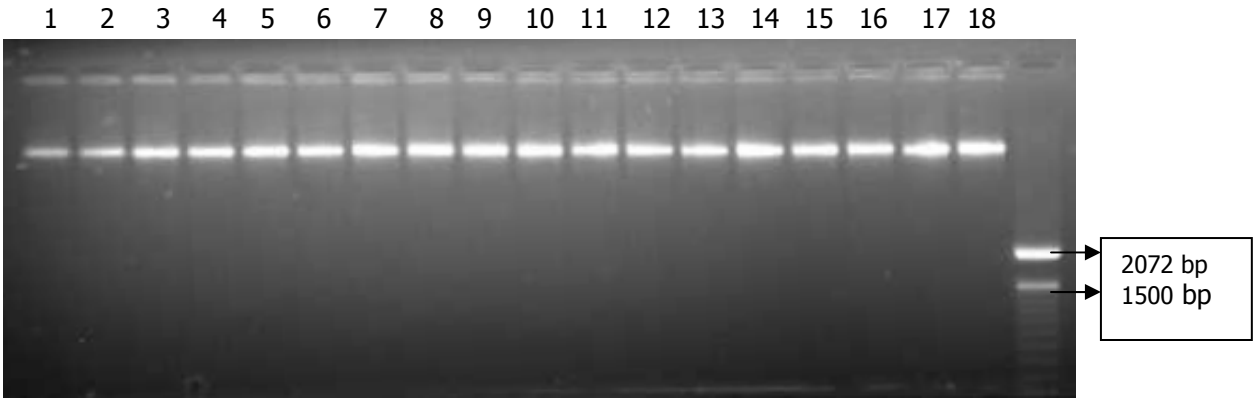
Table 1 lists the DNA concentrations and DNA yield of all 18 DNA extractions. The average DNA concentration was 457 ± 31 ng / µl.
The average DNA extraction efficiency was 91.4 ± 6.1 µg DNA / g of sample, the relative Repeatability Standard Deviation (RSDr) was 6.7%.

Therefore, the soybean DNA extraction procedure is sufficiently efficient and yields adequate amounts of genomic DNA with a sufficient level of repeatability.

4.3 Analysis of DNA fragmentation

Analysis of DNA fragmentation was performed by ethidium bromide-stained agarose gel electrophoresis. One microliter of each undiluted DNA was analyzed on a 1% agarose gel (TBE buffer), including a molecular weight marker (Figure 1).

Figure 1. Agarose gel electrophoresis of eighteen genomic DNA samples extracted from soybean seeds (1 µl undiluted DNA).



The 18 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 18 genomic DNA samples showed indications of significant degradation.

4.4 Evidence of the absence of PCR inhibitory compounds

The absence of PCR inhibitory compounds in the DNA preparations was demonstrated by Real-time PCR using the oligonucleotides directed to the endogenous control gene *lectin* on serial dilutions of the DNA preparations.

All DNA extracts were adjusted to a concentration of 40 ng/µl. From this sample (named "undiluted sample"), a fourfold serial dilution series was prepared with water (1:4, 1:16, 1:64, 1:256).
To assess the presence of inhibitors, the Ct values (y axis) of the dilution series were plotted against the logarithm of the DNA amount (x axis). By linear regression, a trend line ($y = ax + b$) was calculated, as well as a correlation coefficient, r^2, as a measure of linearity (Table 2).
The ideal slope value, 'a', (optimal PCR efficiency) than becomes a = -3.32 (typically 'a' values between -3.1 and -3.6 indicate excellent PCR efficiencies).

Correlation coefficients of r^2 >0.98 indicate an excellent linear relationship, and thus, equally efficient PCR amplification over the measured dynamic range.
The Ct value for the "undiluted sample" (40 ng/µl, 200 ng/reaction) was extrapolated from the equation calculated by linear regression. Subsequently the extrapolated Ct for the undiluted

sample was compared with the measured Ct. Differences between measured and extrapolated Ct of < 0.5 are indicative of low level or absence of inhibition.

The results reported in Table 2 show no evidence of the presence of PCR inhibitors; in fact all 18 ΔCt values are < 0.5, suggesting absence of PCR inhibitors.

Table 2. Results of the analysis for PCR inhibitory compounds; comparison of extrapolated Ct values versus measured Ct values.

DNA	Slope, a	Intercept, b	Linearity, R^2	$Ct_{measured}$	$Ct_{extrapolated}$	ΔCt ($Ct_{measured}$- $Ct_{extrapolated}$)
A1	-3.4477	30.1770	0.9997	22.16	22.24	-0.08
A2	-3.5102	30.3226	0.9999	22.35	22.25	0.10
A3	-3.5644	30.2912	0.9995	22.38	22.09	0.29
A4	-3.4918	30.2546	0.9999	22.46	22.22	0.24
A5	-3.4705	30.2414	0.9977	22.34	22.26	0.09
A6	-3.5217	30.4561	0.9999	22.55	22.35	0.20
B1	-3.3889	30.2363	0.9999	22.55	22.44	0.11
B2	-3.4336	30.5378	0.9992	22.79	22.64	0.15
B3	-3.4494	30.6269	0.9999	22.71	22.69	0.02
B4	-3.5080	30.7840	0.9996	23.06	22.71	0.35
B5	-3.3633	30.5680	0.9999	22.93	22.83	0.10
B6	-3.3576	30.5944	0.9995	22.80	22.87	-0.07
C1	-3.4920	30.7847	0.9999	22.79	22.75	0.04
C2	-3.5200	30.7951	0.9996	22.93	22.70	0.24
C3	-3.4440	30.7391	0.9997	22.84	22.81	0.03
C4	-3.4819	30.8506	0.9999	23.08	22.84	0.24
C5	-3.4039	30.6721	0.9999	22.98	22.84	0.15
C6	-3.4822	30.7781	0.9999	22.86	22.77	0.10

5. Experimental 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 "Dellaporta-derived" 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.

5.1 Preparation of samples

About 200 g of soybean seed material were ground using a GRINDOMIX GM 200 (Retsch GmbH) mixer.

5.2 DNA extraction

DNA was extracted following the "Dellaporta-derived" 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.

5.3 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 3.

Table 3. 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	197
2	188
3	182
4	186
5	190
6	150
1	172
2	147
3	125
4	99
5	91
6	91
1	187
2	159
3	164
4	163
5	177
6	177

- ✓ DNA concentration (ng/μl)

Overall average of all samples:	158 ng/μl
Standard deviation of all samples	34.7 ng/μl
Coefficient of variation	21.9 %

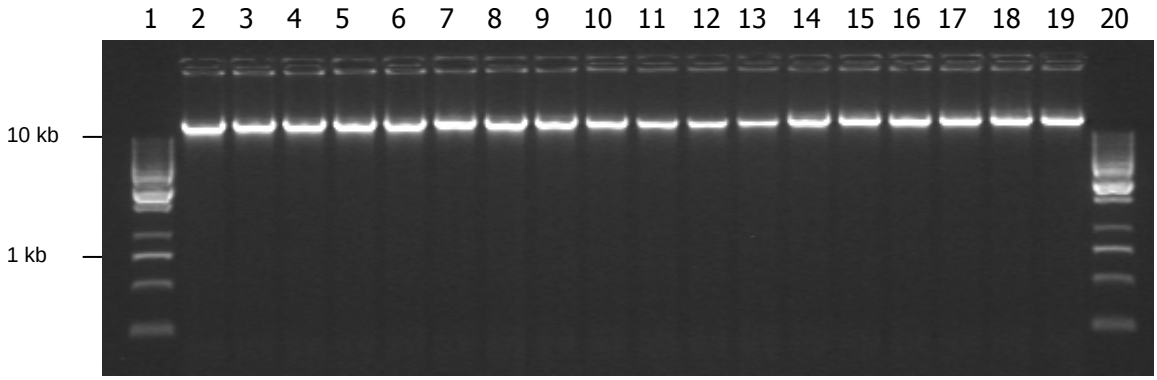
- ➢ Yield (total volume of DNA solution: 200 μl)

Overall average of all samples:	31.6 μg
Standard deviation	6.9 μg
Coefficient of variation	21.9 %

5.4 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).

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: 1kb DNA Marker.



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').

5.4 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 40 ng/µl (hereafter referred as "undiluted" samples).

Subsequently fourfold serial dilutions of each extract were prepared with pure water (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, *Le1*.

The Ct values obtained for "undiluted" and diluted DNA samples are reported in the Table 4.

Table 4. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of soybean lectin 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.

	Undiluted	Diluted			
DNA extract	(40 ng/µl)	1:4	1:16	1:64	1:256
1	20.77	22.80	24.90	26.79	29.39
2	22.53	24.63	26.43	28.79	31.11
3	21.07	22.97	25.14	27.03	29.26
4	20.94	22.75	24.78	27.22	29.73
5	21.07	22.99	24.95	26.90	29.38
6	20.94	22.77	24.79	27.02	28.75
1	22.56	24.58	26.86	28.99	31.14
2	20.84	22.99	24.91	27.07	29.31
3	22.68	24.86	26.82	28.87	31.08
4	21.24	23.29	25.33	27.57	29.56
5	21.15	23.13	25.32	27.36	29.67
6	21.15	23.20	25.39	27.53	29.70
1	22.68	24.70	26.78	28.92	31.20
2	22.65	24.88	26.82	28.89	31.38
3	22.78	24.91	26.98	29.22	31.44
4	22.75	24.79	26.78	29.05	31.22
5	22.12	24.15	26.21	28.37	30.65
6	22.53	24.66	26.84	29.06	31.33

Table 5 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 (40 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 5. Comparison of extrapolated Ct values versus measured Ct values (amplification of soybean lectin gene, *Le1*)

DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**
1	0.994	-3.6	20.56	20.77	0.21
2	0.996	-3.6	22.28	22.53	0.24
3	0.997	-3.4	20.91	21.07	0.16
4	0.987	-3.9	20.27	20.94	0.67
5	0.995	-3.5	20.77	21.07	0.30
6	0.993	-3.3	20.79	20.94	0.15
1	0.998	-3.6	22.44	22.56	0.12
2	0.997	-3.5	20.79	20.84	0.05
3	0.999	-3.4	22.72	22.68	0.04
4	0.995	-3.5	21.18	21.24	0.06
5	0.999	-3.6	20.96	21.15	0.19
6	0.996	-3.6	21.04	21.15	0.11
1	0.999	-3.6	22.49	22.68	0.20
2	0.996	-3.6	22.60	22.65	0.05
3	1.000	-3.6	22.68	22.78	0.10
4	0.999	-3.6	22.57	22.75	0.19
5	0.999	-3.6	21.93	22.12	0.19
6	1.000	-3.7	22.41	22.53	0.12

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, except the sample number 4 extracted on day 1, with a value of 0.67.

R2 of linear regression is > 0.99 for all DNA samples except one slightly below (0.987).

Curve slopes are all between -3.1 and -3.6, with two exceptions: sample 4 extracted on day 1, with a value of -3.9 (same sample showing a ΔCt of 0.67) and sample 6 extracted on day 3, with a value of -3.7.

6. 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.

7. 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)]

8. References

1. Dellaporta *et al*. [1983] Plant Molecular Biology Reporter 1, nr 4, pp. 19-21.

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

European Commission

EUR 22910 EN – DG Joint Research Centre, Institute for Health and Consumer Protection
Title: Event-specific Method for the Quantification of Soybean Line A2704-12 Using Real-time PCR – Validation Report and Protocol - Soybean Seeds Sampling and DNA Extraction
Authors: M. Mazzara, C. Delobel, E. Grazioli, S. Larcher, C. Savini and G. Van den Eede
Luxembourg: Office for Official Publications of the European Communities
2007 – 47pp. – 21 x 29,7 cm
EUR - Scientific and Technical Research series; ISSN 1018-5593
ISBN 978-92-79-06928-4

Abstract

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 A2704-12 transformation event in soybean DNA (unique identifier ACS-GMØØ5-3). 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 Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Bayer CropScience provided the detection method and the samples (genomic DNA extracted from wild-type and 100% soybean A2704-12 event). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The international collaborative trial involved thirteen laboratories from ten 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/.

.

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

LB-NA-22910-EN-C

ISBN 978-92-79-06928-4

Publications Office