Report on the Verification of the Performance of MON 89034, 1507, MON 88017 and 59122 Event-specific Methods on the Maize Event MON 89034 x 1507 x MON 88017 x 59122 Using Real-Time PCR

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Joint Research Centre
Institute for Health and Consumer Protection
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Executive Summary

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, has carried out a verification study to assess the performance of four quantitative event-specific methods on the maize event MON 89034 x 1507 x MON 88017 x 59122 (unique identifier MON-89Ø34-3 x DAS-Ø15Ø7-1 x MON-88Ø17-3 x DAS-59122-7) which combines the MON 89034, 1507, MON 88017 and 59122 transformation events. The four methods have been validated individually on single-trait events, to detect and quantify each event in maize samples. This study was conducted according to internationally accepted guidelines [1, 2].

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto and Dow AgroSciences provided the detection methods and the control samples: genomic DNA from homogenised seeds of MON 89034 x 1507 x MON 88017 x 59122 maize (258-4CC, 258-4JJ, 258-4B, 258-4N) and from homogenised seeds of conventional maize (10001262-V). The EURL-GMFF prepared the verification samples (calibration samples and blind samples at different GM percentages).

The results of the verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and to the validation results on the individual parental events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results of this EURL-GMFF verification study are publicly available at http://gmo-crl.jrc.ec.europa.eu/.

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Report on Steps 1-3 of the Validation Process

Monsanto and Dow AgroSciences submitted the detection methods and control samples of the maize event MON 89034 x 1507 x MON 88017 x 59122 (unique identifier MON-89Ø34-3 x DAS-Ø15Ø7-1 x MON-88Ø17-3 x DAS-59122-7) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the EURL-GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/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/doc/Method%20requirements.pdf) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed and one request of complementary information was addressed to the applicant. Upon reception of the complementary information, the scientific assessment of the detection method for maize MON 89034 x 1507 x MON 88017 x 59122 was positively concluded in January 2009.

The event-specific detection methods for the four maize lines hosting the single events MON 89034, 1507, MON 88017 and 59122 were validated by the EURL-GMFF following the conclusion of the respective international collaborative studies and the publication of the validation reports (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). Hence, the detection methods applied on the maize event MON 89034 x 1507 x MON 88017 x 59122 did not undergo a full validation process. The EURL-GMFF performed a verification of the detection methods to verify that they exhibit a comparable performance on samples of event MON 89034 x 1507 x MON 88017 x 59122 combining the four traits (as provided in accordance to Annex I.2.C.2 of Commission Regulation (EC) No 641/2004).

In February 2009, the EURL-GMFF concluded the experimental verification of the methods (step 3, experimental testing of the samples and methods) by quantifying, with each specific method, five blind GM-levels within the range 0.09%-8% on a copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were within the limits established by the ENGL.

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

Content

1. Introduction

Monsanto and Dow AgroSciences submitted the detection methods and control samples of the maize event MON 89034 x 1507 x MON 88017 x 59122 (unique identifier MON-89Ø34-3 x DAS-Ø15Ø7-1 x MON-88Ø17-3 x DAS-59122-7) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed, established by Regulation (EC) 1829/2003, carried out a verification of the four event-specific methods for the detection and quantification of MON 89034, 1507, MON 88017 and 59122 in the MON 89034 x 1507 x MON 88017 x 59122 maize event combining the four traits. The single methods had been previously validated by international collaborative studies on the single-trait maize events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Upon reception of methods, samples and data (step 1), the EURL-GMFF carried out the assessment of the documentation (step 2) and the verification of the methods (step 3) according to the requirements of Regulation (EC) 641/2004 and following EURL-GMFF procedures. The EURL-GMFF method verification was concluded in February 2009.

A method submitted by the applicant for DNA extraction from maize seeds was also evaluated by the EURL-GMFF in order to confirm its performance characteristics. The protocol for DNA extraction is available at http://gmo-crl.jrc.ec.europa.eu/.

The procedure of verification consisted of a quantitative real-time Polymerase Chain Reaction (PCR). The methodology consists of four event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of events MON 89034, 1507, MON 88017 and 59122 DNA to total maize DNA in the MON 89034 x 1507 x MON 88017 x 59122 maize event. The procedures are simplex systems, in which the events MON 89034, 1507, MON 88017 and 59122 were quantified in reference to the maize *hmg* (*high mobility group*) taxon-specific endogenous gene.

The study was carried out in accordance to the following internationally accepted guidelines:

- ISO 5725: 1994
- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies".

2. Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenised seeds of MON 89034 x 1507 x MON 88017 x 59122 maize (258-4CC, 258-4JJ, 258-4B, 258-4N),
- genomic DNA extracted from homogenised seeds of conventional maize (10001262-V).

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 MON 89034 x 1507 x MON 88017 x 59122 and non-GM maize genomic DNA at different GMO levels were prepared in a constant amount of total maize DNA.

The validated methods for the individual MON 89034, 1507, MON88017 and 59122 events were applied in the verification as published and available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

In Table 1 are reported the five GM levels used in the verification of the MON 89034, 1507, MON 88017 and 59122 methods.

Table 1. MON 89034, 1507, MON 88017 and 59122 GM contents (%) in maize event MON 89034 x 1507 x MON 88017 x 59122

MON 89034 (GM DNA / Non-GM DNA x 100)	**1507** (GM DNA / Non-GM DNA x 100)	**MON 88017** (GM DNA / Non-GM DNA x 100)	**59122** (GM DNA / Non-GM DNA x 100)
0.09	0.1	0.09	0.1
0.4	0.5	0.5	0.4
0.9	0.9	0.9	0.9
3.0	2.0	5.0	2.0
8.0	5.0	8.0	4.5

3. Experimental design

Eight runs for each event-specific method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the target taxon-specific assay (*hmg*). Five GM levels per run were examined and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. In total, for each method (MON 89034, 1507, MON 88017 and 59122), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level.

4. Methods

To detect MON 89034, 1507, MON 88017 and 59122 in maize event MON 89034 x 1507 x MON 88017 x 59122, four specific fragments of the integration regions of 77 bp, 58 bp, 95 bp and 86 bp respectively, were amplified using specific primers.

For relative quantification of events MON 89034, 1507, MON 88017 and 59122, a maize-specific target-taxon system which amplifies a 79 bp fragment of the maize gene *hmg* (high mobility group), using *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM and TAMRA, were used.

Standard curves are generated for each GM specific system (MON 89034, 1507, MON 88017 and 59122), by plotting the Ct values of the calibration samples against the logarithm of the DNA copy numbers of MON 89034, 1507, MON 88017 or 59122, and fitting a linear regression into these data. Thereafter, the normalised Ct values of the blind samples are measured and, by means of the regression function, the relative amount of MON 89034, 1507, MON 88017 or 59122 DNA is estimated.

Detailed information on standard curve samples preparation is available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

5. Deviations reported

For the 1507 method, only seven runs were carried out, instead of eight. Therefore, the quantification of the five GM levels was performed on fourteen replicates per GM level instead of sixteen.

6. Results

PCR efficiency and linearity

PCR efficiency was calculated using the formula $[10^{(-1/slope)} - 1] \times 100$, and the R^2 (expressing the linearity of the regression) is reported for all PCR systems in the eight runs. Values of the standard curves slopes for MON 89034, 1507, MON 88017 and 59122 methods are presented in Tables 2, 3, 4 and 5, respectively.

Table 2. Values of standard curve slope, PCR efficiency and R^2 of the MON 89034 method on event MON 89034 x 1507 x MON 88017 x 59122.

Run	MON 89034			*hmg*		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.48	94	1.00	-3.21	105	0.99
2	-3.55	91	1.00	-3.20	105	1.00
3	-3.57	91	1.00	-3.13	109	0.99
4	-3.47	94	1.00	-3.19	106	1.00
5	-3.56	91	1.00	-3.23	104	0.99
6	-3.43	96	1.00	-3.17	107	1.00
7	-3.56	91	1.00	-3.14	108	1.00
8	-3.39	97	1.00	-3.20	105	1.00
Mean	-3.50	93	1.00	-3.18	106	1.00

Table 3. Values of standard curve slope, PCR efficiency and R^2 of the 1507 method on event MON 89034 x 1507 x MON 88017 x 59122.

Run	1507			*hmg*		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.21	105	0.99	-3.15	108	1.00
2	-3.24	103	0.99	-3.10	110	1.00
3	-3.15	108	0.99	-3.14	108	1.00
4	-3.13	109	1.00	-3.11	110	1.00
5	-3.20	105	0.99	-3.15	108	1.00
6	-3.35	99	0.99	-3.10	110	1.00
7	-3.21	105	0.99	-3.15	108	1.00
Mean	-3.22	105	0.99	-3.13	109	1.00

Table 4. Values of standard curve slope, PCR efficiency and R^2 of the MON 88017 method on event MON 89034 x 1507 x MON 88017 x 59122.

Run	MON 88017			*hmg*		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.16	107	1.00	-3.16	107	1.00
2	-3.36	99	1.00	-3.20	105	1.00
3	-3.27	102	1.00	-3.18	106	1.00
4	-3.25	103	1.00	-3.20	105	1.00
5	-3.23	104	1.00	-3.16	107	0.99
6	-3.31	100	1.00	-3.16	107	1.00
7	-3.29	101	1.00	-3.21	105	0.99
8	-3.39	97	0.99	-3.19	106	1.00
Mean	-3.28	102	1.00	-3.18	106	1.00

Table 5. Values of standard curve slope, PCR efficiency and R^2 of the 59122 method on event MON 89034 x 1507 x MON 88017 x 59122.

Run	59122			*hmg*		
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.48	94	1.00	-3.25	103	1.00
2	-3.43	96	0.99	-3.31	100	1.00
3	-3.22	104	1.00	-3.28	102	1.00
4	-3.45	95	1.00	-3.32	100	1.00
5	-3.41	97	1.00	-3.28	102	1.00
6	-3.32	100	1.00	-3.32	100	1.00
7	-3.46	95	1.00	-3.33	100	1.00
8	-3.25	103	1.00	-3.34	99	1.00
Mean	-3.38	98	1.00	-3.30	101	1.00

The mean PCR efficiencies of the GM and target taxon-specific systems were 93% and 106% for MON 89034, 105% and 109% for 1507, 102% and 106% for MON 88017, and 98% and 101% for 59122, respectively. The R^2 of the methods was 0.99 or 1.00 for both systems in all cases. Overall, the data reported confirmed the appropriate PCR efficiency and linearity of the four methods tested on MON 89034 x 1507 x MON 88017 x 59122 maize samples.

7. Method performance requirements

The results of the verification study for the MON 89034, 1507, MON 88017 and 59122 detection methods applied to event MON 89034 x 1507 x MON 88017 x 59122 maize DNA are reported in Tables 6, 7, 8 and 9, respectively. Results were evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by the EURL-GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm, see also Annex 1). In addition, Tables 6 to 9 report the trueness and repeatability standard deviation for each GM level for all methods.

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 89034 method on event MON 89034 x 1507 x MON 88017 x 59122 maize DNA.

MON 89034					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.4**	**0.9**	**3.0**	**8.0**
Mean	0.09	0.38	0.84	2.69	7.52
SD	0.01	0.05	0.07	0.31	0.90
RSD_r (%)	12	14	8.0	11	12
Bias (%)	-2.2	-4.2	-6.2	-10	-6.1

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 1507 method on event MON 89034 x 1507 x MON 88017 x 59122 maize DNA.

1507					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**0.9**	**2.0**	**5.0**
Mean	0.09	0.41	0.81	1.64	4.78
SD	0.01	0.06	0.07	0.10	0.47
RSD_r (%)	13	15	8.4	6.3	9.8
Bias (%)	-12	-18	-9.8	-18	-4.4

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 88017 method on event MON 89034 x 1507 x MON 88017 x 59122 maize DNA.

MON 88017					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.5**	**0.9**	**5.0**	**8.0**
Mean	0.07	0.43	0.75	4.66	6.89
SD	0.01	0.05	0.07	0.54	0.57
RSD_r (%)	9.3	11	8.9	12	8.2
Bias (%)	-24	-15	-16	-6.8	-14

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 59122 method on event MON 89034 x 1507 x MON 88017 x 59122 maize DNA.

59122					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.4**	**0.9**	**2.0**	**4.5**
Mean	0.12	0.41	1.02	1.97	4.63
SD	0.02	0.03	0.06	0.09	0.29
RSD_r (%)	15	8.4	5.8	4.7	6.4
Bias (%)	24	1.3	14	-1.3	3.0

The trueness of the method is estimated using the measures of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method, measured as bias from the accepted value, should be ± 25% across the entire dynamic range. As shown in tables 6, 7, 8 and 9, the values ranged from -10% to -2.2% for MON 89034, from -18% to -4.4% for 1507, from -24% to -6.8% for MON 88017, and from -1.3% to 24% for 59122. Therefore, the four methods satisfied the above mentioned requirement throughout their respective dynamic ranges.

Tables 6 to 9 further document the relative repeatability standard deviation (RSD_r) as estimated for each GM level. In order to accept methods for collaborative trial evaluation, the EURL-GMFF requires that RSD_r values are below 25%. As it can be observed from Tables 6 to 9, the values ranged between 8.0% and 14% for MON 89034, between 6.3% and 15% for 1507, between 8.2% and 12% for MON 88017, and between 4.7% and 15% for 59122. Therefore, the four methods satisfied this requirement throughout their respective dynamic ranges.

8. Comparison of method performance between event MON 89034 x 1507 x MON 88017 x 59122 and the single trait events

An indicative comparison of the four methods performances on the maize event MON 89034 x 1507 x MON 88017 x 59122 and the single trait events is shown in Tables 10, 11, 12 and 13. The performance of the methods on the single lines was previously assessed though international collaborative trials.

Table 10. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 89034 detection method on event MON 89034 x 1507 x MON 88017 x 59122 and on event MON 89034.

Trueness and repeatability of MON 89034 quantification on MON 89034 x 1507 x MON 88017 x 59122			**Trueness and repeatability of MON 89034 quantification on single event MON 89034***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.09	-2.2	12	**0.09**	25	18
0.4	-4.2	14	**0.4**	6.4	13
0.9	-6.2	8.0	**0.9**	4.3	17
3.0	-10	11	**3.0**	-5.8	12
8.0	-6.1	12	**8.0**	-11	9.5

*method validated (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 11. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the 1507 detection method on event MON89034 x 1507 x MON88017 x 59122 and on event 1507.

Trueness and repeatability of 1507 quantification on MON 89034 x 1507 x MON 88017 x 59122			**Trueness and repeatability of 1507 quantification on single event 1507***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	-12	13	**0.1**	6.0	18
0.5	-18	15	**0.5**	-4.0	12
0.9	-9.8	8.4	**0.9**	3.7	7.7
2.0	-18	6.3	**2.0**	-1.7	8.5
5.0	-4.4	9.8	**5.0**	8.4	14

*method validated (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 12. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 88017 detection method on event MON 89034 x 1507 x MON 88017 x 59122 and on event MON 88017.

Trueness and repeatability of MON 88017 quantification on MON 89034 x 1507 x MON 88017 x 59122			**Trueness and repeatability of MON 88017 quantification on single event MON 88017***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.09	-24	9.3	**0.09**	-2.6	28
0.5	-15	11	**0.5**	2.9	13
0.9	-16	8.9	**0.9**	-9.6	19
5.0	-6.8	12	**5.0**	-4.8	19
8.0	-14	8.2	**8.0**	-7.6	18

*method validated (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 13. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the 59122 detection method on event MON 89034 x 1507 x MON 88017 x 59122 and on event 59122.

Trueness and repeatability of 59122 quantification on MON 89034 x 1507 x MON 88017 x 59122			**Trueness and repeatability of 59122 quantification on single event 59122***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	24	15	**0.1**	29	18
0.4	1.3	8.4	**0.4**	15	14
0.9	14	5.8	**0.9**	9.0	16
2.0	-1.3	4.7	**2.0**	7.0	14
4.5	3.0	6.4	**4.5**	-1.0	8.5

*method validated (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Regarding trueness, the MON 89034 method (Table 10), when applied to event MON 89034 x 1507 x MON 88017 x 59122 and compared to the single line, showed lower bias at 0.09%, 0.4% and 8% GM levels and higher bias at 0.9% and 3% GM levels. The 1507 event-specific method (Table 11) showed generally lower bias when applied to the single event, apart from the highest GM level (5.0%). The MON 88017 even-specific method (table 12) showed lower bias through the entire dynamic range when applied to the single line compared to the stacked event. Finally, the 59122 method (Table 13) when applied to the stack event showed lower bias at the 0.1%, 0.4 and 2.0% GM levels and higher at 0.9% and 4.5%, compared with the single line. In all cases, the trueness of the four event-specific methods when applied to the stacked event was within the acceptance range set by ENGL (± 25%) on the whole dynamic ranges studied.

Concerning the relative repeatability standard deviation (RSD_r %), the MON 89034 method (Table 10) showed lower values at 0.09%, 0.9% and 3.0% GM levels when applied to the stacked and compared to the single event, and higher values at 0.4% and 8%. The 1507 method (Table 11), showed lower values at 0.1%, 2.0% and 5.0% and higher to 0.5% and 0.9%, when applied to the stacked event. The MON 88017 and the 59122 methods (Tables 12 and 13) showed better precision over the whole dynamic range when applied to the stacked event compared to the single lines. In all cases, the relative repeatability standard deviation (RSD_r %) of the four event-specific methods, when applied to the stack event, was below the ENGL acceptance level established at maximum 25%.

Therefore, the method verification has demonstrated that the MON 89034, 1507, MON 88017 and 59122 detection methods, developed to detect and quantify the single events, can be equally applied for the quantification of the respective events combined in event MON 89034 x 1507 x MON 88017 x 59122.

9. Conclusions

The overall method performance of the four event-specific methods for the quantitative detection of events MON 89034, 1507, MON 88017 and 59122 combined in maize event MON 89034 x 1507 x MON 88017 x 59122 have been evaluated with respect to the method acceptance criteria and the method performance requirements recommended by the ENGL (as detailed under http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), and to the validation results obtained for the single trait events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results obtained during the present verification study indicate that the analytical modules of the methods submitted by the applicant comply with ENGL performance criteria. The methods are 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 EURL-GMFF carries out its operations according to ISO 9001 (certificate number: CH-32231) and ISO 17025 (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.

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 ≤ 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.