Event-specific Method for the Quantification of Cotton Line MON 1445 Using Real-time PCR

Validation Report

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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 MON 1445 transformation event in cotton DNA (unique identifier MON-∅1445-2). 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 (cotton seeds containing the transformation event and conventional cotton seeds). 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 mostly met the ENGL performance requirements. The method is therefore considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

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

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Report on Steps 1-3 of the Validation Process

Monsanto submitted the detection method and control samples for cotton event MON 1445 (unique identifier MON-∅1445-2) under Article 8 and 20 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, five 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 MON 1445 was positively concluded in June 2006.

Starting from April 2006, the CRL-GMFF verified experimentally the method characteristics (step 3, experimental testing of samples and methods) by quantifying five blind GM-levels within the range 0.1%-6.0% 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 by the CRL-GMFF 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

Monsanto submitted the detection method and control samples for cotton event MON 1445 (unique identifier MON-∅1445-2) under Article 8 and 20 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute for Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Regulation EC No 1829/2003) organised the international collaborative study for the event-specific method for the detection and quantification of MON 1445 cotton. The study involved twelve 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) No 641/2004 and following its operational procedures.

The internal in-house experimental evaluation of the method was carried out in April 2006.

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 between June and July 2006.

A method for DNA extraction from cotton seeds, submitted by the applicant, was evaluated by the CRL-GMFF; laboratory testing of the method was carried out 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 MON 1445 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton *acp 1* (*acyl carrier protein*) endogenous assay (reference gene) and the target assay (MON 1445) 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 ENGL laboratories to determine its performance. Clear guidance was given to the laboratories with regards to the standard operational procedures to follow for the 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 MON 1445

Laboratory	**Country**
Bavarian Health and Food Safety Authority	Germany
Crop Research Institute - Reference Laboratory for GMO Detection and DNA fingerprinting	Czech Rep.
Institute of Chemical Technology Prague	Czech Rep.
LGC	UK
National Centre for Food, Spanish Food Safety Agency	Spain
National Institute of Engineer, Technology and Innovation - Food Industry Laboratory	Portugal
RIKILT Institute of Food Safety	The Netherlands
Scientific Institute of Public Health (IPH)	Belgium
Service Commun des Laboratoires du MINEFI - Laboratoire de Strasbourg	France
The Food and Consumer Product Safety Authority	The Netherlands
Veterinary Public Health Institute for Lazio and Toscana Regions; National Reference Centre for GMO Analysis	Italy
Walloon Agricultural Research Centre (CRA-W) - Department Quality of Agricultural Products	Belgium

3. Materials

For the validation of the quantitative event-specific method, genomic DNA was extracted from samples consisting of:

i) Seeds of cotton harbouring the MON 1445 event (Line DP5690RR, Lot number GLP-0403-14812-S) and;

ii) Seeds of conventional cotton (Line ST474, lot number GLP-0403-14786-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% MON 1445 cotton 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 (160 µL of DNA solution each) for the preparation of the standard curve, labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (80 µL of DNA solution each), labelled from U1 to U20.
- ✓ Reaction reagents:
    - ☐ Universal PCR Master Mix 2X, 3 vials: 5 mL each
    - ☐ Sterile distilled water, one vial: 12.2 mL

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

    acp 1 reference system

    - ☐ *acp 1* primer forward (10 µM): 240 µL
    - ☐ *acp 1* primer reverse (10 µM): 240 µL
    - ☐ *acp 1* TaqMan® probe (5 µM): 160 µL

    MON 1445 system

    - ☐ MON 1445 primer forward (10 µM): 240 µL
    - ☐ MON 1445 primer reverse (10 µM): 240 µL
    - ☐ MON 1445 TaqMan® probe (5 µM): 160 µL

4. Experimental design

Twenty unknown samples (labelled from U1 to U20), representing five GM levels, were used in the validation study (Table 2). On each PCR plate, the samples were analysed either for the MON 1445 specific system or the *acp 1* specific system. In total, two plates were run per participating laboratory and 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 1445 GM contents

MON 1445 GM% (GM copy number/cotton genome copy number *100)
6.0
2.5
0.9
0.5
0.1

5. Method

Description of operational steps followed

For the specific detection of event MON 1445, an 87-bp fragment of the integration region of the construct inserted into the plant genome (5′ insert-to-plant junction) 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 FAM dye at its 5′-end and TAMRA as quencher dye at 3′-end.

For the relative quantification of event MON 1445 DNA, a cotton-specific reference system amplifies a 76-bp fragment of the cotton endogenous gene *acp 1* (*Acyl carrier protein*) using two *acp 1* gene-specific primers and an *acp 1* gene-specific probe labelled with FAM and TAMRA.

Standard curves are generated for both the MON 1445 and the *acp 1* 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 1445 DNA in a test sample, the MON 1445 copy number is divided by the copy number of the cotton reference gene (*acp 1*) and multiplied by 100 to obtain the percentage value (GM % = MON 1445 / *acp 1* x 100).

Calibration sample S1 was prepared by mixing the appropriate amount of MON 1445 DNA in control non-GM cotton DNA to obtain a 10% GM MON 1445 in a total of 200 ng cotton DNA. Sample S2 was prepared as a 1:2 dilution from S1, sample S3 as a 1:5 dilution from S2, sample S4 as a 1:3 dilution from S3 and sample S5 as a 1:4 dilution from S4.

The absolute copy numbers in the calibration curve samples are determined by dividing the sample DNA weight (in picograms) 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 (ng/4 µL)	200	100	20	6.68	1.68
Cotton genome copies	85830	42910	8580	2860	710
MON 1445 cotton copies	8583	4291	858	286	71

6. Deviations reported

Ten laboratories reported no deviations from the protocol.

One laboratory received thawed samples and reagents due to improper storage and late delivery of the samples by the courier. This occurrence was considered as a major violation of the validation protocol and as such the data from this laboratory were not taken into account in the statistical analysis carried out at the CRL-GMFF and in the results of the ring-trial produced in the present validation report.

One laboratory could not quantify the U1 sample as the vial was reported as empty and no replacement material has been requested during the study.

One laboratory performed twice the second run i.e. ‘B’ plate, due to abnormal values for the reference system of two ‘unknown’ samples in the first ‘B’ plate. Only the data from the second ‘B’ plate were considered for analysis.

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 1445 system and the *acp 1* reference system are summarised in Table 4.

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

		MON 1445			***acp 1***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.11	109.67	0.97	-3.26	102.81	0.97
	B	-3.28	101.69	0.97	-3.17	106.78	0.98
2	A	-3.02	114.44	0.98	-3.14	108.00	0.99
	B	-3.31	100.62	0.98	-3.20	105.14	0.99
3	A	-3.38	97.75	0.98	-3.30	101.06	0.99
	B	-3.04	113.46	0.98	-3.16	107.27	0.98
4	A	-3.30	101.05	0.99	-3.53	91.95	0.99
	B	-3.15	107.72	0.98	-3.37	97.85	0.99
5	A	-3.22	104.61	0.99	-3.36	98.42	0.97
	B	-3.34	99.36	1.00	-5.80	48.77	0.99
6	A	-3.13	108.91	0.99	-3.18	106.42	0.99
	B	-3.23	104.20	0.99	-3.19	105.76	0.99
7	A	-3.41	96.57	0.96	-2.93	119.69	0.92
	B	-3.46	94.44	0.98	-2.93	119.38	0.96
8	A	-3.43	95.73	1.00	-3.37	98.18	0.99
	B	-3.38	97.58	0.99	-3.33	99.51	0.99
9	A	-3.28	101.90	0.99	-3.14	108.03	0.99
	B	-3.13	108.87	0.99	-3.28	101.62	0.99
10	A	-3.40	96.99	0.98	-3.35	98.95	0.99
	B	-3.23	104.07	0.99	-3.47	94.23	0.99
11	A	-	-	-	-	-	-
	B	-	-	-	-	-	-
12	A	-3.20	105.24	0.98	-3.29	101.56	0.99
	B	-3.09	110.44	0.99	-3.11	109.64	0.99
	Mean	-3.25	103.5	0.98	-3.37	101.4	0.98

The mean PCR efficiency was 103.5% for the MON 1445 and 101.4 % for the *acp 1* system. The linearity of the method was on average 0.98 for both systems. Data reported confirm the appropriate performance characteristics of the method tested in terms of efficiency and linearity.

GMO quantification

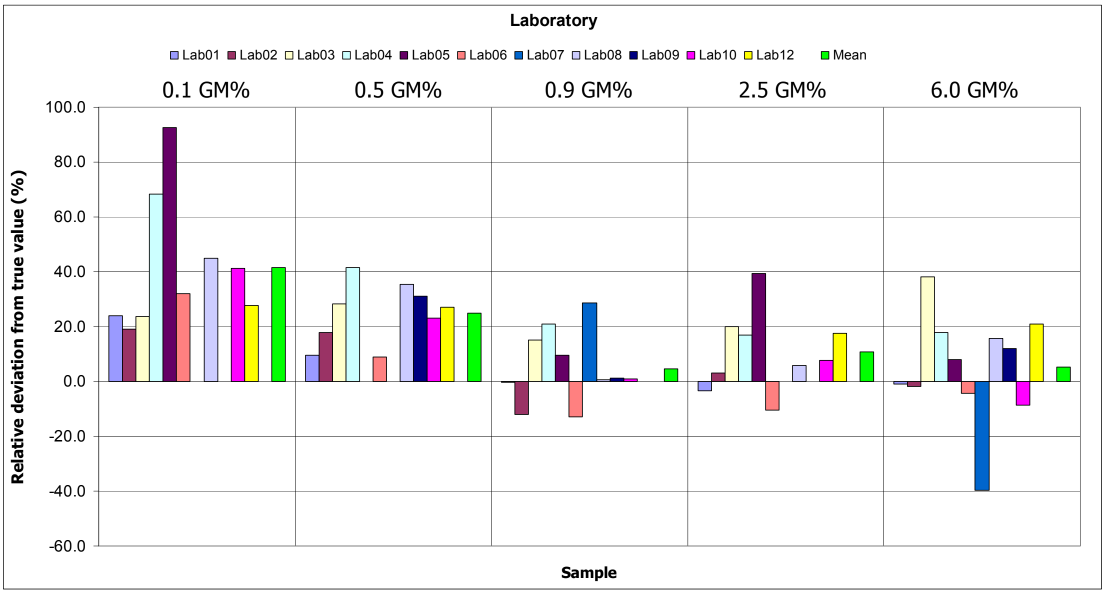
Table 5 shows the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

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

	Sample GMO content (GM% = GM copy number/cotton genome copy number x 100)																			
Lab	**0.1**				**0.5**				**0.9**				**2.5**				**6.0**			
	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	0.12	0.11	0.14	0.13	0.59	0.56	0.52	0.53	0.84	1.11	0.83	0.81	2.57	2.34	2.33	2.42	6.21	6.53	5.85	5.16
2	0.12	0.12	0.12	0.12	0.50	0.52	0.67	0.67	0.67	1.04	0.69	0.77	2.48	2.69	2.11	3.03	6.52	5.84	6.34	4.86
3	0.14	0.14	0.11	0.10	0.54	0.78	0.49	0.77	1.13	0.85	1.02	1.14	2.44	2.86	3.73	2.97	6.67	9.59	8.12	8.75
4	0.19	0.20	0.14	0.15	0.67	0.93	0.60	0.62	1.06	1.18	1.05	1.06	2.87	2.89	3.21	2.71	6.74	7.49	6.99	7.08
5	0.18	0.17	0.19	0.23	0.93	1.49	0.63	0.49	0.88	1.00	1.10	0.96	3.65	3.55	3.78	2.96	7.08	5.94	5.84	7.03
6	0.12	0.10	0.14	0.17	0.50	0.45	0.53	0.71	0.67	0.86	0.81	0.79	2.36	2.34	2.03	2.22	4.60	6.03	5.93	6.43
7	-	0.32	0.05	0.06	1.09	0.89	0.34	0.70	1.42	1.15	1.20	0.87	1.87	5.09	2.16	2.55	3.54	1.03	4.44	5.47
8	0.13	0.17	0.15	0.14	0.63	0.50	0.77	0.81	0.98	0.88	0.95	0.81	3.03	2.38	2.81	2.37	7.05	6.99	7.23	6.50
9	0.10	0.09	0.13	0.21	0.62	0.72	0.56	0.73	0.91	0.95	1.07	0.71	2.00	2.71	4.13	3.94	4.70	8.99	6.46	6.74
10	0.15	0.15	0.13	0.14	0.61	0.68	0.58	0.59	0.92	0.92	1.01	0.79	2.70	2.55	2.88	2.62	4.07	5.44	6.19	6.26
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	0.15	0.14	0.11	0.11	0.71	0.76	0.42	0.66	0.86	0.98	0.91	0.85	3.13	3.01	2.94	2.68	8.04	5.77	7.45	7.78

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, as well as the mean value (represented by the green bar).

Figure 1. Relative deviation (%) from the true value of MON 1445 for all laboratories



As observed in Figure 1, relative deviations from the true values are mainly positive at the lowest GM levels (0.1% and 0.5%), meaning that the GM content of unknown samples tends to be overestimated at these GM levels. In particular, at GM level 0.1% six laboratories overestimated the GM content of more than 25%.

The relative deviations from the true values are modest at the highest GM levels (0.9%, 2.5% and 6.0% respectively) and appear to be randomly distributed.

Overall, the average relative deviation was evident at the 0.1% GM level, but acceptable at the higher GM levels, indicating a satisfactory accuracy of the method from the 0.5% GM level up.

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 24% at the 6.0% GM level, thus within the acceptance criterion.

Table 6. MON 1445: summary of validation results.

Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**0.9**	**2.5**	**6.0**
Laboratories having returned valid results	11	11	11	11	11
Samples per laboratory	4	4	4	4	4
Number of outliers	2	2	-	2	-
Reason for exclusion	2 C	2C	-	2C	-
Mean value	0.14	0.62	0.94	2.77	6.31
Relative repeatability standard deviation, RSD_r (%)	14	18	13	11	17
Repeatability standard deviation	0.02	0.11	0.13	0.30	1.07
Relative reproducibility standard deviation, RSD_R (%)	21	18	17	16	24
Reproducibility standard deviation	0.03	0.11	0.16	0.45	1.52
Bias (absolute value)	0.04	0.12	0.04	0.27	0.31
Bias (%)	41	25	4.7	11	5.2

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 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 demonstrates a relative repeatability standard deviation well within the acceptance criterion over the dynamic range with a maximum of 18% at the 0.5% 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 from 0.5% to 6.0% of the dynamic range where the trueness is within the acceptance criterion. However, the method bias % exceeds the ENGL requirement at the 0.1% GM level, being equal to 41%.

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 with the deviation of the method bias at the 0.1%. This divergence does not affect the estimation of the GM content at the higher GM levels where the trueness is within the acceptance criterion.

Being both the relative reproducibility and repeatability standard deviations within the acceptance limits over the dynamic range, 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, 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 = $[10^{(-1/\text{slope})}] - 1$

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

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 1/10th 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. 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 1/20th 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.

Acceptance Criterion: The response of an assay with respect to these small variations should not deviate more than ± 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 ± 25% of the accepted reference value over the whole dynamic range.