Event-specific Method for the Quantification of Soybean CV127 Using Real-time PCR

Validation Report

20 September 2011

Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

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 CV-127 transformation event in soybean DNA (unique identifier BPS-CV-127-9). 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", BASF Plant Science GmbH provided the detection method and the samples [genomic DNA from soybean seeds harbouring the CV127 event (line 127) and from conventional soybean seeds (line Conquista)]. 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 ten 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

BASF Plant Science GmbH provided the detection method and control samples for soybean event CV127 (unique identifier BPS-CV127-9) according to Articles 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/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 soybean event CV127 and positively concluded in March 2009.

Between March and July 2009, the EURL-GMFF verified experimentally the purity of the control samples provided and conducted the in-house experimental testing of samples and methods (step 3). The method characteristics were verified by quantifying five blind GM levels within the range 0.09%-4.5% on a copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision were within the limits established by the ENGL. The DNA extraction module of the method was also tested on samples of food and feed and a report is published on the EURL-GMFF website (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

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

Content

1. Introduction

BASF Plant Science GmbH provided the detection method and control samples for soybean event CV127 (unique identifier BPS-CV127-9) according to 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 organised the international collaborative study for the event-specific method for the detection and quantification of soybean CV127. 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 methods, 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.

The internal experimental evaluation of the method was carried out between March and July 2009.

Following the evaluation of the data and the results of the internal tests, the international collaborative study was organised (step 4) and took place in July 2009.

A CTAB method for DNA extraction from ground seeds followed by anion exchange chromatography, 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 validating a quantitative real-time PCR (Polymerase Chain Reaction) method. The method is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event CV127 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean *le1* (*lectin 1*) endogenous assay, and the target assay (CV127) are performed in separate wells.

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

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

2. List of participating laboratories

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

On 30th June 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 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 CV127.

Thirty-four laboratories expressed in writing their willingness to participate, five declined the invitation, while thirty-one 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 CV127.

Laboratory	Country
Agricultural Institute of Slovenia	SI
Austrian Agency for Health and Food Safety	AT
Danish Plant Directorate	DK
Finnish Customs Laboratory	FI
Laboratory Agroalimentary of the Ministry of the Environment	ES
Laboratory for the Detection of GMO in Food	DE
Laboratory of DNA analysis	EE
LGC Limited	UK
Lower Saxony Federal State Office for Consumer Protection and Food Safety	DE
National Centre for Food, Spanish Food Safety Agency	ES
National Diagnostic Centre of Food and Veterinary Service	LV
Walloon Agricultural Research Centre (CRA-W)	BE

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 CV127 (line 127), and
ii) genomic DNA extracted from conventional soybean seeds (Conquista).

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 soybean CV127 and non-GM soybean genomic DNA at different GMO percentages 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 (200 µL of DNA solution each) labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (100 µL of DNA solution each) labelled from U1 to U20.
- ✓ Reaction reagents:
  - Universal PCR Master Mix (2x), two bottles: 5 mL each
  - distilled sterile water, one tube: 3.8 mL

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

  le1 taxon-specific assay
  - *Lec F* (10 µM): 120 µL
  - *Lec R* (10 µM): 120 µL
  - *Lec P* (10 µM): 40 µL

  CV127 assay
  - SE-127-f4 (10 µM): 320 µL
  - SE-127-r2 (10 µM): 320 µL
  - SE-127-p3 (10 µM): 320 µL

4. Experimental design

Twenty unknown samples (labelled from U1 to U20), representing five GM levels, were included in the validation study (Table 2). On each PCR plate, the samples were analysed for the CV127 specific system and for the *le1* taxon-specific system. In total, two plates were run per participating laboratory and four replicates for each GM level were analysed. PCR 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 application provided.

Table 2. CV127 GM contents

CV127 GM% (GM copy number/soybean genome copy number x 100)
0.09
0.30
0.90
2.50
4.50

5. Method

For the detection of event CV127, an 88-bp fragment of the region spanning the 3' plant-to-insert junction is amplified. 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 GM event CV127, a soybean-specific reference system amplifies a 74-bp fragment of the soybean endogenous gene *le1*, using *le1* specific primers and a *le1* specific probe labelled with FAM as reporter dye and TAMRA as quencher dye.

Standard curves are generated for both the CV127 and the *le1* 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 CV127 DNA in a test sample, the CV127 copy number is divided by the copy number of the soybean reference gene (*le1*) and multiplied by 100 to obtain the percentage value (GM% = CV127/*le1* x 100).

The calibration sample S1 was prepared by mixing the appropriate amount of CV127 DNA in control non-GM soybean DNA to obtain a 5% GM CV127. Calibration samples S2-S5 were prepared by 2.8-fold dilution from the S1 sample.

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (nanograms) by the published average 1C value for the soybean genome (1.15 pg) [(3)]. The copy number values used in the quantification, the GMO contents of the calibration samples and total DNA quantity used in PCR are listed in Table 3.

Table 3. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	100	36	12.8	4.6	1.6
Soybean genome copies	86960	31300	11140	4000	1400
CV127 GM copies	4348	1565	557	200	70

6. Deviations reported

Eight laboratories reported no deviations from the protocol.

One laboratory repeated plate A due to swapping in the position of several samples.

One laboratory repeated plate A for the lectin assay due a technical problem occurred to the last point (S5) of the calibration curve.

One laboratory swapped the position of the calibration samples of the GM and of the taxon-specific assay on the plates.

One laboratory loaded the samples U1 to U4 in the wells assigned to S1 to S4 in one plate.

One laboratory swapped sample U4 with U5 in the loading order in one plate.

7. 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 standard curve and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for the CV127 and the *le1* assays are reported in Table 4.

The mean PCR efficiency was 90% for the CV127 assay and 98% for the *le1* assay, with both values within the ENGL acceptance criteria. The R^2 of the method was 0.99 and 1.00 for CV127 and *le1* assays, respectively.

The results confirm the appropriate performance characteristics of the method tested in terms of efficiency and linearity.

Table 4. Values of slope, PCR efficiency and R^2 obtained during the validation study

		CV127			*le1*		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.59	90	1.00	-3.34	99	1.00
	B	-3.61	89	1.00	-3.34	99	0.98
2	A	-3.47	94	0.99	-3.23	104	0.98
	B	-3.50	93	1.00	-3.35	99	0.99
3	A	-4.00	78	1.00	-3.52	92	1.00
	B	-3.56	91	1.00	-3.43	96	1.00
4	A	-3.58	90	1.00	-3.40	97	1.00
	B	-3.57	91	1.00	-3.38	98	1.00
5	A	-3.57	90	1.00	-3.36	99	1.00
	B	-3.51	93	1.00	-3.36	99	1.00
6	A	-3.57	91	1.00	-3.32	100	1.00
	B	-3.52	92	1.00	-3.32	100	1.00
7	A	-3.51	93	1.00	-3.46	95	1.00
	B	-3.51	93	1.00	-3.43	96	1.00
8	A	-3.56	91	1.00	-3.35	99	1.00
	B	-3.73	86	1.00	-3.41	96	1.00
9	A	-3.38	97	0.99	-3.52	92	1.00
	B	-3.50	93	1.00	-3.17	107	0.99
10	A	-3.65	88	0.99	-3.34	99	1.00
	B	-3.50	93	1.00	-3.12	109	1.00
11	A	-3.63	88	1.00	-3.34	99	1.00
	B	-3.60	90	1.00	-3.37	98	1.00
12	A	-3.71	86	1.00	-3.46	94	1.00
	B	-3.72	86	1.00	-3.48	94	0.99
	Mean	-3.59	90	1.00	-3.37	98	1.00

GMO quantification

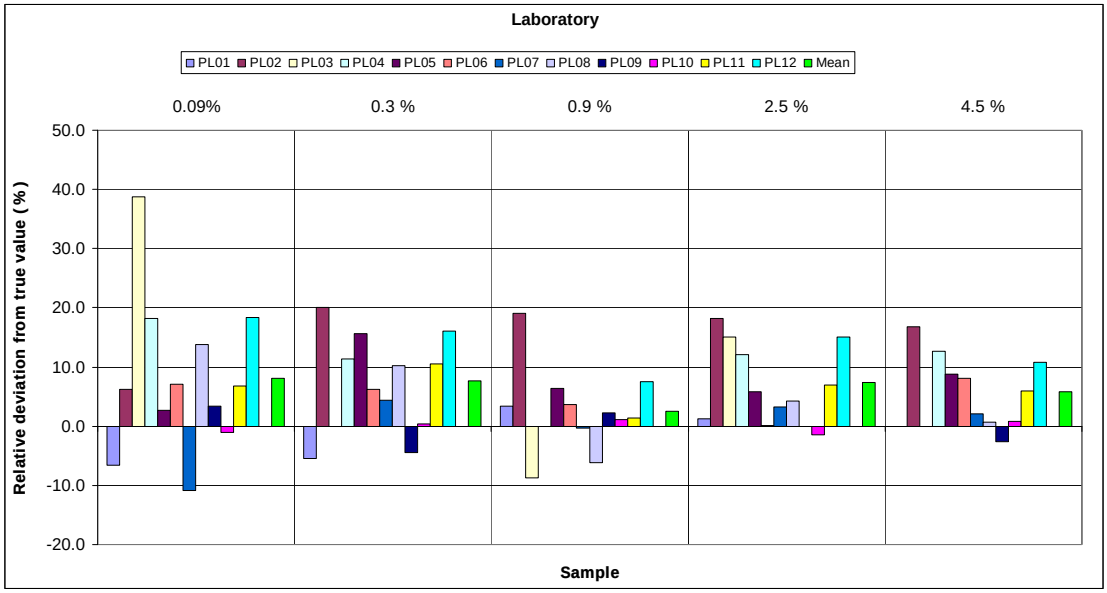
Table 5 reports the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

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

	GMO content (GMO% = GMO copy number / soybean genome copy number x 100)																			
LAB	0.09				0.3				0.9				2.5				4.5			
	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	0.09	0.07	0.08	0.29	0.31	0.29	0.24	0.89	0.91	1.00	0.92	2.48	2.40	2.61	2.64	4.29	4.60	4.76	4.35
2	0.08	0.10	0.11	0.10	0.43	0.32	0.35	0.33	0.89	1.01	1.26	1.12	2.80	2.73	2.98	3.32	4.95	5.18	5.81	5.08
3	0.00	0.10	0.14	0.14	0.29	0.34	0.05	0.39	0.46	0.81	0.80	1.21	3.45	2.51	2.77	2.78	0.39	1.44	5.11	0.24
4	0.11	0.10	0.12	0.09	0.36	0.35	0.34	0.29	0.80	0.95	0.80	1.05	2.95	2.60	2.88	2.78	4.94	5.18	5.10	5.06
5	0.09	0.08	0.10	0.10	0.33	0.32	0.39	0.36	0.94	0.90	0.97	1.03	2.67	2.68	2.51	2.72	5.20	4.64	4.63	5.12
6	0.09	0.08	0.11	0.10	0.32	0.33	0.31	0.31	0.83	1.06	0.82	1.03	2.66	2.45	2.45	2.45	5.21	4.50	4.98	4.76
7	0.08	0.08	0.08	0.08	0.32	0.33	0.31	0.30	0.88	0.90	0.95	0.86	2.44	2.64	2.59	2.65	5.15	4.91	4.31	4.00
8	0.12	0.10	0.10	0.10	0.35	0.36	0.32	0.29	0.88	0.84	0.80	0.86	2.48	2.73	2.87	2.35	4.45	4.75	4.74	4.19
9	0.09	0.09	0.09	0.10	0.26	0.20	0.37	0.32	0.90	0.58	1.09	1.11	3.42	2.19	2.28	2.31	4.78	4.21	3.16	5.37
10	0.09	0.08	0.09	0.10	0.28	0.29	0.32	0.31	0.91	0.76	0.98	0.99	2.55	2.32	2.49	2.49	4.64	4.81	4.60	4.10
11	0.10	0.09	0.10	0.09	0.34	0.34	0.34	0.30	0.92	0.88	0.83	1.02	2.83	2.71	2.59	2.56	4.34	5.05	5.10	4.57
12	0.09	0.12	0.11	0.10	0.36	0.40	0.34	0.30	0.86	1.01	0.87	1.13	2.93	2.70	2.84	3.04	4.79	5.30	5.50	4.35

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 CV127 for all laboratories



The mean relative deviations from the true values are positive for all GM levels, being well within the ENGL acceptance criterion of maximum 25%. One laboratory only overestimated the DNA content of sample 0.09% by almost 40%.

The average bias generated by all laboratories is modest, being equal or below 8.1%, 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 16% at the 0.9% GM level, thus well within the acceptance criterion.

Table 6. CV127: summary of validation results.

	Expected value (GMO %)				
unknown sample GMO %	**0.09**	**0.3**	**0.9**	**2.5**	**4.5**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	1	0	1	1
Reason for exclusion	-	C	-	C	C
Mean value	0.10	0.32	0.92	2.68	4.76
Relative repeatability standard deviation, RSD_r (%)	11	11	16	7.1	9.2
Repeatability standard deviation	0.010	0.036	0.144	0.189	0.439
Relative reproducibility standard deviation, RSD_R (%)	15	12	16	8.8	9.9
Reproducibility standard deviation	0.014	0.040	0.144	0.236	0.470
Bias (absolute value)	0.01	0.02	0.02	0.18	0.26
Bias (%)	8.1	7.8	2.5	7.3	5.9

C = Cochran's 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, the EURL-GMFF requires that the RSD_r value is below 25%, as indicated by the 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 16% at 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 ± 25% across the entire dynamic range. In this case, the method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 8.1% at the 0.09% 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. 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$_r$)

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

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

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

Limit of Quantitation (LOQ)

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

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

Limit of Detection (LOD)

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

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

Robustness

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

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

Method Performance Requirements

Dynamic Range

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

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

Reproducibility Standard Deviation (RSD_R)

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

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

Trueness

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

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