Event-specific Method for the Quantification of Soybean MON 87701 Using Real-time PCR

Validation Report

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**Joint Research Centre
Institute for Health and Consumer Protection
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Executive Summary

The European Union Reference Laboratory for GM Food and Feed (EURL-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 event MON 87701 (unique identifier MON-877∅1-2) in soybean DNA samples. The collaborative trial was conducted according to internationally accepted guidelines [1, 2].

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed" and with Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003", Monsanto provided the detection method and the control samples: genomic DNA from soybean seeds harbouring the MON 87701 event and from conventional soybean seeds. The EURL-GMFF 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 Regulation (EC) No 641/2004.

The results of the collaborative study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm .

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Report on Steps 1-3 of the Validation Process

Monsanto submitted the detection method and control samples for soybean event MON 87701 (unique identifier MON-877∅1-2) 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 European Union Reference Laboratory for GM Food and Feed (EURL-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 EURL-GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/doc/Description%20CRL%20validation%20process.pdf).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories (ENGL) 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 MON 87701 and positively concluded in September 2009.

Between October and November 2009, the EURL-GMFF experimentally verified the purity of the control samples provided. The method characteristics were verified by quantifying five blind GM levels within the range 0.085% - 8.1% on a genome copy number basis (step 3, experimental testing of samples and methods). The experiments were performed under repeatability conditions and demonstrated that the PCR (Polymerase Chain reaction) 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 EURL-GMFF website (http://gmo-crl.jrc.ec.europa.eu/summaries/40-3-2_DNAExtr_report.pdf).

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

Content

1.   Introduction

Monsanto submitted the detection method and control samples for soybean event MON 87701 (unique identifier MON-877∅1-2) 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 European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, organised the international collaborative study for the validation of the event-specific method for detection and quantification of soybean MON 87701. The study involved twelve laboratories, among those listed in Annex II ("National reference laboratories assisting the EURL for testing and validation of methods for detection") of Regulation (EC) No 1981/2006 of 22 December 2006.

Upon reception of method, samples and related data (step 1), the EURL-GMFF 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 experimental evaluation of the method was carried out between October and November 2009.

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 December 2009.

A method for DNA extraction from seeds and grains followed by PEG precipitation, submitted by the applicant, was evaluated by the EURL-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/statusofdoss.htm.

The collaborative study aimed at assessing the performance of a 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 MON 87701 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean assay for the lectin (*Le1*) endogenous gene and the target assay for the event MON 87701 are performed in separate wells.

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

- ISO 5725 (1994).[1]
- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995). [2]

2. List of participating laboratories

As part of the international collaborative study the method was tested in twelve laboratories to determine its performance.

In November 2009, the EURL-GMFF invited all National Reference Laboratories nominated under Regulation (EC) No 1981/2006 of 22 December 2006 and listed in Annex II ("National reference laboratories assisting the EURL 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 MON 87701.

Thirty-seven laboratories expressed in writing their willingness to participate, one responded after the deadline, one declined the invitation, while thirty did not answer. The EURL-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 MON 87701

Laboratory	Country
Center for Agricultural Technology Augustenberg	DE
Central Agricultural Office, Food and Feed Safety Directorate, Central Feed Investigation Laboratory	HU
Federal Institute for Risk Assessment	DE
Finnish Customs Laboratory	FI
Institute of Molecular Biology of the Slovak Academy of Sciences	SK
Laboratory for the detection of GMO in Food	DE
Laboratory of DNA analysis, Department of Gene Technology	EE
National Research Institute of Animal Production, National Feed Laboratory in Lublin	PL
National Institute of Biological Resources – Laboratory for the Characterization of Plant Propagation Materials	PT
Service Commun des laboratories du MINEFI – Laboratoire de Strasbourg	FR
State Institute of Chemical and Veterinarian Analysis	DE
The Food and Environment Research Agency	UK

3.   Materials

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

i)   genomic DNA extracted from homozygous soybean seeds harbouring the event MON 87701 (Seed Lot No. 10001043), and

ii)   genomic DNA extracted from conventional soybean seeds (Seed Lot No. 10000909).

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

Samples containing mixtures of 100% soybean MON 87701 and non-GM soybean genomic DNA at different GMO concentrations were prepared by the EURL-GMFF, using the control samples provided, in a constant amount of total soybean DNA.

Participants received the following materials:

- ✓ Five calibration samples (140 µL of DNA solution each) labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (70 µL of DNA solution each) labelled from U1 to U20.
- ✓ Reaction reagents as follows:
  - ▪ universal PCR Master Mix (2x), three bottles: 5 mL each
  - ▪ distilled sterile water, one tube: 12 mL

- ✓ Primers and probes (1 tube each) as follows:
  Le1 reference system
  - ▪ *Le1* primer 1   (10 µM):   240 µL
  - ▪ *Le1* primer 2   (10 µM):   240 µL
  - ▪ *Le1* probe   (5 µM):   160 µL

  MON 87701 system
  - ▪ MON 87701 primer 1   (20 µM):   480 µL
  - ▪ MON 87701 primer 2   (20 µM):   480 µL
  - ▪ MON 87701 probe   (5 µM):   800 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 MON 87701 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.

Table 2. MON 87701 GM contents

MON 87701 GM%
(GM copy number/soybean genome copy number x 100)
0.085
0.26
0.90
2.70
8.10

5.   Method

Description of operational steps followed

For the specific detection of event MON 87701 genomic DNA, an 89-bp fragment of the region that spans the 5' plant-to-insert junction in soybean MON 87701 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 (6-carboxyfluorescein) as reporter dye at its 5' end and TAMRA (carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of event MON 87701 DNA, a soybean-specific reference system amplifies a 74-bp fragment of the endogenous gene lectin *Le1*, using two *Le1* specific primers and a *Le1* specific probe labelled with FAM as a reporter dye and TAMRA as a quencher dye.

Standard curves are generated for both the MON 87701 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 MON 87701 DNA in a test sample, the MON 87701 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 to obtain the percentage value (GM % = MON 87701/*Le1* x 100).

Calibration sample S1 was prepared by mixing the appropriate amount of MON 87701 DNA in control non-GM soybean DNA to obtain a 9% GM MON 87701. Sample S2 was prepared by three-fold dilution from the S1 sample; sample S3 was prepared by three-fold dilution from the S2 sample; sample S4 was prepared by three-fold dilution of the 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 mass (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	200	66.7	22.2	7.41	1.48
Soybean genome copies	176991	58997	19666	6555	1311
MON 87701 GM soybean copies	15929	5310	1770	590	118

6.   Deviations reported

Eight laboratories reported no deviations from the protocol.

One laboratory performed PCR reactions in 25 µL of total volume because a RotorGene (Qiagen) instrument was used. Final concentrations of PCR reagents remained unchanged.

7.   Summary of results

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula $((10^{(-1/slope)})-1) \times 100)$ of the reference curve and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for the MON 87701 system and the *Le1* reference system are summarised in Table 4.

The mean PCR efficiency was 93 % for the MON 87701 system and 97 % for the *Le1* system, with both values within the European Network of GMO Laboratories (ENGL) acceptance criteria. The linearity of the method was 0.99 and 1.00 for the MON 87701 and the *Le1* systems, respectively.

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)

| Lab	Plate	MON 87701			Le1		
		Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.46	95	1.00	-3.43	96	1.00
	B	-3.48	94	1.00	-3.37	98	1.00
2	A	-3.80	83	0.97	-3.21	105	0.99
	B	-3.85	82	0.97	-3.55	91	1.00
3	A	-3.54	92	1.00	-3.42	96	1.00
	B	-3.44	95	1.00	-3.27	102	0.99
4	A	-3.65	88	1.00	-3.53	92	1.00
	B	-3.70	86	1.00	-3.50	93	1.00
5	A	-3.40	97	1.00	-3.28	102	0.99
	B	-3.36	98	1.00	-3.37	98	0.99
6	A	-3.43	96	1.00	-3.48	94	0.99
	B	-3.37	98	0.99	-3.42	96	1.00
7	A	-3.81	83	0.99	-3.40	97	1.00
	B	-3.40	97	1.00	-3.44	95	0.99
8	A	-3.27	102	1.00	-3.09	111	0.98
	B	-3.34	99	1.00	-3.30	101	1.00
9	A	-3.60	89	1.00	-3.42	96	1.00
	B	-3.46	95	1.00	-3.44	95	1.00
10	A	-3.58	90	1.00	-3.39	97	1.00
	B	-3.53	92	1.00	-3.37	98	1.00
11	A	-3.51	93	0.99	-3.46	95	1.00
	B	-3.16	107	1.00	-3.41	97	1.00
12	A	-3.48	94	1.00	-3.47	94	0.99
	B	-3.47	94	1.00	-3.46	95	0.99
	Mean	-3.50	93	0.99	-3.39	97	1.00

GMO quantification

Table 5 shows the mean values for the four replicates of 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)																			
	0.085				0.26				0.9				2.7				8.1			
	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	0.10	0.10	0.08	0.10	0.31	0.32	0.22	0.26	0.95	1.00	1.00	0.88	3.07	3.00	2.40	2.28	8.95	7.27	7.08	6.96
2	0.13	0.13	0.08	0.09	0.27	0.26	0.23	0.29	1.05	0.56	0.93	0.64	2.21	2.09	1.54	1.79	4.33	4.99	6.82	3.33
3	0.08	0.07	0.08	0.12	0.32	0.27	0.24	0.26	0.62	1.04	0.80	1.10	2.62	2.96	2.45	2.87	10.77	6.64	6.02	9.68
4	0.12	0.10	0.09	0.10	0.32	0.31	0.29	0.31	0.97	1.05	1.01	1.04	2.88	2.78	3.00	2.61	8.05	7.40	7.72	7.54
5	0.08	0.06	0.07	0.08	0.20	0.25	0.22	0.20	0.94	0.83	1.04	0.70	2.57	2.68	2.00	2.12	7.12	7.51	7.77	6.87
6	0.09	0.09	0.08	0.07	0.45	0.32	0.21	0.30	0.64	0.89	1.14	1.05	2.94	3.65	2.21	2.42	9.01	9.26	8.89	8.05
7	0.10	0.12	0.09	0.09	0.45	0.32	0.19	0.35	0.88	0.92	1.98	0.93	3.57	3.88	2.63	2.46	7.12	12.88	11.72	7.34
8	0.08	0.08	0.08	0.07	0.23	0.24	0.25	0.21	0.98	0.90	1.06	1.05	2.86	2.80	2.47	3.16	8.36	8.83	8.16	7.38
9	0.09	0.12	0.10	0.11	0.32	0.27	0.22	0.35	0.94	1.09	1.13	0.96	3.06	3.36	2.72	2.53	8.50	7.42	8.98	7.37
10	0.10	0.10	0.08	0.10	0.31	0.30	0.27	0.31	0.93	1.08	1.00	0.97	3.05	2.75	2.53	2.72	8.84	7.96	8.24	7.72
11	0.09	0.07	0.14	0.06	0.20	0.34	0.22	0.17	0.93	1.09	1.07	0.83	3.01	2.88	2.98	3.05	9.49	9.21	8.46	6.67
12	0.11	0.18	0.10	0.20	0.67	0.37	0.39	0.51	1.11	1.33	2.22	1.73	4.98	7.41	4.42	3.98	11.38	11.86	12.74	11.94

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

Figure 1. Relative deviation (%) from the true value of MON 87701 for all laboratories
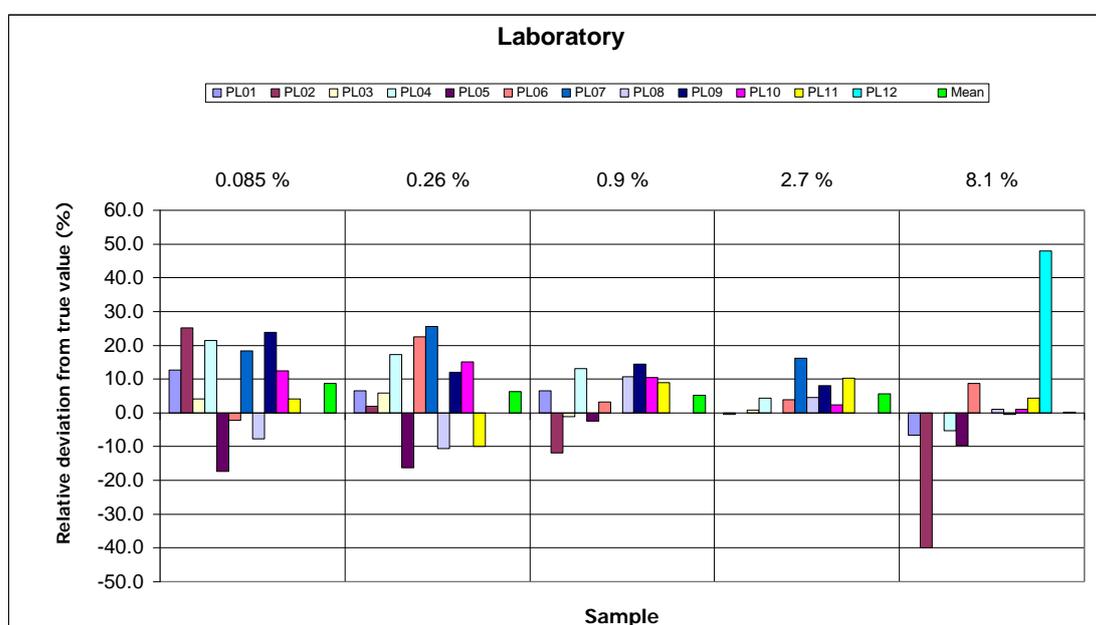
As observed in Figure 1, the mean relative deviations from the true values are positive for all GM levels tested apart from the 8.1% GM level, where it is close to zero. This means that the GM content tends to be over-estimated at most GM levels. For samples between 0.085% and 0.9 % GM levels, single laboratories showed deviations from the true value above 25%. For the 8.1% GM level, two laboratories showed deviations from the true value above 25%. However,

the average relative deviations for all GM levels are well below 25%, which is the performance limit set by the ENGL.

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 EURL-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 23% at the 8.1% GM level, thus well within the acceptance criterion.

Table 6. MON 87701: summary of validation results

unknown sample GMO %	Expected value (GMO%)				
	0.085	0.26	0.9	2.7	8.1
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	1	1	2	3	2
Reason for exclusion	1 C	1G	1C 1G	1 C 2 DG	2 C
Mean value	0.09	0.28	0.95	2.85	8.11
Relative repeatability standard deviation, RSD_r (%)	18	21	15	14	10
Repeatability standard deviation	0.02	0.06	0.15	0.40	0.82
Relative reproducibility standard deviation, RSD_R (%)	20	22	15	14	23
Reproducibility standard deviation	0.02	0.06	0.15	0.40	1.88
Bias (absolute value)	0.007	0.02	0.05	0.15	0.01
Bias (%)	8.6	6.4	5.2	5.6	0.1

C = Cochran's test; G = Grubbs' test; DG = double 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 EURL-GMFF requires that the RSD_r value is below25 %, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). As it can be observed from the values reported, the

method showed a repeatability standard deviation below 25% at all GM levels, with the highest value of RSD_r (%) of 21% at the 0.26% 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. As reported in table 6, the method satisfies this requirement across the dynamic range tested; in fact the highest bias was equal to 8.6% at the 0.085% GM level.

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

11.   References

1. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
2. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem.*, 67, 331-343.
3. Arumuganathan K. and Earle E. D., 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 the 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. cost/sample) of the method.

Acceptance criteria: The method should generally be practicable in line with other methods for a similar purpose. More specifically the method is deemed unacceptable, unless suitable justification is supplied, if:

- it requires a new type of apparatus (not generally available) or expensive equipment; or

- the resources required to perform the method (time, workload, reagents, costs) are considerably higher than the resources required to perform other methods for similar purpose.

Other practicability considerations may also deem the method impracticable.

DNA Extraction and Purification

The aim of a DNA extraction procedure is to provide DNA of suitable quality for subsequent analysis. DNA quality depends on the average length, structural integrity and chemical purity of the extracted DNA.

It is recognised that highly fragmented DNA and co-extracted impurities of a DNA preparation may hinder the correct process of detecting and quantifying genetically modified DNA. Food and feed made of various ingredients may exert a matrix effect, depending on the DNA extraction method employed, and impair the sensitivity of the following analytical approach. For this purpose, critical steps of DNA extraction and purification should be clearly highlighted in the technical documentation accompanying a method and acceptance criteria are established to allow objective determination of PCR quality of DNA

extracts which can be considered suitable for subsequent detection experiments (e.g. qualitative and/or quantitative PCR).

DNA extraction procedures should result in repeatable recovery, fragmentation profile, concentration and PCR quality of DNA extracts. As such, it is recommended to process the given DNA extraction protocol on different days (e.g. 3 days) with an adequate number of test portions (e.g. 6 per day).

In agreement with international guidelines (e.g. EN ISO 21571[Error! Reference source not found.], EN ISO 24276[Error! Reference source not found.]) the following criteria are used to assess method performance.

a) DNA concentration

Definition: amount of an analyte per unit volume of solution

Acceptance criterion: The DNA extraction method employed shall be appropriate to obtain the quantity of nucleic acid required for the subsequent analysis. The DNA concentration measured as weight of the analyte/volume of solution should be higher than the working concentration described in the protocol of the detection method.

Example: if the RT-PCR protocol indicates 40 ng/µL as the DNA concentration of the DNA solution to be added to the master-mix, the concentration of the DNA extract should be > 40 ng/µL.

b) DNA fragmentation state

Definition: Breakage of genomic (high molecular weight) DNA into smaller DNA fragments

Acceptance criterion: For quantitative (real time-based) analysis, the molecular weight of the extracted DNA sample should be at least higher than the amplicon size produced by the event specific and the taxon specific reference systems as established by comparison with a reference nucleic acid marker.

For qualitative analysis, in case of DNA suspensions to be used in qualitative analysis, the presence of a certain proportion of DNA molecules of molecular weight lower than the amplicon size produced by the method may be considered acceptable.

c) Purity of DNA extracts

Definition: the absence of co-extracted compounds in a DNA sample impairing the efficiency of the PCR reactions and leading to a delay in the onset of the exponential phase of the amplification profile

Acceptance criterion: The difference (ΔCt) average between the measured Ct value and the extrapolated Ct value of the first diluted sample of the inhibition test should be <0.5. [(measured Ct – extrapolated Ct)] <0.5 and the slope of the inhibition curve should be within -3.6 and -3.1.

The preferred PCR assay for the inhibition test is the internal control assay (e.g. the taxon specific reference system). The total DNA amount in the first sample of the dilution series should be not less than the total DNA amount used in the submitted method (e.g. the DNA amount indicated in the PCR protocol of the taxon specific reference system).

Specificity

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

Acceptance Criterion: The method should not produce amplification signals with target sequences different for the target sequence for which the method was developed. This should be demonstrated by similarity searches against databases (e.g. EMBL, GenBank, Patent, etc.) and with empirical results from testing the method with non-target transgenic events and non-transgenic material.

For detection of specific GM events, the target sequence shall be event specific.

For taxon specific target sequences (target sequence), the absence of allelic and copy-number variation across a globally representative and diverse sample of the species variety shall be demonstrated. Allelic and/or copy-number variation in other lines shall be reported if such variation is known by the applicant. The specificity of the target sequence shall be verified by *in silico* studies against publicly available sequence databases (e.g. EMBL, GenBank, etc.) and experimentally by demonstrating the absence of amplification products when the target sequence specific assay is applied to individual PCRs of pure genomic DNA of a representative sample of the closest relatives to the target taxa as well as of the most important food crops.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of trueness 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 range of the standard curve(s) for real-time PCR should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) ends.
Example: 0.09% and 4.5% for a 0.9% GMO concentration or 50 and 2500 genome copies if the target is 500 copies.

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 shall 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.

Precision: Relative 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 Quantification (LOQ)

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

Acceptance Criterion: LOQ should be less than 1/10th of the value of the target concentration with an RSD$_r$ $\leq$ 25 %. Target concentration should be intended as the threshold relevant for legislative requirements.

Limit of Detection (LOD)

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

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

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.
Note: The adequacy of the robustness testing needs to be demonstrated on a method-by-method basis. For instance, for a real-time PCR method, the following factors and their origin/source shall be taken into account: different thermal cycler models, DNA polymerase, uracyl-n-glycosylase, magnesium chloride concentration, primer forward and reverse concentration, probe concentration, temperature profile, time profile, dNTP including dUTP concentrations.
Acceptance Criterion: The response of an assay with respect to these small changes shall not deviate more than ±30%.
Alternatively, robustness can be demonstrated through the application of formal robustness tests using factorial designs such as those published by Plackett Burman or Youden.

Method Performance Requirements

Precision: Relative Reproducibility Standard Deviation (RSD$_R$)

Definition: The relative 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 RSD_R should be <35% over the whole dynamic range. However, at concentrations <0.2% then RSD_R values <50% are deemed acceptable.

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.