Event-specific Method for the Quantification of Cotton MON 88913 Using Real-time PCR

Validation Report

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Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics 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 MON 88913 transformation event in cotton DNA (unique identifier MON-88913-8). The collaborative trial was conducted according to internationally accepted guidelines [1, 2].

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed" and with Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003", Monsanto provided the detection method and the samples: genomic DNA from cotton seeds harbouring the MON 88913 event (line ST 4664) and from conventional cotton seeds (line ST 474). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from nine 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/ .

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Report on Steps 1-3 of the Validation Process

Monsanto submitted the detection method and control samples for cotton event MON 88913 (unique identifier MON-88913-8) 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, two scientific assessments were performed for cotton event MON 88913 and positively concluded in August 2007.

Between August 2007 and August 2008, the CRL-GMFF experimentally verified the purity of the control samples provided, and requests for replacement samples were made. The method characteristics were verified by quantifying five blind GM levels within the range 0.09%-8.0% 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 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 10th June 2008 (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

Monsanto submitted the detection method and control samples for cotton event MON 88913 (unique identifier MON-88913-8) 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, Molecular Biology and Genomics Unit of the Institute for Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Regulation (EC) No 1829/2003) organised the international collaborative study for the event-specific method for the detection and quantification of cotton MON 88913. 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 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 Regulation (EC) No 641/2004 and following its operational procedures.

The internal experimental evaluation of the method was carried out between August 2007 and August 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 September 2008.

A method for DNA extraction from seeds and grains followed by PEG precipitation, 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 MON 88913 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton endogenous gene, *acp1* (reference gene) and the target assay (MON 88913) 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 August 2008, the CRL-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 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 cotton event MON 88913.

Thirty-two laboratories expressed in writing their willingness to participate, two declined the invitation, while thirty-eight 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 cotton line MON 88913.

Laboratory	Country
Central Agricultural Office, Food and Feed Safety Directorate, Central Feed Investigation Laboratory	HU
Central Science Laboratory	UK
Crop Research Institute - Reference Laboratory for GMO Detection and DNA fingerprinting	CZ
Federal Institute for Risk Assessment	DE
Groupement d'Intérêt Public – Groupe d'Etude et de contrôle des Variétés et des Productions Agricoles	FR
Institute for National Investigation for the Health and Veterinarian Nature Saxonia	DE
Institute of Chemical Technology Prague	CZ
LGC Limited	UK
National Centre for Food, Spanish Food Safety Agency	ES
National Institute of Biology	SI
Scientific Institute of Public Health (IPH)	BE
Veterinary Public Health Institute for Lazio and Toscana Regions; National Reference Centre for GMO Analysis	IT

3. Materials

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

i) genomic DNA extracted from homozygous cotton seeds harbouring the event MON 88913 (Lot No. GLP-0602-16861-S), and
ii) genomic DNA extracted from conventional cotton seeds (Lot No. GLP-0602-16862-S).

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

Samples containing mixtures of 100% cotton MON 88913 and non-GM cotton genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the control samples provided, in a constant amount of total cotton 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), two bottles: 5 mL each
  - distilled sterile water, one tube: 4 mL

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

  acp1 reference system

  - *acp1* primer 1 (10 µM): 240 µL
  - *acp1* primer 2 (10 µM): 240 µL
  - *acp1* probe (10 µM): 160 µL

  MON 88913 system

  - MON 88913 primer 1 (10 µM): 800 µL
  - MON 88913 primer 2 (10 µM): 800 µL
  - MON 88913 probe (10 µM): 320 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 88913 specific system and for the *acp1* 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. MON 88913 GM contents

MON 88913 GM% (GM copy number/cotton genome copy number x 100)
0.09
0.30
0.90
3.00
8.00

5. Method

Description of operational steps followed

For the specific detection of event MON 88913 genomic DNA, a 94-bp fragment of the region that spans the 5' plant-to-insert junction in cotton MON 88913 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 MGBNFQ as quencher dye at its 3' end.

For the relative quantification of event MON 88913 DNA, a cotton-specific reference system amplifies a 76-bp fragment of *acp1*, a cotton endogenous gene encoding an acyl carrier protein, using two *acp1* gene-specific primers and an *acp1* gene-specific probe labelled with FAM dye and TAMRA as quencher dye.

Standard curves are generated for both the MON 88913 and the *acp1* 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 88913 DNA in a test sample, the MON 88913 copy number is divided by the copy number of the cotton reference gene (*acp1*) and multiplied by 100 to obtain the percentage value (GM% = MON 88913/*acp1* x 100).

Calibration sample S1 was prepared by mixing the appropriate amount of MON 88913 DNA in control non-GM cotton DNA to obtain a 10% GM MON 88913. Sample S2 was prepared by two-fold dilution from the S1 sample; sample S3 was prepared by five-fold dilution from S2 sample; sample S4 was prepared by three-fold dilution of sample S3 and sample S5 was prepared by four-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 cotton genome (2.33 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	100	20	6.68	1.68
Cotton genome copies	85837	42919	8584	2861	715
MON 88913 GM cotton copies	8584	4292	858	286	72

6. Deviations reported

Seven laboratories reported no deviations from the protocol.

Two laboratories reported a different plate set-up from the protocol, with no consequences in the analysis since the results were correctly attributed to the corresponding samples.

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 minor amplification in a reagent control sample, as consequence of an experimental error, introducing a tip containing the U12 unknown sample in the control sample well already filled. However, this had no effect in the results.

One laboratory reported not centrifuging the plates prior to PCR analysis, as established in the protocol.

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) x 100) of the reference curve and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for the MON 88913 system and the *acp1* reference system are summarised in Table 4.

The mean PCR efficiency was 105% for the MON 88913 system and 101% for the *acp1* system, with both values within the ENGL acceptance criteria. The linearity of the method was 0.99 and 1.00 for MON 88913 and *acp1* 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)

		MON 88913			*acp1*		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.38	98	0.99	-3.29	101	1.00
	B	-3.32	100	1.00	-3.32	100	1.00
2	A	-3.31	101	1.00	-3.41	97	1.00
	B	-3.31	100	1.00	-3.36	99	1.00
3	A	-3.37	98	1.00	-3.31	100	1.00
	B	-3.41	96	1.00	-3.30	101	1.00
4	A	-3.20	105	1.00	-3.32	100	1.00
	B	-3.26	103	0.99	-3.23	104	1.00
5	A	-3.43	96	1.00	-3.34	99	1.00
	B	-3.40	97	1.00	-3.35	99	1.00
6	A	-3.30	101	0.99	-3.22	104	1.00
	B	-3.40	97	0.99	-3.24	103	1.00
7	A	-3.24	103	1.00	-3.33	100	1.00
	B	-3.34	99	1.00	-3.36	98	1.00
8	A	-3.32	100	1.00	-3.32	100	1.00
	B	-3.30	101	1.00	-3.14	108	0.99
9	A	-3.26	103	1.00	-3.29	101	1.00
	B	-3.30	101	0.99	-3.24	104	1.00
10	A	-3.12	109	0.98	-3.22	105	0.99
	B	-2.85	125	0.99	-3.35	99	1.00
11	A	-2.67	137	0.94	-3.19	106	1.00
	B	-2.65	139	0.89	-3.22	105	1.00
12	A	-3.31	100	0.99	-3.44	95	0.99
	B	-2.87	123	0.99	-3.14	108	1.00
	Mean	-3.22	105	0.99	-3.29	101	1.00

GMO quantification

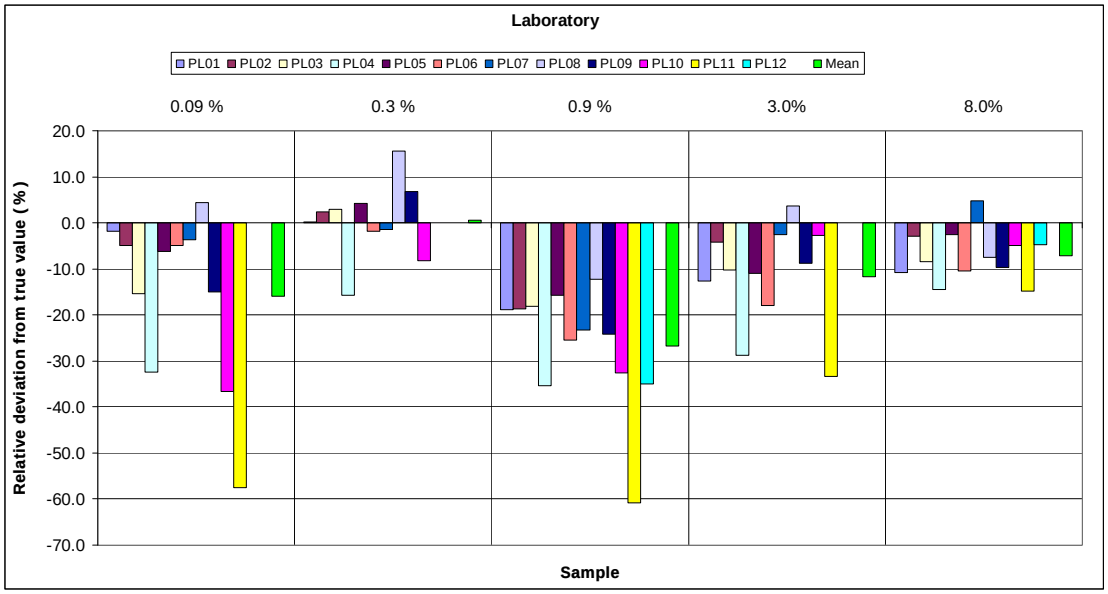
Table 5 shows the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

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

	GMO content (GMO% = GMO copy number/cotton genome copy number x 100)																			
LAB	0.09				0.3				0.9				3.0				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	0.11	0.09	0.07	0.08	0.30	0.32	0.26	0.33	0.74	0.81	0.64	0.73	2.47	2.94	2.53	2.54	6.99	8.08	7.27	6.21
2	0.07	0.10	0.09	0.09	0.31	0.33	0.28	0.30	0.69	0.76	0.67	0.81	2.98	2.80	2.91	2.82	7.45	8.02	7.47	8.11
3	0.08	0.08	0.08	0.07	0.30	0.31	0.32	0.30	0.79	0.76	0.69	0.70	2.65	2.94	2.85	2.33	6.95	7.52	7.73	7.13
4	0.07	0.05	0.06	0.05	0.27	0.21	0.24	0.28	0.63	0.52	0.57	0.60	1.74	2.05	2.00	2.76	6.55	6.43	5.85	8.57
5	0.09	0.08	0.09	0.09	0.29	0.30	0.34	0.32	0.72	0.75	0.79	0.76	2.77	2.69	2.63	2.58	7.36	7.57	7.36	8.88
6	0.10	0.09	0.08	0.08	0.29	0.32	0.26	0.31	0.74	0.66	0.57	0.72	2.59	2.49	2.30	2.47	7.11	6.89	7.72	6.98
7	0.09	0.09	0.09	0.08	0.30	0.34	0.26	0.29	0.73	0.73	0.64	0.67	3.39	2.62	2.72	2.97	8.63	8.23	8.44	8.21
8	0.07	0.10	0.09	0.11	0.37	0.38	0.33	0.31	0.93	0.78	0.73	0.72	3.78	2.74	2.81	3.10	7.31	7.25	7.52	7.53
9	0.08	0.08	0.08	0.07	0.35	0.32	0.29	0.32	0.76	0.60	0.65	0.72	2.89	2.79	2.64	2.62	6.82	6.83	7.36	7.91
10	0.06	0.07	0.06	0.04	0.33	0.26	0.22	0.29	0.71	0.70	0.47	0.53	2.50	3.15	3.11	2.92	6.65	6.93	7.24	9.58
11	0.03	0.03	0.05	0.04	0.08	0.20	0.10	0.13	0.41	0.21	0.34	0.46	2.35	1.64	2.08	1.93	5.90	5.33	9.50	6.50
12	0.02	0.02	0.11	0.11	0.11	0.11	0.37	0.27	0.47	0.39	0.73	0.75	3.10	1.29	1.24	2.52	7.86	6.21	8.07	8.33

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 88913 for all laboratories



As observed in Figure 1, the mean relative deviations from the true values are negative for most GM levels apart from 0.3% GM level, where it is positive. This means that the GM content tends to be under-estimated at most GM levels. For 0.9% GM level, five laboratories showed deviation from the true value above 25%, with an overall average relative deviation of 27%. For samples at 0.09% and 3.0% GM levels, some laboratories also showed deviations from the true value above 25%, however, the average relative deviations for those levels were well below 25%.

Overall, the average relative deviation is within the acceptance criterion at all GM levels tested, except for 0.9% GM level for which the value was slightly above the acceptance limit (27%), 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 25% at the 0.09% GM level, thus well within the acceptance criterion.

Table 6. MON 88913: summary of validation results.

unknown sample GMO %	Expected value (GMO%)				
	0.09	0.3	0.9	3.0	8.0
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	1	2	0	1	0
Reason for exclusion	1 C	1 C 1G	-	1 C	-
Mean value	0.08	0.30	0.66	2.65	7.42
Relative repeatability standard deviation, RSD_r (%)	13	10	13	11	12
Repeatability standard deviation	0.01	0.03	0.09	0.28	0.89
Relative reproducibility standard deviation, RSD_R (%)	25	12	21	16	12
Reproducibility standard deviation	0.02	0.04	0.14	0.42	0.89
Bias (absolute value)	-0.01	0.001	-0.24	-0.35	-0.58
Bias (%)	-16	0.5	-27	-12	-7.2

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

Table 6 further documents the relative repeatability standard deviation (RSD_r), as estimated for each GM level. In order to accept methods for collaborative study evaluation, the CRL requires that 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). As 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 13% at 0.09% and 0.9% GM levels.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. In this case, the method satisfies this requirement across the dynamic range tested, apart from the 0.9% GM level that shows a bias of -27%.

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 mostly 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.
2. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
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 a method can be applied.

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

Practicability

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

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

Specificity

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

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

Dynamic Range

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

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

Accuracy

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

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

Amplification Efficiency

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

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

R^2 Coefficient

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

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

Repeatability Standard Deviation (RSD,)

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

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

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

Limit of Quantitation (LOQ)

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

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

Limit of Detection (LOD)

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

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

Robustness

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

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

Method Performance Requirements

Dynamic Range

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

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

Reproducibility Standard Deviation (RSD_R)

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

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

Trueness

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

Acceptance Criterion: the trueness should be within $\pm$ 25% of the accepted reference value over the whole dynamic range.