Event-specific Method for the Quantification of Maize Line MON 89034 Using Real-time PCR

Validation Report

21 October 2008

**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 89034 transformation event in maize DNA (unique identifier MON-89034-3). The collaborative trial was conducted according to internationally accepted guidelines [1, 2].
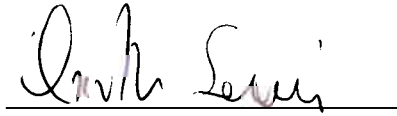
In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and with Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto provided the detection method and the samples (genomic DNA extracted from conventional seeds and from 100% maize containing the event MON 89034). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from nine European countries.

The results of the international collaborative trial met the ENGL performance requirements. The method is therefore considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

The results of the collaborative study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/.
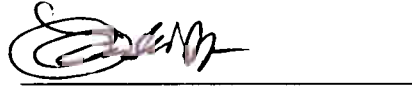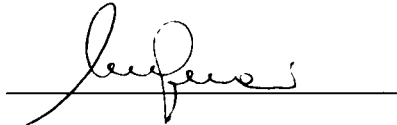
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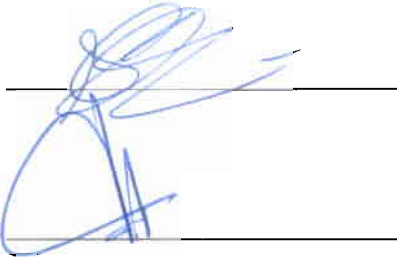2) M. Querci

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Report on Steps 1-3 of the Validation Process

Monsanto submitted the detection method and control samples for maize event MON 89034 (unique identifier MON-89034-3) 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.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, 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 maize MON 89034 was positively concluded in June 2007.

Between April and October 2007, 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 0.09%-8% on a copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision of the quantifications were within the limits established by the ENGL.

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 maize event MON 89034 (unique identifier MON-89034-3) 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 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 89034 maize. The study involved twelve laboratories from nine European countries, as listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for detection") of Commission Regulation (EC) No 1981/2006 of 22 December 2006.

Upon reception of method, samples and related data (step 1), the JRC carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3) according to the requirements of Regulation (EC) No 641/2004 and following its operational procedures.

The internal in-house experimental evaluation of the method was carried out between April and October 2007.

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 November 2007.

A method for DNA extraction from maize seeds, submitted by the applicant, was evaluated by the CRL-GMFF; laboratory testing of the method was carried out between April and October 2007 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.ec.europa.eu/statusofdoss.htm.

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 maize line MON 89034 DNA to total maize DNA. The procedure is a simplex system, in which a maize *hmg* (high mobility group) endogenous assay (reference gene) and the target assay (MON 89034) 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.

On 22nd October 2007 the CRL-GMFF invited all National Reference Laboratories nominated under Commission 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 GM event DP-356043-5.

Thirty-six laboratories expressed in writing their willingness to participate, four declined the invitation, while thirty-three did not answer. The CRL-GMFF performed a random selection of twelve laboratories out of those that responded positively to the invitation, making use of a validated software application.

Clear guidance was given to the selected laboratories with regards to the standard operational procedures to follow for the execution of the protocol. The participating laboratories are listed in Table 1.

Table 1. Laboratories participating in the validation of the detection method for maize line MON 89034.

Laboratory	Country
Bavarian Health and Food Safety Authority	Germany
Central Agricultural Office, Directorate Food and Feed Safety, Central Feed Investigation Laboratory - National Reference Laboratory	Hungary
Crop Research Institute - Reference Laboratory for GMO Detection and DNA fingerprinting	Czech Rep.
Federal Environment Agency Austria	Austria
Finnish Customs Laboratory	Finland
Institute for Agricultural and Fisheries Research (ILVO)	Belgium
Institute for Consumer Protection. Department 3 – Food Safety	Germany
Institute for Hygiene and Environment	Germany
Institute of Biochemistry and Biophysics Polish Academy of Sciences	Poland
National Food Administration	Sweden
Scottish Agricultural Science Agency	UK
Walloon Agricultural Research Centre (CRA-W) - Department Quality of Agricultural Products	Belgium

3.   Materials

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

i)   genomic DNA of maize harbouring the MON 89034 event in a hemizygous status (Lot number: GLP-0612-17873-S) and;

ii)   genomic DNA of conventional maize (Lot number: GLP-0612-17871-S)

Samples were provided by the applicant in accordance with 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 89034 maize DNA and non-GM maize genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the control samples provided, in a constant amount of total maize 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:
    - ❑ TaqMan$^®$ 2X PCR Master Mix, 2 vials: 5 mL each
    - ❑ TaqMan$^®$ buffer A (10X), 1 vial: 800 µL
    - ❑ MgCl$_2$ (25 mM), 1 vial: 2.1 mL
    - ❑ dNTP mix (10 mM each), 1 vial: 160 µL
    - ❑ AmpliTaq Gold$^®$ polymerase (5U/ µL), 1 vial: 80 µL
    - ❑ Sterile distilled water, 1 vial: 8 mL

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

    hmg reference system
    - ❑ hmg primer 1   (10 µM):   240 µL
    - ❑ hmg primer 2   (10 µM):   240 µL
    - ❑ hmg probe   (5 µM):   260 µL

    MON 89034 system
    - ❑ MON 89034 primer 1   (10 µM):   720 µL
    - ❑ MON 89034 primer 2   (10 µM):   720 µL
    - ❑ MON 89034 probe   (5 µM):   320 µL

4. Experimental design

Twenty unknown samples, representing five GM levels, were used in the validation study (Table 2). On each PCR plate, the samples were analyzed either for the MON 89034 specific system or the *hmg* 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 89034 GM contents

MON 89034 GM%
(GMO copy number/maize genome copy number x 100)
0.09
0.40
0.90
3.00
8.00

5. Method

Description of operational steps followed

For the specific detection of event MON 89034 DNA, a 77-bp fragment of the integration region of the construct inserted into the plant genome (that spans the 3' 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 and a non fluorescent quencher MGBNFQ.

For the relative quantification of event MON 89034 DNA, a maize-specific reference system amplifies a 79-bp fragment of the maize endogenous gene *hmg* (*high mobility group,* accession number AJ131373), using a pair of *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM and TAMRA.

Standard curves are generated for both the MON 89034 and the *hmg* 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 maize line MON 89034 DNA in a test sample, the MON 89034 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value (GM% = MON 89034/*hmg* x 100).

Calibration sample S1 was prepared by mixing the appropriate amount of MON 89034 DNA in control non-GM maize DNA to obtain a 10% GM of MON 89034 maize line in a total of 200 ng

maize DNA. Samples S2 and S3 were prepared by 1:4 serial dilutions from the S1 sample and samples S4 and S5 were prepared by 1:3 serial dilutions from the S3 sample.

The absolute copy numbers in the calibration curve samples are determined by dividing the sample DNA weight (nanograms) by the published average 1C value for maize genome (2.725) [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 (% GM calculated considering the 1C value for maize genome as 2.725 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)	200	50	12.5	4.2	1.4
maize genome copies	73394	18349	4587	1529	510
MON 89034 maize line copies	7339	1835	459	153	51

6.   Deviations reported

Ten laboratories reported no deviations from the protocol.

One laboratory performed twice run B as no amplification plot was observed for wells corresponding to reference sample S5 in the *hmg* system.

One laboratory did not get signals for one out of the three replicates for sample U3 with both the MON 89034 and the *hmg* system, likely due to a pipetting error.

7.   Summary of results

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula $((10^{(-1/slope)})-1) \times 100$] of the reference curve and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for the MON89034 system and the *hmg* reference system are summarised in Table 4.

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

Lab	Plate	MON 89034			hmg		
		Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.69	86.68	1.00	-3.28	101.65	1.00
	B	-3.50	92.95	1.00	-3.15	107.59	0.99
2	A	-3.70	86.47	0.99	-3.31	100.37	0.99
	B	-3.65	87.77	0.99	-3.29	101.20	0.99
3	A	-3.65	87.81	1.00	-3.34	99.42	1.00
	B	-3.64	88.38	1.00	-3.23	103.94	1.00
4	A	-3.65	87.82	1.00	-3.39	97.30	1.00
	B	-3.60	89.62	1.00	-3.21	105.04	0.99
5	A	-3.75	84.83	1.00	-3.52	92.45	1.00
	B	-3.68	87.10	1.00	-3.49	93.60	1.00
6	A	-3.61	89.13	0.99	-3.12	108.98	0.99
	B	-3.59	89.92	0.99	-3.23	104.20	1.00
7	A	-3.74	84.94	1.00	-3.24	103.70	0.99
	B	-3.66	87.60	1.00	-3.24	103.63	1.00
8	A	-3.53	91.91	1.00	-3.35	99.00	1.00
	B	-3.56	90.79	0.99	-3.26	102.70	1.00
9	A	-3.79	83.69	0.99	-3.40	96.78	1.00
	B	-3.58	90.11	1.00	-3.30	100.88	1.00
10	A	-3.39	97.32	0.98	-3.36	98.39	0.99
	B	-3.48	93.91	0.99	-3.31	100.29	0.99
11	A	-3.56	90.99	1.00	-3.23	104.03	0.99
	B	-3.67	87.40	1.00	-3.28	101.61	1.00
12	A	-3.71	85.90	0.99	-3.29	101.19	1.00
	B	-3.78	83.79	0.99	-3.33	99.74	1.00
	Mean	-3.63	88.6	0.99	-3.30	101.1	1.00

The mean PCR efficiency was 88.6% for the MON 89034 system and 1001.1% for the hmg reference system. The linearity of the method was 0.99 and 1.00 for MON 89034 and hmg, respectively. 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.

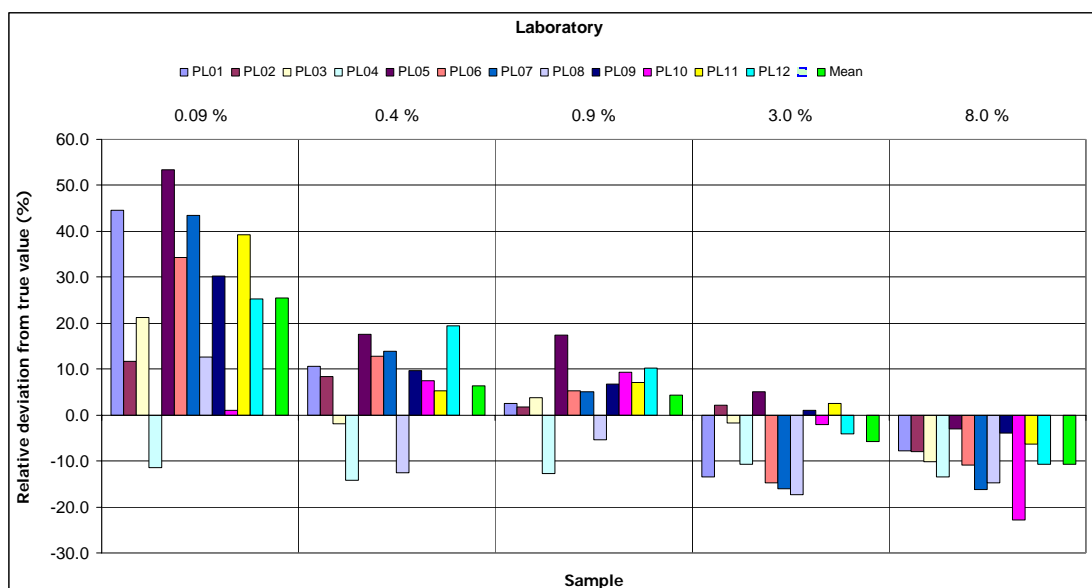Table 5. GM% mean values determined by laboratories for unknown samples.

LAB	Sample GMO content (GM% = GMO copy number/maize genome copy number x 100)																			
	0.09				0.4				0.9				3.0				8.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.14	0.08	0.15	0.15	0.40	0.45	0.41	0.52	1.12	1.05	0.71	0.81	2.51	2.88	2.50	2.49	7.62	6.77	7.63	7.50
2	0.11	0.08	0.11	0.10	0.40	0.42	0.52	0.40	0.87	0.90	0.92	0.97	3.36	2.80	2.79	3.30	7.19	8.16	7.27	6.82
3	0.13	0.10	0.10	0.11	0.39	0.38	0.42	0.38	1.02	0.93	0.91	0.88	3.35	3.08	2.41	2.95	6.98	7.36	7.12	7.30
4	0.09	0.08	0.08	0.07	0.28	0.32	0.41	0.36	0.72	0.64	0.83	0.95	2.60	2.41	2.81	2.88	7.82	6.57	6.60	6.72
5	0.14	0.14	0.13	0.14	0.48	0.47	0.46	0.47	1.10	0.95	0.96	1.21	2.90	2.95	3.81	2.95	8.53	6.04	7.98	8.50
6	0.13	0.10	0.11	0.13	0.49	0.58	0.33	0.41	0.98	1.36	0.81	0.63	2.90	3.00	1.90	2.43	6.79	6.65	8.37	6.72
7	0.15	0.10	0.13	0.13	0.50	0.47	0.37	0.48	1.08	1.10	0.84	0.77	2.72	2.78	2.35	2.21	6.26	6.53	7.29	6.74
8	0.12	0.07	0.11	0.11	0.39	0.35	0.29	0.37	0.99	1.02	0.69	0.70	2.68	2.70	2.20	2.34	6.64	6.71	6.44	7.50
9	0.12	0.08	0.13	0.13	0.43	0.45	0.44	0.44	1.04	0.96	0.89	0.96	2.90	3.15	3.03	3.04	7.63	7.35	7.78	7.96
10	0.08	0.11	0.10	0.07	0.40	0.41	0.44	0.46	0.84	0.89	1.10	1.11	2.76	2.28	3.45	3.27	7.05	6.02	6.16	5.45
11	0.12	0.10	0.18	0.10	0.45	0.49	0.39	0.36	1.20	0.99	0.87	0.80	3.53	3.33	2.38	3.07	6.87	8.90	6.24	7.96
12	0.13	0.12	0.10	0.11	0.54	0.53	0.39	0.44	0.98	1.02	0.99	0.97	2.91	2.92	2.98	2.69	7.76	6.49	7.43	6.93

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).

As observed in Figure 1, relative deviations from the true values are mainly positive for GM levels of 0.09% and 0.4% and negative at GM level of 3.0% and 8.0%, meaning that the GM content of unknown samples tends to be overestimated at the lowest GM levels and underestimated at the upper end of the dynamic range. More specifically, six laboratories over-estimated significantly (> 25%) the true values at the 0.09% GM level.

Overall, the average deviation from true vale, expressed as bias %, was limited and within the acceptance criterion of 25%.

Figure 1. Relative deviation (%) from the true value of MON 89034 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 22% at the 0.09% level and below 20% over the other GM levels, thus within the acceptance criterion.

Table 6. MON 89034: summary of validation results.

Unknown sample GMO%	Expected value (GMO%)				
	0.09	**0.4**	**0.9**	**3**	**8**
Laboratories having returned results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	-	-	-	-	-
Reason for exclusion	-	-	-	-	-
Mean value	**0.11**	**0.43**	**0.94**	**2.83**	**7.15**
Relative repeatability standard deviation, RSD_r (%)	18	13	17	12	9.5
Repeatability standard deviation	0.020	0.055	0.155	0.350	0.681
Relative reproducibility standard deviation, RSD_R (%)	22	15	17	14	10
Reproducibility standard deviation	0.025	0.064	0.155	0.390	0.740
Bias (absolute value)	0.02	0.03	0.04	-0.18	-0.86
Bias (%)	25	6.4	4.3	-5.8	-11

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 highest relative repeatability standard deviation is 18% at the 0.09% level, therefore well within the acceptance criterion.

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. The method satisfies this requirement across the dynamic range tested; in fact, the highest value of bias (%) is 25% at the 0.09% 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 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, therefore the method is considered applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The CRL-GMFF carries out its 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 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 ≥ 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 1/20th of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring ≤ 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.