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Event-specific Method for the Quantification of Maize MON87460 Using Real-time PCR

Validation Report and Validated Method

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Validation Report

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Executive Summary

The European Union Reference Laboratory for GM Food and Feed (EU-RL 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 87460 transformation event in maize DNA (unique identifier MON-8746Ø-4). The collaborative study 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 Company provided the detection method and the samples (genomic DNA extracted from homogenised seeds containing the transformation event and from conventional homogenised seeds). The EU-RL GMFF prepared the validation samples (calibration samples and blind samples at defined GM percentages [DNA/DNA], unknown to laboratories participating to the collaborative study). The collaborative trial involved twelve laboratories from ten European countries.

The results of the international collaborative study 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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Report on Steps 1-3 of the Validation Process

Monsanto Company provided the detection method and the control samples for maize event MON 87460 (unique identifier MON-8746Ø-4) according to Articles 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EU-RL 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 internal procedures ("Description of the EU-RL 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 (ENGL) and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed and one request for complementary information was addressed to the applicant. Upon reception of complementary information, the scientific evaluation of the detection method for event MON 87460 was positively concluded in May 2009.

In September-November 2009, the EU-RL GMFF verified experimentally the method characteristics (step 3, experimental testing of samples and methods) by quantifying five GM levels within the range 0.09%-8.0% on a copy number basis. The experiments were performed under repeatability conditions and showed that the PCR efficiency, linearity, trueness and precision were within the limits established by the ENGL. The DNA extraction method was previously tested on samples of food and feed and a report was published at http://gmo-crl.jrc.ec.europa.eu/summaries/MON88017_DNAExtr_report.pdf).

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

Content

1. Introduction

Monsanto Company submitted the detection method and control samples for maize event MON 87460 (unique identifier MON-8746Ø-4) according to Articles 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), established by Regulation (EC) No 1829/2003, organised the international collaborative study for the validation of the method of detection and quantification of maize MON 87460. The study involved twelve laboratories from ten European countries among those 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 EU-RL GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3) according to the requirements of Regulation (EC) No 641/2004.

The internal experimental evaluation of the method was carried out in September-November 2009.

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

The collaborative study aimed at validating a quantitative real-time PCR (Polymerase Chain Reaction) method. The method is an event-specific quantitative real-time TaqMan® PCR procedure for the determination of the relative content of MON 87460 DNA to total maize DNA. The procedure is a simplex system, in which a maize *hmg* (high mobility group) endogenous assay (targeting the taxon-specific *hmg* gene) and the target assay (MON 87460) are performed in separate wells.

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

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

2. Selection of participating laboratories

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

On 24th November 2009 the EU-RL GMFF invited the 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 maize event MON 87460.

Thirty-five laboratories expressed in writing their willingness to participate, while thirty-four did not answer. The EU-RL GMFF carried out 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 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 MON 87460.

Laboratory	Country
Central Agricultural Office, Food and Feed Safety Directorate - Laboratory for GMO Food	HU
Central Agricultural Office, Food and Feed Safety Directorate, Central Feed Investigation Laboratory	HU
Crop Research Institute - Reference Laboratory for GMO Detection and DNA fingerprinting	CZ
Danish Plant Directorate, Laboratory for Diagnostics in Plants, Seed, and Feed National Institute of Engineer, Technology and Innovation - Food Industry Laboratory	DK
Federal state agency of analysis and diagnosis for Rhineland-Palatinate – Institute of food chemistry Trier	DE
Food and Environment Research Agency	UK
Italian National Institute for Health - Department of Veterinary Public Health and Food Safety - Unit GMOs and Mycotoxins	IT
Laboratory of DNA analysis	EE
Lower Saxony Federal State Office for Consumer Protection and Food Safety, State Food Laboratory Braunschweig	DE
National Institute of Engineer, Technology and Innovation - Food Industry Laboratory	PT
Service Commun des Laboratoires du MINEFI - Laboratoire de Strasbourg	FR
Walloon Agricultural Research Centre (CRA-W) - Department Quality of Agricultural Products	BE

3. Materials

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

i) genomic DNA extracted from maize seeds hemizygous for event MON 87460. The male parent used in the cross was homozygous for MON87460 insert while the female inbred was a conventional line not carrying the MON 87460 insert;
ii) genomic DNA extracted from conventional maize seeds.

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 maize MON 87460 and non-GM maize genomic DNA at different GM percentages were prepared by the EU-RL GMFF, using the control samples provided, in a constant amount of total maize DNA.

Participants received the following materials:

- ✓ Five calibration samples (150 µL of DNA solution each) for the preparation of the standard curve, labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (75 µL of DNA solution each), labelled from U1 to U20.
- ✓ Reaction reagents:
  - ❑ TaqMan® Universal PCR Master Mix 2x, 3 bottles: 5.0 mL/bottle
  - ❑ Sterile distilled water, 1 tube: 9.5 mL

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

hmg taxon-specific system

❑	hmg primer 1,	(10 µM):	250 µL
❑	hmg primer 2,	(10 µM):	250 µL
❑	hmg probe,	(5 µM):	300 µL

MON87460-specific system

❑	MON 87460 primer 1,	(20 µM):	500 µL
❑	MON 87460 primer 2,	(20 µM):	500 µL
❑	MON 87460 probe,	(5 µM):	1000 µL

4. Experimental design

Twenty unknown samples (labelled from U1 to U20), representing five GM levels, were included in the validation study (Table 2). On each PCR plate, the samples were analysed for the MON 87460 specific system and for the *hmg* taxon-specific system. In total, two plates were run per laboratory and four replicates for each GM level were analysed. PCR was performed in triplicate for all samples. Participating laboratories carried out the estimation of the GM% according to the instructions provided in the protocol and using the application provided.

Table 2. MON 87460 GM contents

MON 87460 GM% [GM copy number/maize genome copy number (x 100)]
0.09
0.40
0.90
3.00
8.00

4. Method

For the detection of event MON 87460, an 82 bp fragment of the region spanning the 5' insert-to-plant junction is amplified. PCR products are measured during each cycle (real-time) by means of a specific oligonucleotide probe labelled with FAM dye (6-carboxyfluorescein) as a reporter at its 5' end and TAMRA (carboxytetramethylrhodamine) as a quencher dye at its 3' end.

For the relative quantification of GM event MON 87460, a maize specific reference system amplifies a 79 bp fragment of the maize endogenous gene *hmg* (High Mobility Group, GenBank accession number AJ131373), using two *hmg* gene-specific primers and an *hmg* probe labelled with FAM and TAMRA.

Standard curves are generated for both the MON 87460 and the *hmg* 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 87460 DNA in a test sample, the MON 87460 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 87460 / *hmg* x 100).

The calibration sample S1 was prepared by mixing the appropriate amount of MON 87460 DNA in control non-GM maize DNA to obtain a 10% MON 87460 in a total of 200 ng maize DNA.

Calibration samples S2 and S3 were prepared by 1:4 serial dilutions from the S1 and S2 samples, respectively. Calibration samples S4 and S5 were prepared by 1:3 serial dilutions from samples S3 and S4, respectively.

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (nanograms) by the published average 1C value for maize genome (2.725 pg) [(3)]. The copy number values used in the quantification, the GMO contents of the calibration samples and the total DNA quantity used in PCR are listed in Table 3.

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

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng/5 µL)	200	50	12.5	4.2	1.4
Maize genome copies	73394	18349	4587	1529	510
MON 87460 copies	7339	1835	459	153	51

6. Deviations reported

Eleven laboratories reported no deviations from the protocol.

One laboratory set the reaction volume at 30 µL for both assays.

7. Results

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula [(10^(-1/slope))-1)*100] of the standard curve and of the R^2 (expressing the linearity of the regression) reported by the laboratories for the MON 87460 and the *hmg* assays are reported in Table 4.

Table 4. Values of slope, PCR efficiency and R^2

	MON 87460			***hmg***		
LAB	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	-3.38	98	1.00	-3.36	98	1.00
	-3.43	96	0.99	-3.36	99	1.00
2	-3.26	103	0.98	-3.45	95	0.99
	-3.35	99	0.99	-3.40	97	1.00
3	-3.12	109	0.99	-3.44	95	1.00
	-3.36	98	1.00	-3.36	99	1.00
4	-3.32	100	1.00	-3.38	98	1.00
	-3.24	104	1.00	-3.23	104	0.99
5	-3.48	94	1.00	-3.39	97	1.00
	-3.37	98	1.00	-3.41	97	1.00
6	-3.32	100	1.00	-3.35	99	1.00
	-3.33	100	1.00	-3.33	100	1.00
7	-3.49	93	0.99	-3.64	88	0.99
	-3.20	105	0.98	-3.42	96	0.99
8	-3.29	102	1.00	-3.33	100	1.00
	-3.20	105	1.00	-3.26	102	1.00
9	-3.47	94	1.00	-3.36	99	1.00
	-3.39	97	1.00	-3.34	99	1.00
10	-3.28	102	0.99	-3.09	111	0.99
	-3.23	104	0.99	-3.08	111	1.00
11	-3.30	101	0.99	-3.33	100	1.00
	-3.17	107	0.99	-3.16	107	1.00
12	-3.28	102	1.00	-3.37	98	1.00
	-3.28	102	0.99	-3.23	104	1.00
Mean	-3.31	101	0.99	-3.34	100	1.00

The mean PCR efficiency was 101% and 100% for the MON 87460 and the *hmg* amplification systems, respectively, corresponding to a mean slope of -3.31 and -3.34. The R^2 of the method was 0.99 and 1.00 for the MON 87460 and the *hmg* assays, respectively. The data confirm the appropriate performance characteristics of the method tested in terms of PCR efficiency and linearity; in fact, the acceptance values set by the ENGL and the EU-RL GMFF are between -3.1 and -3.6 for the slope and ≥0.98 for the correlation coefficient R^2.

GMO quantification

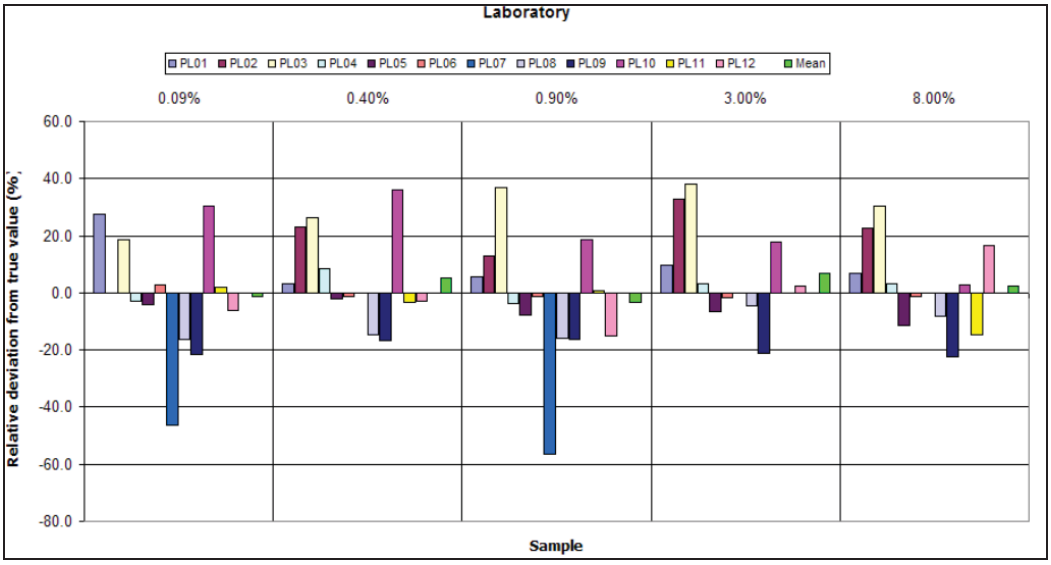
Table 5 reports the mean values of the four replicates for each GM level as estimated by all laboratories. Each mean value is the average of three PCR repetitions.

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

	GMO content (GM% = GMO copy number/maize genome copy number x 100)																			
LAB	**0.09**				**0.40**				**0.90**				**3.00**				**8.00**			
	REP 1	**REP 2**	**REP 3**	**REP 4**	**REP 1**	**REP 2**	**REP 3**	**REP 4**	**REP 1**	**REP 2**	**REP 3**	**REP 4**	**REP 1**	**REP 2**	**REP 3**	**REP 4**	**REP 1**	**REP 2**	**REP 3**	**REP 4**
1	0.10	0.13	0.13	0.10	0.46	0.40	0.37	0.40	1.08	0.90	0.90	0.92	3.51	3.23	3.50	2.92	7.59	8.26	9.39	8.97
2	0.04	0.12	0.19	0.07	0.46	0.54	0.58	0.40	1.30	1.03	0.97	0.77	3.91	4.68	3.99	3.38	10.47	9.82	9.13	9.90
3	0.08	0.10	0.14	0.10	0.50	0.50	0.55	0.47	1.51	1.17	1.00	1.25	4.18	3.94	4.17	4.29	10.38	10.12	10.50	10.69
4	0.10	0.08	0.08	0.09	0.49	0.40	0.41	0.43	0.86	0.85	0.91	0.84	3.49	3.17	2.96	2.75	7.71	8.48	8.26	8.62
5	0.09	0.09	0.07	0.10	0.41	0.40	0.36	0.40	0.81	0.82	0.84	0.85	2.75	2.85	2.83	2.78	6.96	7.07	6.92	7.41
6	0.10	0.09	0.08	0.10	0.41	0.38	0.39	0.40	0.91	0.84	0.95	0.86	3.11	2.81	2.98	2.91	8.25	7.80	7.58	7.93
7	0.06	0.05	0.02	0.07	0.67	0.28	0.21	0.25	0.25	0.39	0.30	0.63	0.89	2.97	4.79	5.70	24.14	3.22	7.22	8.28
8	0.08	0.08	0.06	0.09	0.43	0.25	0.36	0.33	0.66	0.74	0.90	0.73	2.84	2.56	3.12	2.92	7.07	7.36	7.03	7.89
9	0.07	0.07	0.07	0.07	0.33	0.33	0.35	0.33	0.71	0.69	0.82	0.78	2.37	2.63	2.23	2.24	6.60	6.86	5.46	5.85
10	0.10	0.11	0.14	0.12	0.58	0.60	0.51	0.49	1.02	1.19	1.03	1.04	3.82	3.19	3.59	3.54	8.65	8.26	7.78	8.22
11	0.08	0.11	0.08	0.09	0.45	0.45	0.32	0.33	1.04	0.95	0.82	0.82	3.59	2.10	3.23	2.32	7.76	6.05	6.79	6.74
12	0.11	0.06	0.07	0.10	0.36	0.41	0.38	0.40	0.83	0.70	0.71	0.82	2.95	3.15	3.02	3.14	8.76	8.80	9.61	10.09

In Figure 1 the relative deviation from the true value for each GM level tested is shown for each laboratory, following removal of statistical outliers. The coloured bars represent the relative GM quantification obtained by the participating laboratories; the green bar represents the overall mean value for each GM level.

Figure 1. Relative deviation (%) from the true value of MON 87460 for all laboratories



The mean relative deviations from the true values are substantially balanced between positive and negative ones by laboratories at all GM levels, being well within the ENGL acceptance

criterion of maximum 25%. One laboratory seriously underestimated the DNA content of samples 0.09% and 0.90%. However, these deviations (Table 6) did not reach the significance level to qualify the corresponding data as outliers.
The average bias generated by all laboratories is modest, being equal or below 7% at all GM levels tested, indicating a satisfactory trueness of the method.

8. Method performance requirements

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

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

As it can be observed in Table 6, the method satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R (%) is 28% at the 0.09% GM level, thus well within the acceptance criterion.

Table 6. MON 87460: summary of validation results.

	Target value (GMO%)				
Sample	**0.09**	**0.4**	**0.9**	**3.0**	**8.0**
Laboratories having returned results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	1	1	0	2	1
Reason for exclusion	1C	1C	-	2C	1C
Mean value	0.09	0.42	0.87	3.2	8.2
Relative repeatability standard deviation, RSD_r (%)	19	11	14	8.1	6.3
Repeatability standard deviation	0.017	0.048	0.119	0.259	0.513
Relative reproducibility standard deviation, RSD_R (%)	28	19	27	18	17
Reproducibility standard deviation	0.025	0.079	0.231	0.591	1.375
Bias (absolute value)	0.00	0.02	-0.03	0.21	0.18
Bias (%)	-1.4	5.1	-3.4	7.0	2.2

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

Bias is estimated according to ISO 5725 data analysis protocol.

Table 6 further documents the relative repeatability standard deviation (RSD_r), as estimated for each GM level. In order to accept methods for collaborative study, the EU-RL GMFF requires that RSD_r value is below 25%, as indicated by the ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

As it can be observed from the values reported in Table 6, the method shows a relative repeatability standard deviation below 25% over the entire dynamic range with a maximum of 19% at 0.09% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, the trueness should be ± 25% across the entire dynamic range. The method fully satisfies this requirement; indeed, the highest value of bias (%) is 7% at the 3.0% GM level, thus within the acceptance criterion.

9. Conclusions

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

The results obtained during the collaborative study indicate that the analytical module of the method submitted by the applicant complies with ENGL performance criteria.

The method is therefore applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The EU-RL GMFF operates according to ISO 9001:2008 (certificate number: CH-32232) and technical activities under ISO 17025:2005 [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative / quantitative PCR) – Accredited tests available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7]

11. References

1. International Standard (ISO) 5725. 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Swizerland.

2. Horwitz, W. (1995) Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67, 331-343.
3. Arumuganathan K, Earle ED. 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218

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

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

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

Method Acceptance Criteria

Applicability

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

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

Practicability

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

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

Specificity

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

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

Dynamic Range

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

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative

requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

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

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

Amplification Efficiency

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

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

R^2 Coefficient

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

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

Repeatability Standard Deviation (RSD_r)

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

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

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

Limit of Quantitation (LOQ)

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

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

Limit of Detection (LOD)

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

Acceptance Criterion: LOD should be less than $1/20^{th}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring ≤ 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.

Event-specific Method for the Quantification of Maize MON 87460 Using Real-time PCR

Protocol

18 January 2012

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

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Monsanto Company

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Content

1. General information and summary of the methodology

This protocol describes an event-specific quantitative real-time PCR (polymerase chain reaction) procedure for the determination of the relative content of maize event MON 87460 DNA to total maize DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction plates.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in the PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the detection of maize event MON 87460 DNA, an 82 bp fragment of the region spanning the 5′ insert-to-plant junction is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with fluorescent dye FAM (6-carboxyfluorescein) as a reporter at its 5′ end and with TAMRA (carboxytetramethylrhodamine) as a quencher dye at its 3′ end.

For the relative quantification of maize event MON 87460, a maize-specific reference system amplifies a 79 bp fragment of the maize *high mobility group* gene (*hmg*), using specific primers and an *hmg* specific probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of MON 87460 DNA in a test sample, Ct values for the MON 87460 and *hmg* system are determined for the sample. Standard curves are then used to estimate the relative amount of MON 87460 DNA to total maize DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from homogenised maize grains.
The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The method was validated in a collaborative study coordinated by the EU-RL GMFF. The study was undertaken with twelve participating laboratories in December 2009.

Each participant received twenty blind samples containing maize MON 87460 genomic DNA at five GM contents, ranging from 0.09 % to 8.0 %.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each GM level in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.04% in 200 ng of total maize DNA. The relative LOD was not assessed in the collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.085% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.09 %.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence of the recombination region between the insert and the plant genome; the sequence is specific to maize event MON 87460 and thus imparts event-specificity to the method.

The specificity of the GMO assay was assessed by the applicant in real-time PCR against genomic DNA extracted from oilseed rape RT73, RT200; maize GA21, NK603, MON810, MON863, MON88017, LY038, MON89034, MON87460; cotton MON531, MON1445, MON15985, MON88913; soybean GTS 40-3-2, MON89788; wheat MON71800 and of conventional maize, oilseed rape, cotton, soybean, wheat, rice, millet, lentil, sunflower, and oat. According to the method developer, none of the materials tested, except the positive control maize event MON 87460, yielded detectable amplification.

The specificity of the maize reference assay *hmg* was assessed by the applicant in real-time PCR against genomic DNA extracted from oilseed rape RT73, RT200; maize GA21, NK603, MON810, MON863, MON88017, LY038, MON89034, MON87460; cotton MON531, MON1445, MON15985, MON88913; soybean GTS 40-3-2, MON89788; wheat MON71800 and of conventional maize, oilseed rape, cotton, soybean, wheat, rice, millet, lentil, sunflower, and oat. According to the method developer, none of the materials tested, except the positive controls represented by all maize events and conventional maize, yielded detectable amplification.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. "forward flow direction" during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.
- PCR-reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochlorite solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - should be carried out at 0 – 4 °C.
- In order to avoid repeated freeze/thaw cycles, aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of maize event MON 87460

3.2.1 General

The PCR set-ups for the taxon-specific target sequence (*hmg*) and for the GMO (event MON 87460) are carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 50 µL and 25 µL for the MON 87460 and for the *hmg* systems respectively, with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a sample containing 10% MON 87460 maize DNA in a total of 200 ng of maize DNA (corresponding to approximately 73394 maize genome copies with one genome assumed to correspond to 2.725 pg of haploid maize genomic DNA)[(1)]. The other three standard samples are prepared by serial dilution.

A calibration curve is produced by plotting the Ct values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly using the options available with the sequence detection system software.

The copy number measured for the unknown sample DNA is obtained by interpolation from the standard curves.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. Keep thawed reagents at 1-4 °C on ice.
2. To prepare the amplification reaction mixtures add the following components (Tables 1 and 2) in two reaction tubes (one for the MON 87460 assay and one for the *hmg* assay) on ice in the order mentioned below (except DNA).

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 87460 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25.0
MON 87460 forward (20 µM)	600 nM	1.5
MON 87460 reverse (20 µM)	600 nM	1.5
MON 87460 probe (5 µM)	250 nM	2.5
Nuclease free water	#	15.5
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* reference assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.50
hmg primer 1 (10 µM)	300 nM	0.75
hmg primer 2 (10 µM)	300 nM	0.75
hmg-probe (5 µM)	160 nM	0.80
Nuclease free water	#	6.20
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the MON 87460 and one for the *hmg* reaction mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of master mix (e.g. 46 x 3 = 138 µL master mix for three PCR repetitions of the MON 87460 system and 21 x 3 = 63 µL master mix for the *hmg* system). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 µL DNA for three PCR repetitions for both systems). Vortex each tube for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
6. Spin down the tubes in a micro-centrifuge. Aliquot 25 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250x *g* for 1 minute at 4 °C) to spin down the reaction mixture.
7. Place the plate in the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Cycling program for MON 87460/*hmg* assays

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	Decontamination		50 °C	120	No	1
2	Initial denaturation		95 °C	600	No	1
3	Amplification	Denaturation	95 °C	15	No	45
		Annealing & Extension	60 °C	60	Yes	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

a) Set the threshold: display the amplification curves of one system (e.g. MON 87460) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.

b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *hmg* system).

e) Save the settings and export all the data to a text file for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct-values for each reaction.

The standard curves are generated both for the *hmg* and the MON 87460 specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the DNA copy numbers in the unknown sample.

To obtain the percentage value of event MON 87460 DNA in the unknown sample, the MON 87460 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 (GM% = MON 87460/hmg x 100).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction plates (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction plates suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)

- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Micro-centrifuge
- Centrifuge for plates
- Micropipettes
- Vortex
- Rack for reaction tubes
- 0.5/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2x). Applied Biosystems Part No 4304437

4.3 Primers and Probes

Table 4. Primers and probes sequences

Name	Oligonucleotide DNA Sequence (5′ to 3′)	Length (nt)
MON 87460 target sequence		
MON 87460 forward	5′ – CAC gTT gAA ggA AAA Tgg ATT g – 3′	22
MON 87460 reverse	5′ – TCg CgA TCC TCC TCA AAg AC – 3′	20
MON 87460 probe	6 - FAM 5' – Agg gAg TAT gTA gAT AAA TTT TCA AAg CgT TAg ACg gC – 3' TAMRA	38
Taxon specific *hmg* target sequence		
hmg primer 1	5′ – TTg GAC TAg AAA TCT CgT gCT gA – 3′	23
hmg primer 2	5′ – gCT ACA TAg ggA gCC TTg TCC T – 3′	22
hmg-probe	6-FAM 5′ – CAA TCC ACA CAA ACg CAC gCg TA – 3′ TAMRA	23

FAM: 6-carboxyfluorescein; TAMRA: tetramethylrhodamine

5. References

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Abstract

The European Union Reference Laboratory for GM Food and Feed (EU-RL 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 87460 transformation event in maize DNA (unique identifier MON-8746Ø-4). The collaborative study 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 Company provided the detection method and the samples (genomic DNA extracted from homogenised seeds containing the transformation event and from conventional homogenised seeds). The EU-RL GMFF prepared the validation samples (calibration samples and blind samples at defined GM percentages [DNA/DNA], unknown to laboratories participating to the collaborative study). The collaborative trial involved twelve laboratories from ten European countries.

The results of the international collaborative study 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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