

Event-specific Method for the Quantification of Soybean Line A5547-127 Using Real-time PCR

Validation Report and Protocol

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Validation Report

20 January 2009

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 A5547-127 transformation event in soybean DNA (unique identifier ACS-GMØØ6-4). 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", Bayer CropScience provided the detection method and the samples (genomic DNA from leaves of plants harbouring the A5547-127 event and from leaves of conventional A5547 soybean plants). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from eight European countries.

The results of the international collaborative trial met the ENGL performance requirements. The method is, therefore, considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

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

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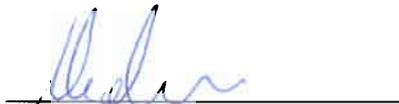
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Report on Steps 1-3 of the Validation Process

Bayer CropScience submitted the detection method and control samples for soybean event A5547-127 (unique identifier ACS-GMØØ6-4) 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 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.ec.europa.eu/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.ec.europa.eu/guidancedocs.htm>) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, a scientific assessment was performed for soybean event A5547-127 and positively concluded in April 2008.

In April-May 2008, the CRL-GMFF experimentally verified the purity of the control samples provided and the method characteristics (step 3, experimental testing of samples and methods) by quantifying five blind GM levels within the range 0.08%-8.0% on a genome copy number basis. The experiments were performed under 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 previously tested on samples of food and feed and a report was published on the CRL-GMFF website on 14th May 2007 (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

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

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1. Introduction

Bayer CropScience submitted the detection method and control samples for soybean event A5547-127 (unique identifier ACS-GMØØ6-4) 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 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 Commission Regulation (EC) No 1829/2003) organised the international collaborative study for the event-specific method for the detection and quantification of soybean A5547-127. The study involved twelve laboratories, among those listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for detection") of Commission Regulation (EC) No 1981/2006 of 22 December 2006.

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 Commission Regulation (EC) No 641/2004 and following its operational procedures.

The internal experimental evaluation of the method was carried out between April and May 2008.

Following the evaluation of the data and the results of the internal laboratory tests, the international collaborative study was organised (step 4) and took place in June 2008.

A method for DNA extraction from soybean seeds, submitted by the applicant, was evaluated by the CRL-GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on method testing are available at <http://gmo-crl.jrc.ec.europa.eu/>.

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

- Quantitative real-time PCR (Polymerase Chain Reaction). The method is an event-specific real-time quantitative TaqMan[®] PCR procedure for the determination of the relative content of event A5547-127 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 (A5547-127) 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 twelve laboratories to determine its performance.

In May 2008 the CRL-GMFF invited all National Reference Laboratories nominated under Commission Regulation (EC) No 1981/2006 of 22 December 2006 and listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for detection") of that Regulation to express the availability to participate in the validation study of the quantitative real-time PCR method for the detection and quantification of soybean event A5547-127.

Thirty-eight laboratories expressed in writing their willingness to participate, three declined the invitation, while thirty-one did not answer. The CRL-GMFF performed a random selection of twelve laboratories out of those that responded positively to the invitation, making use of a validated software application.

Clear guidance was given to the selected laboratories with regards to the standard operational procedures to follow for the execution of the protocol. The participating laboratories are listed in Table 1.

Table 1. Laboratories participating in the validation of the detection method for soybean line A5547-127.

Laboratory	Country
Bavarian Health and Food Safety Authority	DE
Federal State Office of Analysis and Diagnosis for Rhineland-Palatine - Institute of Food Chemistry Trier	DE
Genetically Modified Organism Controlling Laboratory	PL
Groupe d'Intérêt Public – Groupe d'Etude et de contrôle des Variétés et des Productions Agricoles	FR
Institute of Biochemistry and Biophysics Polish Academy of Sciences, Genetic Modifications Analysis Laboratory	PL
Institute of Chemical Technology Prague	CZ
Laboratory of DNA Analysis, Department of Gene Technology (GT)	EE
Lower Saxony Federal State Office for Consumer Protection and Food Safety	DE
National Institute of Biology	SI
National Veterinary Laboratory, GMO Department	LT
Scientific Institute of Public Health (IPH)	BE
State Office for Agriculture, Food safety and Fisheries – Mecklenburg Western Pomerania	DE

3. Materials

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

- i) genomic DNA extracted from leaves of soybean plants harbouring the event A5547-127 homozygously, and dissolved in water, and
- ii) genomic DNA extracted from leaves of conventional A5547 soybean plants, genetically similar to those harbouring the A5547-127 event, and suspended in water.

Samples were provided by the applicant in accordance to the provisions of Commission 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% soybean A5547-127 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 (150 µL of DNA solution each) labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (85 µL of DNA solution each) labelled from U1 to U20.
- ✓ Reaction reagents as follows:
 - universal PCR Master Mix (2x), two bottles: 5 mL each
 - distilled sterile water, one tube: 4 mL
- ✓ Primers and probes (1 tube each) as follows:
 - Le1* reference system
 - KVM164 (10 µM): 160 µL
 - KVM165 (10 µM): 160 µL
 - TM021 (10 µM): 160 µL
 - A5547-127 system
 - SHA003 (10 µM): 320 µL
 - SHA004 (10 µM): 320 µL
 - TM058 (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, the samples were analysed for the A5547-127 specific system and for the *Le1* reference system. Two plates were run per participating laboratory with two replicates for each GM level. 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. A5547-127 GM contents

A5547-127 GM% (GM copy number/soybean genome copy number x 100)
0.08
0.40
0.90
4.00
8.00

5. Method

Description of operational steps followed

For the specific detection of event A5547-127 genomic DNA, a 75-bp fragment of the region that spans the 5' plant-to-insert junction in soybean A5547-127 event 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 FAM as reporter dye at its 5' end and TAMRA as quencher dye at its 3' end.

For the relative quantification of event A5547-127 DNA, a soybean-specific reference system amplifies a 102-bp fragment of the soybean endogenous lectin gene (*Le1*, GenBank K00821 M30884), using two *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with VIC dye and TAMRA as quencher dye.

Standard curves are generated for both the A5547-127 and the *Le1* 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 A5547-127 DNA in a test sample, the A5547-127 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 to obtain the percentage value ($GM\% = A5547-127/Le1 \times 100$).

Calibration sample S1 was prepared by mixing the appropriate amount of A5547-127 DNA in control non-GM soybean DNA to obtain a 10% GM A5547-127. Sample S2 was prepared by three-fold dilution from the S1 sample; sample S3 was prepared by five-fold dilution from S2 sample; sample S4 was prepared by four-fold dilution of sample S3 and sample S5 was prepared by five-fold dilution from the S4 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

pg) ⁽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.

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

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction	300	100	20	5	1
Soybean genome copies	265487	88496	17699	4425	885
A5547-127 GM cotton copies	26549	8850	1770	442	88

6. Deviations reported

Nine laboratories reported no deviations from the protocol.

One laboratory labelled wrongly six wells of one plate, with no consequences since sample label was correctly attributed in subsequent analysis.

One laboratory performed PCR reactions in 20 µL of total volume because a 384-well plate configuration of the ABI 7900HT instrument was used. Final concentrations of PCR reagents remained unchanged.

One laboratory reported a different procedure for saving the results, due to the different processing of data by the ABI 7500 software. However, this had no consequences on data values.

7. Summary of results

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula $((10^{(-1/\text{slope})}-1) \times 100)$ of the reference curve and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for the A5547-127 system and the *Le1* reference system are summarised in Table 4.

The mean PCR efficiency was 93% for the A5547-127 system and 94% for the *Le1* system, with both values within the ENGL acceptance criteria. The linearity of the method was 1.00 for both systems.

Data reported confirm the appropriate performance characteristics of the method tested in terms of efficiency and linearity.

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

		A5547-127			Le1		
Lab	Plate	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.49	93	1.00	-3.46	95	1.00
	B	-3.50	93	1.00	-3.51	93	1.00
2	A	-3.53	92	1.00	-3.47	94	1.00
	B	-3.49	93	1.00	-3.46	94	1.00
3	A	-3.47	94	1.00	-3.45	95	1.00
	B	-3.49	94	1.00	-3.45	95	1.00
4	A	-3.52	92	1.00	-3.55	91	1.00
	B	-3.60	90	1.00	-3.57	91	0.99
5	A	-3.63	89	1.00	-3.42	96	1.00
	B	-3.62	89	1.00	-3.43	96	1.00
6	A	-3.60	90	1.00	-3.62	89	1.00
	B	-3.52	92	1.00	-3.62	89	1.00
7	A	-3.34	99	0.99	-3.33	99	1.00
	B	-3.42	96	1.00	-3.33	100	0.99
8	A	-3.48	94	1.00	-3.36	98	1.00
	B	-3.43	96	1.00	-3.42	96	1.00
9	A	-3.46	95	1.00	-3.38	98	1.00
	B	-3.50	93	1.00	-3.36	99	0.99
10	A	-3.49	93	0.95	-3.47	94	1.00
	B	-3.47	94	0.99	-3.38	98	1.00
11	A	-3.55	91	1.00	-3.62	89	1.00
	B	-3.50	93	1.00	-3.58	90	1.00
12	A	-3.55	91	1.00	-3.58	90	1.00
	B	-3.58	90	1.00	-3.59	90	1.00
	Mean	-3.51	93	1.00	-3.47	94	1.00

GMO quantification

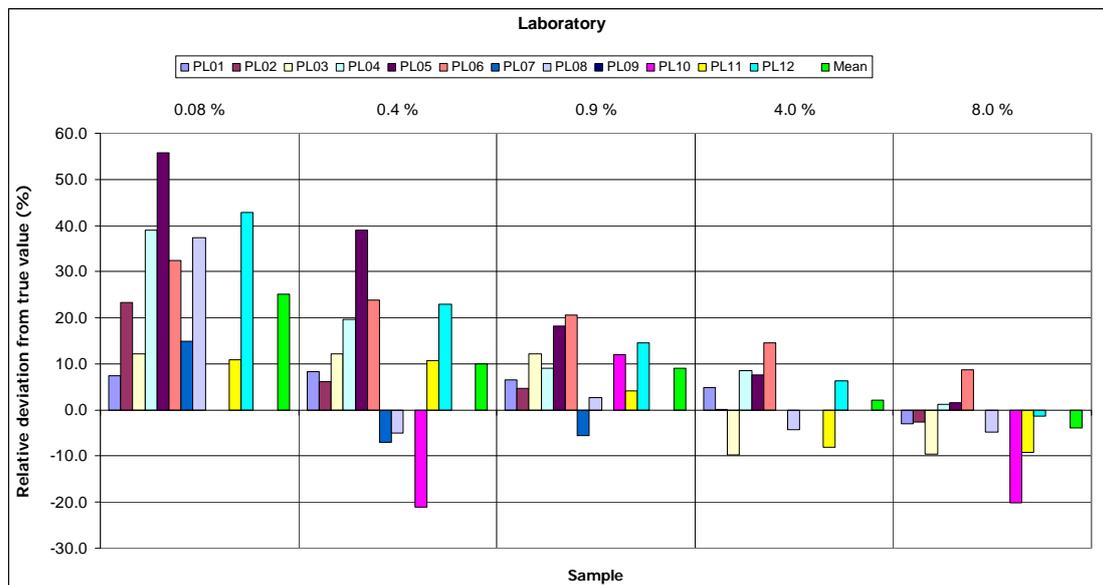
Table 5 shows the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

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

LAB	GMO content (GMO% = GMO copy number/soybean genome copy number x 100)																			
	0.08				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	0.09	0.10	0.08	0.08	0.43	0.42	0.44	0.44	0.89	0.91	1.02	1.01	4.21	4.24	4.09	4.24	7.56	8.04	7.52	7.96
2	0.10	0.10	0.10	0.10	0.42	0.42	0.44	0.42	0.92	0.94	0.99	0.92	3.92	3.95	4.20	3.95	8.07	7.58	7.97	7.53
3	0.08	0.09	0.08	0.10	0.47	0.46	0.45	0.42	1.07	0.92	1.00	1.05	3.94	3.33	3.90	3.29	7.53	6.59	7.37	7.48
4	0.13	0.1	0.11	0.10	0.49	0.52	0.47	0.44	1.06	1.03	0.91	0.92	4.56	4.23	4.11	4.46	8.11	7.91	7.51	8.86
5	0.12	0.13	0.13	0.11	0.54	0.58	0.52	0.59	0.92	1.13	1.04	1.17	4.25	4.43	4.23	4.30	7.77	8.60	7.70	8.43
6	0.10	0.10	0.11	0.11	0.44	0.48	0.51	0.55	1.05	1.05	1.09	1.15	4.48	4.53	4.65	4.66	8.39	9.06	8.44	8.92
7	0.09	0.09	0.09	0.09	0.43	0.34	0.39	0.34	1.00	0.97	0.76	0.66	4.25	4.20	5.13	3.06	6.57	5.71	7.54	9.98
8	0.12	0.11	0.10	0.11	0.38	0.39	0.39	0.37	0.97	0.99	0.88	0.85	3.87	4.25	3.69	3.50	7.99	6.73	7.82	7.92
9	0.15	0.14	0.11	0.09	0.62	0.59	0.44	0.46	1.63	1.82	0.94	1.48	6.22	8.13	3.93	6.46	7.88	19.87	13.95	16.76
10	0.08	0.08	0.09	0.07	0.33	0.36	0.29	0.29	0.77	1.04	0.97	1.25	3.23	3.82	3.32	6.04	6.60	5.50	6.06	7.38
11	0.08	0.08	0.09	0.10	0.43	0.40	0.46	0.48	0.97	0.83	0.96	0.99	3.65	3.50	3.80	3.75	7.47	7.16	7.19	7.25
12	0.11	0.12	0.12	0.10	0.50	0.50	0.46	0.50	0.95	1.01	1.04	1.12	4.16	3.98	4.63	4.25	8.53	7.95	8.00	7.06

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; the green bar represents the overall mean for each GM level.

Figure 1. Relative deviation (%) from the true value of A5547-127 for all laboratories



As observed in Figure 1, the mean relative deviations from the true values are positive for most GM levels apart from 8.0% GM level, where it is negative. This means that the GM content tends to be over-estimated at most GM levels, although the over-estimation gradually decreases with the increasing of the GM levels. For 0.08% GM level, five labs showed deviations from the true value above 25%. The overall average relative deviation for this level was 25%. However, for the rest of the GM levels (0.4, 0.9, 4.0 and 8.0%), only one laboratory showed a deviation from the true value above 25%, at 0.4% GM level.

Overall, the average relative deviation is within the acceptance criterion at all GM levels tested, indicating a satisfactory trueness of the method.

8. Method performance requirements

Among the performance criteria established by ENGL and adopted by the CRL-GMFF (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>, see also Annex 1), repeatability and reproducibility are assessed through an international collaborative trial, carried out with the support of twelve 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 satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R is 16% at the 0.4% GM level, thus well within the acceptance criterion.

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 the RSD_r value is below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>)).

Table 6. A5547-127: summary of validation results.

unknown sample GMO %	Expected value (GMO%)				
	0.08	0.4	0.9	4.0	8.0
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	1	1	1	3	2
Reason for exclusion	1 C	1 C	1 C	3 C	2 C
Mean value	0.10	0.44	0.98	4.09	7.69
Relative repeatability standard deviation, RSD_r (%)	8	7	10	5	6
Repeatability standard deviation	0.01	0.03	0.10	0.21	0.49
Relative reproducibility standard deviation, RSD_R (%)	16	16	11	9	10
Reproducibility standard deviation	0.02	0.07	0.11	0.38	0.75
Bias (absolute value)	0.02	0.04	0.08	0.09	-0.31
Bias (%)	25	10	9	2	-4

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.

As can be observed from the values reported in Table 6, the method showed a repeatability standard deviation below 25% at all GM levels, with the highest value of RSD_r (%) of 10% at the 0.9% GM level.

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 $\pm 25\%$ across the entire dynamic range. In this case the method fully satisfies this requirement across the entire dynamic range tested; in fact, the highest deviation from true value (bias %) is 25% at the 0.08% level, thus 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.ec.europa.eu/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.

Therefore, 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.
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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 $\pm 25\%$ 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 $(-3.1 \geq \text{slope} \geq -3.6)$

R² Coefficient

Definition: the R² coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: the average value of R² 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 $1/10^{\text{th}}$ of the value of the target concentration with an $\text{RSD}_r \leq 25\%$. 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 $1/20^{\text{th}}$ 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.



Event-specific Method for the Quantification of Soybean Event A5547-127 Using Real-time PCR

Protocol

20 January 2009

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

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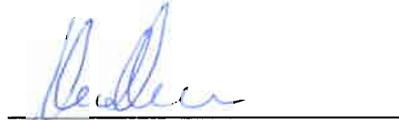
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1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan[®] PCR procedure for the determination of the relative content of soybean event A5547-127 DNA to total soybean DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels.

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 A5547-127 DNA, a 75-bp fragment of the region that spans the 5' plant-to-insert junction in soybean A5547-127 event 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 TAMRA at its 3' end.

For the relative quantification of soybean event A5547-127 DNA, a soybean-specific reference system amplifies a 102-bp fragment of the soybean endogenous *lectin* gene (*Le1*), using two specific primers and a *Le1* gene-specific probe labelled with VIC, as a reporter at its 5' end, and with the non-fluorescent quencher TAMRA at its 3' end.

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 A5547-127 DNA in a test sample, soybean A5547-127 and *Le1* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of soybean event A5547-127 DNA to total soybean DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from soybean leaves, 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 an international collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve participating laboratories in June 2008.

Each participant received twenty blind samples containing soybean A5547-127 genomic DNA at five GM contents, ranging from 0.08% 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 event A5547-127 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at <http://gmo-crl.jrc.ec.europa.eu/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 the collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.08% in 200 ng of total soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.08%.

2.5 Molecular specificity

According to the method developer, 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 A5547-127 and thus imparts event-specificity to the method.

The specificity of event-specific and the soybean-specific assays were experimentally tested by the applicant in real-time PCR against DNA extracted from plant materials containing the specific targets of rice LLRice62; maize T25, MON810, Bt11, GA21, NK603; oilseed rape Ms1, Ms8, Rf1, Rf2, Rf3, Topas 19-2, T45, OXY-235, RT73; soybean A2704-12, Round-Up Ready[®]; cotton lines LLCotton25, GHB614, T304-40, GHB119, MON1445 and conventional soybean.

According to the applicant, the A5547-127 system did not react with any of the plant materials tested, except the positive control soybean line A5547-127; the soybean-specific reference system reacted only with conventional soybean and with all the soybean GM varieties tested.

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 international guidelines, e.g. ISO 24276:2006.
- 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.
- 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 A5547-127

3.2.1 General

The PCR set-up for the taxon specific target sequence (*Le1*) and for the GMO (event A5547-127) 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 curves consist of five samples. The first point of the calibration curves is a 10% A5547-127 in non-GM soybean DNA for a total of 300 ng of DNA (corresponding to approximately 265,487 soybean genome copies with one genome assumed to correspond to 1.13 pg of haploid soybean genomic DNA)⁽¹⁾. The other four standards are prepared by serial dilution.

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 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 each unknown sample DNA is obtained by interpolation from the standard curves.

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 the A5547-127 system and one for the *Le1* system) on ice, add the following components (Table 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 A5547-127 specific system.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
SHA003 primer (10 µM)	400 nM	1
SHA004 primer (10 µM)	400 nM	1
TM058 TaqMan® probe (10 µM)	200 nM	0.5
Nuclease free water	#	5
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 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 TaqMan® probe (10 µM)	200 nM	0.5
Nuclease free water	#	6
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the soybean A5547-127 and one for the *Le1* reaction 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 reaction mix (e.g. 20 μL x 3 = 60 μL reaction mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 μL x 3 = 15 μL DNA for three PCR repetitions). Vortex each tube for approx. 10 s. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
6. Spin down the tubes in a micro-centrifuge. 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 the cycling program described in Table 3.

Table 3. Cycling program for A5547-127 specific system and for the soybean *Le1* reference system.

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

3.3 Data analysis

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

a) Set the threshold: display the amplification curves of one system (e.g. A5547-127) 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 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 *Le1* and the A5547-127 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 DNA copy numbers in the unknown sample.

For the determination of the amount of event A5547-127 DNA in the unknown sample, the A5547-127 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 to obtain the percentage value (GM% = A5547-127/*Le1* x 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
- 0.2/1.5/2.0 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')
A5547-127 target sequence	
SHA003	5' – GCT ATT TGG TGG CAT TTT TCC A – 3'
SHA004	5' – CAC TGC GGC CAA CTT ACT TCT – 3'
TM058 (probe)	FAM - 5' – CCG CAA TGT CAT ACC GTC ATC GTT GT – 3' TAMRA
Reference gene <i>Le1</i> target sequence	
KVM164	5' – CTT TCT CGC ACC AAT TGA CA – 3'
KVM165	5' – TCA AAC TCA ACA GCG ACG AC – 3'
TM021 (probe)	VIC 5' – CCA CAA ACA CAT GCA GGT TAT CTT GG – 3' TAMRA

5. References

1. Arumuganathan K., Earle E.D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter*, 9: 208-218.



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**

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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. Na₂-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 μ l RNase A (10 mg/ml), mix gently and incubate for 20 min at 37°C
31. Add 800 μ l 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 μ l) to a new 2 ml microcentrifuge tube
35. Add 800 μ l 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 μ l) to a new 2 ml microcentrifuge tube containing 90 μ l 3M NaAc
39. Add 600 μ l 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 μ l 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/ μ l) 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 / μ l.

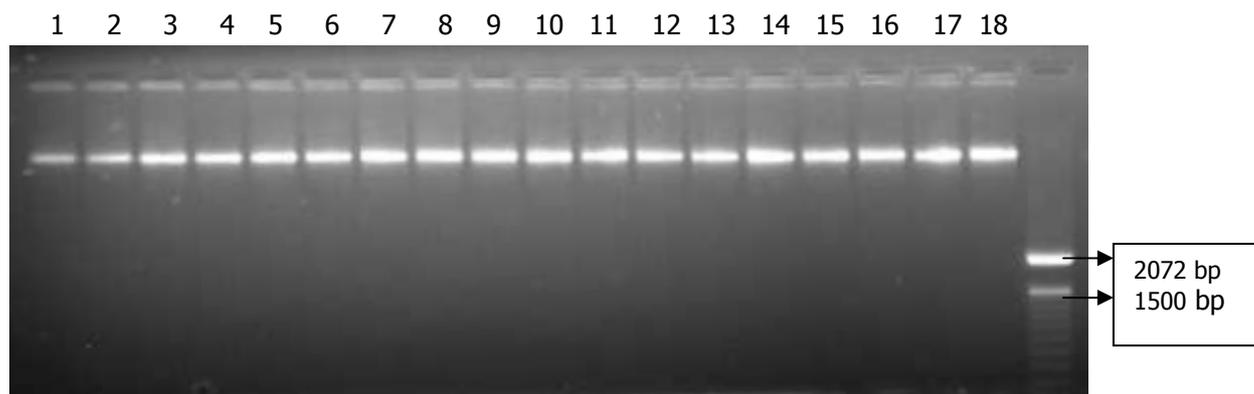
The average DNA extraction efficiency was 91.4 ± 6.1 μ g DNA / g of sample, the relative Repeatability Standard Deviation (RSDr) was 6.7%.

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 μ l 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/ μ l. 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) then 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/ μ l, 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 Δ Ct 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, R²	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/ μ l) 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/ μ l)
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/ μ l)

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

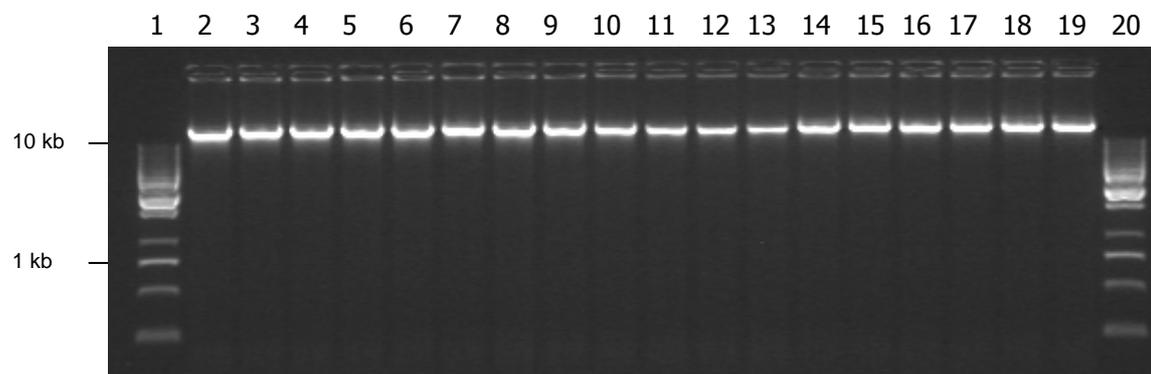
➤ Yield (total volume of DNA solution: 200 μ l)

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

5.4 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 8 μ l 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/ μ l (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.

DNA extract	Undiluted (40 ng/μl)	Diluted			
		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

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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 A5547-127 transformation event in soybean DNA (unique identifier ACS-GMØØ6-4). 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”, Bayer CropScience provided the detection method and the samples (genomic DNA from leaves of plants harbouring the A5547-127 event and from leaves of conventional A5547 soybean plants). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from eight European countries.

The results of the international collaborative trial met the ENGL performance requirements. The method is, therefore, considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

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

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