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DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Event-specific method for the quantitation of maize line MON 863 using real-time PCR

Validation Report

Biotechnology & GMOs Unit
Institute for Health and Consumer Protection
DG JRC

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Executive Summary

The JRC as Community Reference Laboratory (CRL) for the GM Food and Feed (see Regulation EC 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 863 transformation event in maize flour. The collaborative trial was conducted according to internationally accepted guidelines.

Monsanto Company provided the method-specific reagents (primers, probes, reaction master mix), whereas the IRMM/JRC prepared the test samples (GM and non-GM maize flour). The trial involved twelve laboratories from eight European Countries.

The results of the collaborative trial have fully met ENGL's performance requirements and the scientific understanding about satisfactory method performance. Therefore, the JRC as Community Reference Laboratory considers the method validated as fit for the purpose of regulatory compliance.

The results of the collaborative study are publicly available under http://gmo-crl.jrc.it/. The method will also be submitted to CEN, the European Standardisation body, to be considered as international standard.

Contents

Document Approval		
Name / *Function*	**Date**	**Signature**
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1. Introduction

The Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for the GM Food and Feed (see Regulation EC 1829/2003) organised the collaborative trial of the event-specific method for the detection and quantification of MON 863 maize. The study involved twelve European laboratories, members of the European Network of GMO Laboratories (ENGL).

The method was tested by the Community Reference Laboratory in summer 2004. Following the evaluation of the in-house testing results, the ring trial was organized and took place between 28th July and 10th September 2004.

The operational procedure of the collaborative study comprised the following modules:

- ✓ DNA extraction: an enhanced CTAB DNA extraction and purification protocol adopted from the prEN ISO 21571:2002[1].
- ✓ Spectrophotometric quantification of the amount of total DNA extracted, adopted from prEN ISO 21571:2002.
- ✓ Real-time PCR (Polymerase Chain Reaction) monitor run (inhibition test).
- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event MON 863 DNA to total maize DNA. The procedure is a simplex system, in which a maize *Adh*1 endogenous assay (reference gene) and the target assay (MON 863) are performed in separate wells. The PCR assay has been optimised for use in an ABI Prism® 7700 sequence detection system. Although other systems may be used, thermal cycling conditions must be verified.

The ring-trial was carried out in accordance with the following internationally accepted guidelines:

- ✓ ISO 5725, especially considered in relation to the measure of precision (i.e. repeatability and reproducibility) and trueness.
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995).

[1] Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Nucleic Acid Extraction. CEN/TC 275/WG11N0031. Draft November 2002.

2. List of Participants

The method was tested in twelve ENGL laboratories to determine its performance. Each laboratory was requested to carefully follow the protocol provided. The participating laboratories are listed in Table 1.

Table 1. ENGL laboratories participating in the validation study of MON 863.

Laboratory	Country
LAV Sachsen-Anhalt	Germany
Bundesinstitut fuer Risikobewertung (BfR)	Germany
Institute of Public Health	Belgium
National Institute of Biology	Slovenia
Research Institute of Crop Protection, Reference Laboratory of the Ministry of Agriculture	Czech Republic
The Food and Consumer Product Safety Authority	Netherlands
Agricultural Biotechnology Centre	Hungary
Ente Nazionale Sementi Elette, Laboratorio Analisi Sementi	Italy
Istituto Superiore di Sanita', ISS	Italy
Baverian Health and Food Safety Authority	Germany
AGES – Institute for Food Control Vienna	Austria
Department of Plant Genetics and Breeding	Belgium

3. Materials

Samples of maize flour containing wild type maize and MON 863 maize at different concentrations were used. The samples used in the study (GM and non-GM maize flour) were prepared by the Institute for Reference Materials and Measurements (IRMM) of the European Commission Joint Research Centre. Monsanto Company developed and optimised the method and provided the specific reagents.

The participants received the following materials:

- ✓ Calibration maize flour sample 1g (10 % MON 863 maize, candidate CRM 416-3). From this sample, DNA was extracted, quantified and diluted to generate the standard curve.
- ✓ Unknown samples, represented by 10 unknown maize flour samples (candidate CRM samples) labelled from U1 to U10, 1 g each.
- ✓ Negative DNA target control (labelled C1): Bt176 maize DNA (150 µl @20ng/µl).
- ✓ Negative DNA target control (labelled C2): one maize flour sample, (candidate CRM 416-0), nominal 0% MON 863 maize, 1g. In addition to the negative DNA target controls, amplification reagent control (nucleic acid free water provided by each trial participant) was used on each PCR plate.
- ✓ Reaction reagents, primers and probes for the *Adh*1 reference gene and for the MON 863 specific systems as follows:
  - ❑ 4X TaqMan Universal PCR Master Mix (2X) (ABI, Cat No. 4304437): total amount 20 ml.

- ✓ Primers and probes (1 tube each) as follows:
  - ❑ MON 863 primer F 300 µl 5-GTAGGATCGGAAAGCTTGGTAC -3`
  - ❑ MON 863 primer R 300 µl 5- TGTTACGGCCTAAATGCTGAACT -3`
  - ❑ MON 863 probe PR 200 µl 6-FAM-TGAACACCCATCCGAACAAGTAGGGTCA -TAMRA
  - ❑ *Adh*1 primer F 500 µl 5-CCAGCCTCATGGCCAAAG-3`
  - ❑ *Adh*1 primer R 500 µl 5-CCTTCTTGGCGGCTTATCTG-3`
  - ❑ *Adh*1 probe PR 330 µl 6-FAM-CTTAGGGGCAGACTCCCGTGTTCCCT- TAMRA

Table 2 shows the GM value over the dynamic range for the unknown samples.

Table 2. MON 863 GM values.

MON 863 GM concentration (w/w)
0.00
0.10
1.00
5.00 ([1])
10.00

([1]) This GM level was prepared by IRMM for the purpose of this study.

4. Experimental design

Ten unknown samples representing five GM levels, were used to extract DNA. The two replicates for each GM level were analyzed on two PCR plates. The PCR analysis was triplicated for standard curve and control samples, but quadruplicated for unknown samples. Thus, each unknown sample was quantified based on four repetitions. Each participating laboratory carried out the determination of the GM% according to the instructions provided in the protocol.

5. Methods

Description of the operational steps

DNA was extracted from the flour samples by using an enhanced CTAB DNA extraction and purification protocol adopted from the prEN (ISO 21571:2002)[2]. Subsequently, purified DNA was quantified by means of spectrophotometry in order to determine the amount of DNA to be analysed in real-time PCR. The procedure "Basic ultraviolet spectrometric method" was adopted from the Annex B "Methods for the quantification of the extracted DNA" of the prEN ISO 21571:2002. The method has been widely used and ring-tested in the past (Anon, 2002). After the DNA quantification, a real-time PCR monitor run was carried out to provide data about possible PCR inhibition.

For specific detection of event MON 863 genomic DNA, a 84-bp fragment of the region that spans the 5′ insert-to-plant junction in maize event MON 863 is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For relative quantification of event MON 863 DNA, a maize-specific reference system amplifies a 70-bp fragment of *Adh*1, a maize endogenous gene, using a pair of *Adh*1 gene-specific primers and an *Adh*1 gene-specific probe labelled with FAM and TAMRA.

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 event MON 863 DNA in a test sample, the Ct-values of a certain sample for maize event MON 863 and *Adh*1 are determined. Standard curves are then used to calculate relative content of event MON 863 DNA to total maize DNA.

The first standard curve point S1 was derived from the 10% MON 863 candidate CRM (IRMM-416-3). This corresponds to 10,275 GM copies in 280 ng of DNA when 4 µl per reaction/well are used (70 ng/µl). Standard curve points S2 – S5 were obtained by serial dilution of the 10% standard S1.

[2] Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Nucleic Acid Extraction. CEN/TC 275/WG11N0031. Draft November 2002.

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 genomes (2.725 pg). The copy number values, which were used in the quantification, are provided in Table 3.

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

Sample code	S1	S2	S3	S4	S5
Maize genome copies	102,752	25,688	6,422	1,606	401
MON 863 GM copies	10,275	2,569	642	161	40

6. Deviations reported

Five laboratories reported no deviations from the operational steps described in the validation protocol.

During DNA extraction, one laboratory could not perform a gentle agitation of DNA solution at 4°C; one laboratory dissolved the DNA in water instead of TE buffer.

One laboratory performed the spectrophotometric measurement of the DNA extracted in a capillary system rather than using the vessels suggested by the protocol.

One participant measured the concentration of DNA extracted with a fluorometric system (PicoGreen® ds DNA Quantitation) instead of using a spectrophotometer, as required by the protocol; data from this laboratory were not included in Table 4.

Two laboratories noticed problems of inhibition during the monitor run due to a defective batch of *Adh*1 primers; the problem was solved by using a replacement batch of primers.

Three laboratories reported deviations from the plate layout in loading one or more samples during monitor run and quantitation runs; however, these small modifications did not affect the correct calculation of the results.

7. Summary of results

DNA extraction

Table 4 reports the values of DNA concentration after extraction from the ten maize flour samples provided to the participating laboratories. Two replicate samples for each GM level (total of five GM levels) were extracted and the DNA concentration determined by spectrophotometric measurement (the detailed protocol is available under http://gmo-crl.jrc.it/). The concentration of DNA reported in the table is expressed as ng/µl.

Table 4. DNA concentration (ng/μl) after extraction from validation samples at different GM% (w/w) by all laboratories.

		Samples (% GM)							
		0.0	0.1	1.0	5.0	10			
LAB	REPLICATE	DNA concentration (ng/μl)					MEAN	SD	CV %
1	1	206	228	226	172	288			
	2	351	333	514	449	449	**322**	**118**	**37**
2	1	200	200	181	194	256			
	2	160	192	150	193	192	**192**	**28**	**15**
3	1	115	110	153	134	126			
	2	125	130	135	124	114	**127**	**13**	**10**
4	1	280	327	94	123	284			
	2	294	284	305	329	240	**256**	**82**	**32**
5	1	174	171	152	191	190			
	2	158	127	158	157	118	**160**	**24**	**15**
6	1	-	-	-	-	-			
	2	-	-	-	-	-			
7	1	145	158	168	167	183			
	2	164	165	162	154	173	**164**	**10**	**6**
8	1	191	201	164	221	220			
	2	195	220	216	212	333	**217**	**44**	**20**
9	1	-	-	-	-	-			
	2	-	-	-	-	-			
10	1	257	303	328	264	256			
	2	294	281	308	335	308	**293**	**28**	**10**
11	1	132	122	107	134	148			
	2	128	133	121	138	145	**131**	**12**	**9**
12	1	173	140	165	173	191			
	2	155	199	150	231	221	**180**	**30**	**17**
	Mean	**195**	**201**	**198**	**205**	**222**			
	SD	**66**	**71**	**99**	**84**	**83**			
	CV %	**34**	**35**	**50**	**41**	**38**			

The average yield of DNA extraction is very similar for each GM % (vertical columns of table 4, mean valued highlighted in pale blue) across all laboratories: the mean values range from 195 to 222 ng/μl; the variability around this means is instead rather large, with a coefficient of variation ranging from 34 to 50%. These data suggest that the method provides constantly high quantity of DNA for all GM % levels tested, with a significant variability of DNA yield among laboratories.

High differences in DNA quantity extracted can also be noted by comparing concentration mean values among laboratories regardless the GM % extracted (horizontal rows of table 4, mean values highlighted in yellow): in this case the mean values among laboratories range from 131 to 322 ng/μl of DNA extracted.

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 curves and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for both PCR systems and runs (reference gene and GM specific, plate A and B), are summarised in Table 5.

Table 5. Values of standard curve slope, PCR efficiency and linearity (R^2) for the reference gene (*Adh*1) and the GM specific systems (MON 863)

		*Adh*1			MON 863		
LAB	**PLATE**	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**
1	**A**	- 3.16	93	0.99	- 3.77	84	0.99
	B	- 3.39	97	0.99	- 3.72	86	1.00
2	**A**	- 3.32	100	0.99	- 3.81	83	0.99
	B	- 3.19	94	0.99	- 3.44	95	0.99
3	**A**	- 3.72	86	0.99	- 4.02	77	0.98
	B	- 3.73	85	0.99	- 4.17	74	0.99
4	**A**	- 3.77	84	0.99	- 3.71	86	0.99
	B	- 3.77	84	0.99	- 4.00	78	0.99
5	**A**	- 3.29	99	0.99	- 3.54	92	1.00
	B	- 3.37	98	1.00	- 3.61	89	1.00
6	**A**	- 3.04	87	0.97	- 3.42	96	0.99
	B	- 3.08	89	0.97	- 3.41	96	0.99
7	**A**	- 3.50	93	0.99	- 3.58	90	0.99
	B	- 3.49	93	0.98	- 3.68	87	0.99
8	**A**	- 3.33	99	0.99	- 3.62	89	0.99
	B	- 3.82	83	0.98	- 3.70	86	0.99
9	**A**	- 3.29	99	0.99	- 3.75	85	0.98
	B	- 3.50	93	0.99	- 3.62	89	0.99
10	**A**	- 2.94	81	0.99	- 3.53	92	0.99
	B	- 3.95	79	0.93	- 3.69	87	0.99
11	**A**	- 3.32	100	0.99	- 3.73	85	0.99
	B	- 3.33	99	0.99	- 3.84	82	0.99
12	**A**	- 5.68	50	0.77	- 5.50	52	0.72
	B	- 5.94	47	0.74	- 5.93	47	0.72
	Mean	**- 3.62**	**88**	**0.97**	**- 3.87**	**84**	**0.97**

The PCR efficiency of the *Adh*1 system was on average 88%, while the one of the MON 863 system was 84%; it should be noted, however, that one laboratory (number 12) reported values of efficiency and linearity that are clearly deviating (being much lower) compared to all other laboratories. It derives that the average values of PCR efficiency of the two systems was respectively 92% and 87% if data of laboratory 12 are not considered.

The linearity of both reference gene and GM specific systems was on average equal to 0.97; also in this case, only the values reported by one laboratory (number 12) are much lower than the average of all other laboratories; the mean values of linearity of the *Adh*1 reference gene and MON 863 systems were respectively 0.984 and 0.990 if laboratory 12 is not included.

GMO quantitation

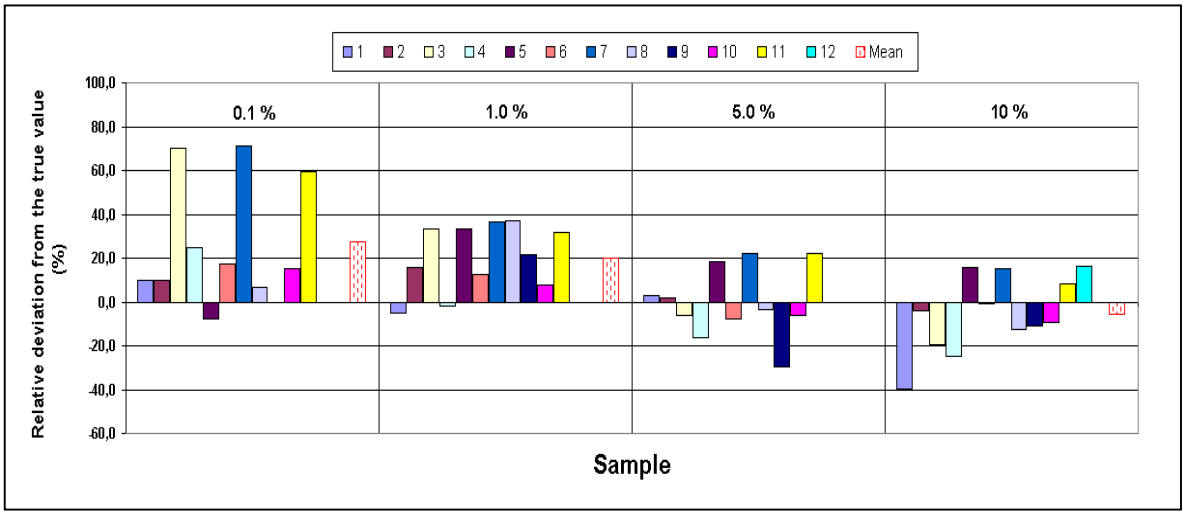
Table 6 shows the mean values of both replicates for each GM level as provided by all laboratories. Each mean value is the average of four PCR repetitions.

Table 6. Replicates' mean value by laboratories and by all unknown samples.

LAB	GM content of unknown samples (w/w)									
	0.00		0.10		1.00		5.00		10.00	
	REP 1	REP 2	REP 1	REP 2	REP 1	REP 2	REP 1	REP 2	REP 1	REP 2
1	0.00	0.00	0.12	0.10	1.12	0.78	5.38	4.94	4.09	7.97
2	0.00	0.00	0.15	0.07	1.33	0.99	5.33	4.84	9.25	9.92
3	0.00	0.00	0.12	0.22	1.11	1.56	5.06	4.32	7.00	9.10
4	0.00	0.00	0.15	0.10	0.87	1.09	4.27	4.12	8.08	6.98
5	0.00	0.00	0.07	0.11	1.08	1.59	6.42	5.43	11.88	11.25
6	0.00	0.00	0.10	0.10	1.20	1.00	4.40	4.80	10.80	9.00
7	0.00	0.00	0.23	0.11	1.25	1.48	6.89	5.35	11.64	11.38
8	0.00	0.00	0.12	0.09	1.38	1.36	4.73	4.93	9.27	8.20
9	0.00	0.00	0.00	0.12	1.24	1.19	4.00	3.03	7.68	10.09
10	0.00	0.00	0.09	0.14	0.91	1.25	4.75	4.64	9.74	8.43
11	0.00	0.00	0.16	0.16	1.29	1.35	5.90	6.29	10.51	11.15
12	0.09	0.59	1.60	2.68	4.77	5.58	8.72	9.79	11.07	12.13

In Figure 1 the deviation from the true value for each GM level tested is shown for each laboratory. Most of the laboratories overestimated the true value of MON 863 content at 0.1% and 1.0%, although the mean value of this bias (represented by the red dashed bar) is within the level accepted as performance requirement. On the other hand, the average relative deviation from the true value is exactly zero at 5% and very limited at 10% GM level.

Figure 1. Relative deviation (%) from the true value for all laboratories and MON 863 levels



8. Method performance requirements

The results of the collaborative trial are reported in table 7. These are evaluated with respect to the method acceptance criteria and to the method performance requirements, as established by ENGL and adopted by CRL. In table 7 estimates of both repeatability and reproducibility for each GM level are reported, after identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

Table 7. MON 863 validation data.

Unknown sample GM%	**Expected value (% GM w/w)**				
	0.00	**0.10**	**1.00**	**5.00**	**10.00**
Laboratories having returned results	12	11	12	12	12
Samples per laboratory	2	2	2	2	2
Laboratories excluded	1	1	1	1	0
Reason for exclusion	G. test	G. test	G. test	G. test	-
Mean value	**0.000**	**0.128**	**1.202**	**4.994**	**9.444**
Repeatability relative standard deviation (%)	0.00	34.51	17.43	10.13	12.80
Repeatability standard deviation	0.00	0.04	0.21	0.51	1.21
Reproducibility relative standard deviation (%)	0.00	34.51	17.81	17.73	20.64
Reproducibility standard deviation	0.00	0.04	0.21	0.89	1.95
Bias (absolute value)	0.00	0.028	0.202	0.006	0.556
Bias (%)	0	28	20	0	- 6

C. test = Cochran's test; G. test = Grubbs' test

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 5, the method fully satisfies this requirement at all GM levels tested; in fact, the values of RSD_R range from 17.73 to 34.51 (not considering the value of zero for the 0% GM level)

In the same table are also reported the relative repeatability standard deviation (RSD_r) values estimated from ring trial results for each GM level. In order to accept methods for collaborative trial evaluation, the CRL requires that RSD_r is below 30%, as indicated by ENGL. As it can be observed from the values reported in table 5, the method satisfies this requirement throughout the whole dynamic range tested, with the exception of the sample 0.1% for which the RSD_r is slightly above the level (34.51).

In table 5 measures of method bias, which allow estimating trueness, are also shown for each GM level. Bias is estimated according to ISO 5725 data analysis protocol. According to ENGL method performance requirements, trueness should be ±25% throughout the whole dynamic range. In this case the method satisfies such requirement for all GM values tested, with the exception of a minor deviation from the requirement for the sample at 0.1% GM level (bias = 28); however, this small deviation is not seen as sufficient to consider the method unsatisfactory.

9. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (available under http://gmo-crl.jrc.it). The method acceptance criteria were reported by the applicant and used to evaluate the method prior the collaborative study.

The results obtained during the collaborative trial indicate that the method can be considered as fit for enforcement purposes with respect to its intra and inter-laboratory variability, and trueness.

In conclusion, the method is considered fully complying with the current labeling requirements in Europe.

10. References

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