EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Community Reference Laboratory
CRL
GM Food and Feed

Event-specific Method for the Quantification of Cotton Line 'LLCotton25' Using Real-time PCR

Validation Report

14 March 2007

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

Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the LLCotton25 transformation event in cotton DNA (unique identifier ACS-GH∅∅1-3). The collaborative trial was conducted according to internationally accepted guidelines [1, 2].

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and with Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Bayer CropScience provided the detection method and the samples (genomic DNA extracted from wild-type and 100% LLCotton25 event). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved eleven laboratories from seven European countries.

The results of the international collaborative trial met the ENGL performance requirements and the scientific understanding about satisfactory method performance. Therefore, the CRL-GMFF considers the method validated as fit for the purpose of regulatory compliance.

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

Drafted by:
C. Savini

Report Verification Team:
1) W. Moens

2) M. Querci

Scientific and technical approval:
M. Mazzara

Compliance with CRL Quality System:
S. Cordeil

Authorisation to publish:
G. Van den Eede

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

Report on Steps 1-3 of the Validation Process

Bayer CropScience submitted the detection method and control samples for cotton event LLCotton25 (unique identifier ACS-GH∅∅1-3) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Community Reference Laboratory for GM Food and Feed (CRL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance 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.it/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.it/guidancedocs.htm) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2 and step 3 (experimental testing of the samples and methods), four scientific assessments were performed and requests of complementary information addressed to the applicant. Upon reception of complementary information, the scientific evaluation of the detection method for event LLCotton25 was positively concluded in November 2006.

Between October 2005 and November 2006, the CRL-GMFF verified experimentally the method characteristics (step 3) by quantifying five blind GM-levels within the range 0.15%-3.30% on a copy number basis. The experiments were performed in repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision of the quantifications were within the limits established by the ENGL. The DNA extraction module of the method was tested on samples of food and feed.

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

Content

1. INTRODUCTION 5

2. LIST OF PARTICIPATING LABORATORIES 6

3. MATERIALS 7

4. EXPERIMENTAL DESIGN 8

5. METHOD 8

DESCRIPTION OF OPERATIONAL STEPS FOLLOWED 8

6. DEVIATIONS REPORTED 9

7. SUMMARY OF RESULTS 9

PCR EFFICIENCY AND LINEARITY 9
GMO QUANTIFICATION 10

8. METHOD PERFORMANCE REQUIREMENTS 12

9. CONCLUSIONS 13

10. QUALITY ASSURANCE 13

11. REFERENCES 13

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

1. Introduction

Bayer CropScience submitted the detection method and control samples for cotton event LLCotton25 (unique identifier ACS-GH∅∅1-3) in accordance 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 Directorate General-Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute for Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Regulation EC 1829/2003) organised the international collaborative study for the event-specific method for the detection and quantification of LLCotton25. The study involved eleven laboratories, all members of the European Network of GMO Laboratories (ENGL).

Upon reception of method, samples and related data (step 1), the JRC carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3), according to the requirements of Regulation (EC) 641/2004 and following its operational procedures.
The internal in-house experimental evaluation of the method was carried out between October 2005 and November 2005.

Following the evaluation of the data and the results of the in-house laboratory tests, the international collaborative study was organised (step 4) and took place in December 2005.

A method for DNA extraction from LLCotton25 seeds, submitted by the applicant, was evaluated by the CRL-GMFF; laboratory testing of the method was carried out between January and November 2006 in order to confirm its performance characteristics. The protocol for DNA extraction and a report on method testing is available at http://gmo-crl.jrc.it/.

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

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event LLCotton25 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton *AdhC* (*Alcohol dehydrogenase*) endogenous assay (reference gene) and the target assay (LLCotton25) 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 eleven ENGL (European Network of GMO Laboratories) laboratories to determine its performance. Clear guidance was given to the laboratories with regards to the standard operational procedures to follow for the common execution of the protocol. The participating laboratories are listed in alphabetical order in Table 1.

Table 1. Laboratories participating in the validation of the detection method for cotton line 'LLCotton25'.

Laboratory	Country
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit	Germany
Bundesinstitut für Risikobewertung (BfR)	Germany
Centro Nacional de Alimentación - Agencia Española de Seguridad Alimentaria	Spain
Chemisches und Veterinäruntersuchungsamt Freiburg	Germany
Danish Plant Directorate - Laboratory for diagnostics in Plants, Seed and Feed	Denmark
Ente Nazionale Sementi Elette/ Laboratorio Analisi Sementi	Italy
Finnish Customs Laboratory - Tullilaboratorio	Finland
Institut für Hygiene und Umwelt der Hansestadt Hamburg	Germany
Istituto Superiore di Sanita' (ISS)	Italy
National Institute for Food Safety and Nutrition	Hungary
Umweltbundesamt GmbH	Austria

3. Materials

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

i) a DNA stock solution homozygous for the GM-event LLCotton25 (Lot Number 32RRMM0025)

and

ii) non-GM DNA stock solution (Lot Number 32RRMM0019) extracted from a genetically similar wild-type line

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

Samples containing mixtures of 100% LLCotton25 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 (200 µl of DNA solution each) for the preparation of the standard curve, denominated from S1 to S5.
- ✓ Twenty unknown DNA samples (100 µl of DNA solution each), denominated from U1 to U20.
- ✓ Amplification reagent control for use on each PCR plate.
- ✓ Reaction reagents, primers and probes for the *AdhC* reference gene and for the LLCotton25 specific systems as follows:

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

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

    AdhC reference system

    - ❑ KVM 158 primer(10 µM): 160 µl
    - ❑ KVM 157 primer (10 µM): 160 µl
    - ❑ TM012 TaqMan® probe (10 µM): 160 µl

    LLCotton25 system

    - ❑ KVM156 primer (10 µM): 320 µl
    - ❑ KVM155 primer (10 µM): 320 µl
    - ❑ TM018 TaqMan® probe (10 µM): 160 µl

Table 2 shows the GM contents of the unknown samples (denominated from U1 to U20) distributed to study participants.

Table 2. LLCotton25 GM contents

LLCotton25 GM % (GM copy number/cotton genome copy number *100)
0.15
0.40
0.90
2.00
3.30

4. Experimental design

Twenty unknown samples (denominated from U1 to U20), representing five GM levels, were used in the validation study. On each PCR plate, samples were analysed in parallel with both the LLCotton25 and *AdhC* specific systems. In total, two plates were run per participating laboratory, with two replicates for each GM level analysed on each run. In total, four replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. Participating laboratories carried out the determination of the GM% according to the instructions provided in the protocol and using the electronic tool provided (Excel spreadsheet).

5. Method

Description of operational steps followed

For the specific detection of event LLCotton25 DNA, a 79-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM is used as the reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For the relative quantification of event LLCotton25 DNA, a cotton-specific reference system amplifies a 73-bp fragment of the cotton endogenous gene *AdhC* (*Alcohol dehydrogenase C,* accession number AF036569), using a pair of *AdhC* gene-specific primers and an *AdhC* gene-specific probe labelled with FAM and TAMRA.

For relative quantification of event LLCotton25 DNA in a test sample, the normalised ΔCt values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCt values against the logarithm of the amount of LLCotton25 event DNA). The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of LLCotton25 event DNA is estimated.

Calibration samples denominated from S1 to S5 were prepared by mixing the appropriate amount of LLCotton25 DNA from the stock solution with non-GM cotton DNA to obtain the following relative contents of LLCotton25: 3.6%, 1.8%, 0.9%, 0.45% and 0.09%. Total DNA amount per reaction was 200 ng, when 5 µl of a DNA solution at the concentration of 40 ng/µl were loaded.

The GM contents of the calibration samples and total DNA quantity used in PCR are provided in Table 3 (%GM calculated considering the 1C value for cotton genome as 2.33 pg) [(3)].

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

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng/5 µl)	200	200	200	200	200
% GM (DNA/DNA)	3.60	1.80	0.90	0.45	0.09

6. Deviations reported

Eight laboratories reported no deviations from the protocol.

One laboratory inverted the loading order for the first plate, thus loading the upper half of the plate with the reference system and the lower half with the event-specific system.

One laboratory did not centrifuge the plates but loaded all the samples at the bottom of the reaction tube.

One laboratory froze samples and reagents in the elapse between the two PCR runs.

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)*100] of the reference curve and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for both runs (plates A and B) are summarised in Table 4.

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

LAB	PLATE	Slope	PCR Efficiency (%)	Linearity (R^2)
1	A	-3.59	90.03	0.99
	B	-3.58	90.41	0.97
2	A	-3.30	99.22	0.99
	B	-3.32	100	1.00
3	A	-3.57	90.52	0.89
	B	-3.58	90.23	0.88
4	A	-3.32	99.78	0.98
	B	-3.43	95.78	0.98
5	A	-3.83	82.42	0.99
	B	-3.59	89.91	0.95
6	A	-3.83	82.45	0.98
	B	-3.78	83.93	0.99
7	A	-3.47	94.20	0.99
	B	-3.62	88.80	1.00
8	A	-3.42	96.23	0.99
	B	-3.11	90.31	0.98
9	A	-3.67	87.33	0.97
	B	-3.64	88.26	0.96
10	A	-3.00	84.77	0.99
	B	-3.46	94.66	0.99
11	A	-3.28	98.29	0.98
	B	-3.48	93.74	0.99
	Mean	**-3.49**	**91.4**	**0.97**

The mean PCR efficiency was above 91%. The linearity of the method was on average 0.97. However, the poor linearity in both runs reported by one laboratory (number 3) contributed significantly to the reduction of the overall mean. If the linearity shown in the two said runs is eliminated, the average value raises to 0.98, in accordance with the ENGL criteria for linearity.
Therefore, the data reported confirm the appropriate performance characteristics of the method tested.

GMO quantification

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

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

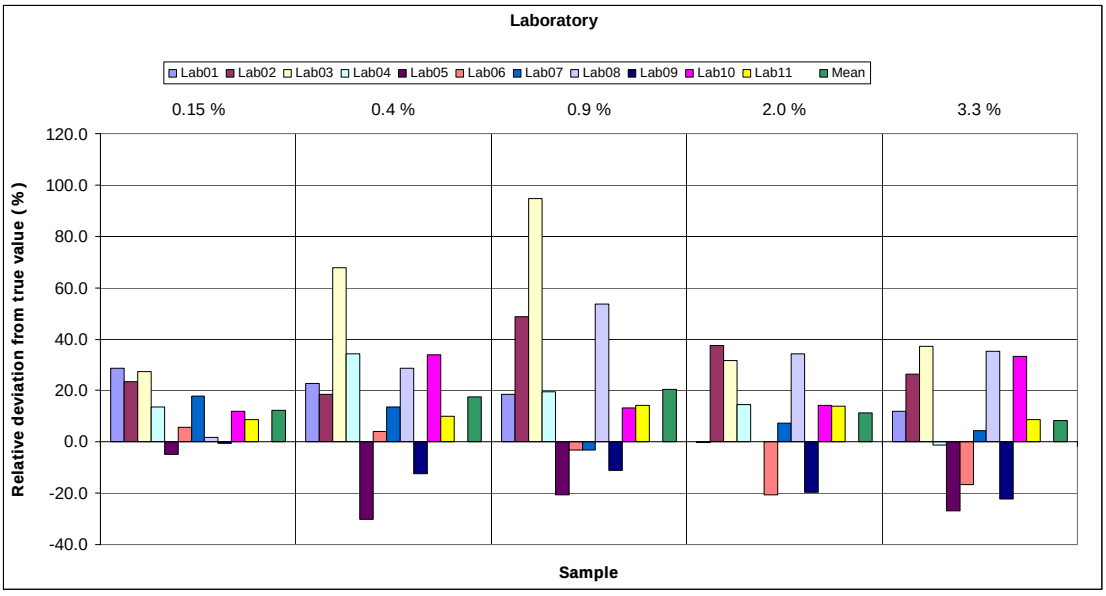
	Sample GMO content (GM% = GM copy number/cotton genome copy number *100)																			
LAB	0.15				0.40				0.90				2.00				3.30			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.24	0.18	0.18	0.17	0.57	0.60	0.35	0.44	1.01	1.33	1.03	0.89	2.35	2.91	1.34	1.39	4.48	4.22	2.79	3.28
2	0.24	0.18	0.16	0.16	0.49	0.45	0.45	0.50	1.15	1.90	1.15	1.15	2.51	2.66	2.97	2.86	4.11	4.83	4.06	3.66
3	0.18	0.14	0.23	0.21	0.45	0.65	0.54	1.04	1.60	1.64	2.12	1.65	2.25	3.06	2.42	2.81	3.05	4.11	4.10	6.87
4	0.15	0.15	0.18	0.20	0.43	0.44	0.41	0.87	1.26	1.25	0.74	1.04	1.73	2.47	2.48	2.48	3.71	3.98	1.83	3.51
5	0.11	0.23	0.16	0.08	0.32	0.26	0.25	0.29	0.87	0.73	0.81	0.44	2.01	12.89	2.19	2.01	2.47	2.84	2.75	1.59
6	0.15	0.06	0.24	0.18	0.42	0.42	0.34	0.47	0.92	0.89	0.89	0.79	2.23	1.95	1.02	1.15	3.33	1.90	2.71	3.05
7	0.18	0.17	0.18	0.18	0.45	0.50	0.52	0.36	0.84	0.72	0.82	1.10	2.27	2.11	2.03	2.18	3.51	3.66	3.30	3.29
8	0.15	0.18	0.14	0.14	0.43	0.58	0.40	0.65	1.28	1.42	1.46	1.37	2.07	3.01	2.64	3.02	3.54	4.31	4.00	5.99
9	0.15	0.11	0.18	0.15	0.33	0.33	0.37	0.37	0.87	0.79	0.81	0.73	1.96	1.87	1.24	1.37	2.80	1.93	2.98	2.57
10	0.18	0.19	0.16	0.14	0.63	0.42	0.35	0.73	0.84	0.90	1.03	1.30	2.38	2.37	2.13	2.25	5.03	5.29	2.58	4.67
11	0.16	0.17	0.17	0.15	0.42	0.46	0.42	0.45	1.12	1.05	1.03	0.91	2.31	2.48	2.35	1.97	3.61	4.14	3.14	3.45

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; green bars represent the overall mean.

As observed in Figure 1, nine out of eleven laboratories overestimated the true value at 0.1%; GM levels of 0.4% and 0.9 were overestimated by at least seven laboratories, with three laboratories with a relative deviation from the true value over 40% for GM level 0.9%; GM levels of 2.0% and 3.3% were also overestimated by the majority of the laboratories.

Overall, the average relative deviation (green bar) was acceptable at all GM levels tested, indicating a satisfactory accuracy of the method.

Figure 1. Relative deviation (%) from the true value of LLCotton25 for all laboratories



8. Method performance requirements

Among the performance criteria established by ENGL and adopted by the CRL-GMFF (http://gmo-crl.jrc.it/guidancedocs.htm, see also Annex 1), repeatability and reproducibility are assessed through an international collaborative trial, carried out with the support of ENGL laboratories (see Table 1). Table 6 illustrates the estimation of repeatability and reproducibility at various GM levels, according to the range of GM percentages tested during the collaborative trial.

The *relative reproducibility standard deviation (RSD_R),* that describes the inter-laboratory variation, should be below 33% at the target concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 6, the method fully satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R (%) is 32% at the 0.4% and 0.9% levels, thus within the acceptance criterion

Table 6. LLCotton25: summary of validation results.

	Expected value (GMO %)				
Unknown sample GM%	0.15	0.40	0.90	2.00	3.30
Laboratories having returned results	11	11	11	11	11
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	0	1	0
Reason for exclusion	-	-	-	1C	-
Mean value	0.17	0.47	1.08	2.23	3.57
Relative repeatability standard deviation, RSD_r (%)	23	28	18	18	24
Repeatability standard deviation	0.04	0.13	0.20	0.41	0.87
Relative reproducibility standard deviation, RSD_R (%)	23	32	32	24	30
Reproducibility standard deviation	0.04	0.15	0.35	0.54	1.07
Bias (absolute value)	0.02	0.07	0.18	0.23	0.27
Bias (%)	12	17	20	11	8.1

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

Bias is estimated according to ISO 5725 data analysis protocol.

Table 6 further documents the *relative repeatability standard deviation (RSD_r)*, as estimated for each GM level. In order to accept methods for collaborative study evaluation, the CRL requires that RSD_r values be below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.it/guidancedocs.htm).

As it can be observed from the values reported in Table 6, the method satisfies this requirement throughout the dynamic range tested with the minor exception of an RSD_r value of 28% at the 0.40% 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 entire dynamic range tested; in fact, the highest value of bias (%) is 20 at the 0.90% level, well within the acceptance criterion.

9. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (as detailed under http://gmo-crl.jrc.it/guidancedocs.htm). The method acceptance criteria were reported by the applicant and used to evaluate the method prior to the international collaborative study (see Annex 1 for a summary of method acceptance criteria and method performance requirements).

The results obtained during the collaborative study indicate that the analytical module of the method submitted by the applicant complies with ENGL performance criteria. The method is therefore applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32232) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)]

11. References

1. Horwitz, W. (1995) Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67, 331-343.
2. International Standard (ISO) 5725. 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Swizerland.
3. Arumuganathan K, Earle ED. 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: The description of analytes, matrices, and concentrations to which a method can be applied.

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

Practicability

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

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

Specificity

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

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

Dynamic Range

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

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below. The range of the

standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

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

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

Amplification Efficiency

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

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of (-3.1 ≥ slope ≥ - 3.6)

R^2 Coefficient

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

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

Repeatability Standard Deviation (RSD_r)

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

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

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

Limit of Quantitation (LOQ)

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

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

Limit of Detection (LOD)

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

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

Robustness

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

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

Method Performance Requirements

Dynamic Range

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

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

Reproducibility Standard Deviation (RSD_R)

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

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

Trueness

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

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