EUROPEAN COMMISSION
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 DAS-59122-7 using real-time PCR

Validation Report

Biotechnology & GMOs Unit
Institute for Health and Consumer Protection
DG Joint Research Centre

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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 59122 transformation event in maize DNA (unique identifier DAS-59122-7). The collaborative trial was conducted according to internationally accepted guidelines.

Pioneer provided the method-specific samples (seeds of maize line 59122, 100% event 59122 and seeds of non-GM maize line with comparable genetic background, 0% event 59122) whereas the JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage). The trial involved 14 laboratories from eight European countries.

The results of the collaborative trial 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		
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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 59122 maize. The study involved fourteen laboratories, members of the European Network of GMO Laboratories (ENGL).

Upon reception of methods, samples and related data, the JRC carried out the scientific evaluation of documentation and the in-house testing of the methods, according to the requirements of Regulation (EC) 641/2004 and following its operational procedures.
The internal tests were carried out in April-June 2005.
Following the evaluation of the data and the results of the laboratory tests, the ring trial was organized and took place in July-August 2005.

A method for DNA extraction from maize seeds, submitted by the applicant, was evaluated by the JRC; laboratory testing of the method was carried out in order to confirm its performance. The protocol was employed for the extraction of DNA samples used in this validation study. The protocol for DNA extraction and a report on method testing is available under http://gmo-crl.jrc.it/.

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

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event 59122 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 (59122) are performed in separate wells. The PCR assay has been optimised for use in real-time PCR instruments for plastic reaction vessels.

The ring-trial 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 Participants

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

Table 1. ENGL laboratories participating in the validation study of 59122.

Laboratory	Country
Agricultural Biotechnology Center	Hungary
Behoerde fuer Umwelt und Gesundheit	Germany
Chemisches und Veterinäruntersuchungsamt Freiburg	Germany
CRA-W, Dépt. Qualité des productions agricoles	Belgium
Department of Plantgenetics and Breeding	Belgium
Dr E Wessling Cemical Laboratory	Hungary
Ente Nazionale Sementi Elette	Italy
INETI – LIA (Instituto Nacional de Engenharia Tecnologia e Inovaçao)	Portugal
Institute of Public Health	Belgium
Istituto Zooprofilattico Sperimentale Lazio e Toscana	Italy
Laboratoire de la DGCCRF	France
Laboratoire National de la Protection des Végétaux	France
LGC	UK
National Institute of Biology	Slovenia

3. Materials

For the validation of the quantitative event-specific method, seeds from maize line 59122 (Lot n. PIV1PNE11040-00) constituted the positive control and conventional maize seeds (Lot n. PIP20LBN) was the negative control. Following DNA extraction, samples containing mixtures of 0% and 100% 59122 maize genomic DNA at different GMO concentrations were prepared by the JRC.

The participants received the following materials:

- ✓ Four calibration samples (180 µl of DNA solution each) labelled from S1 to S4.
- ✓ Twenty unknown DNA samples (100 µl of DNA solution each), labelled from U1 to U20.
- ✓ Amplification reagent control was used on each PCR plate.
- ✓ Reaction reagents as follows:

❑ PCR buffer II 10X, one tube	1.6 ml
❑ Rox Reference Dye (50x), one tube	200 µl
❑ Tween-20 (1%), one tube	145 µl
❑ Glycerol (20%), one tube	565 µl
❑ $MgCl_2$ (100mM), one tube	740 µl
❑ dATP (10mM), one tube	285 µl
❑ dCTP (10mM), one tube	285 µl
❑ dGTP (10mM), one tube	285 µl
❑ dUTP (20mM), one tube	285 µl
❑ Ampli Taq Gold (5U/µl), one tube	115 µl
❑ Distilled sterile water, one tube	6 ml

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

Hmg system

❑ MaiJ-F2 primer (10 µM)	282 µl
❑ mhmg-Rev (10 µM)	282 µl
❑ mhmg probe (10 µM)	110 µl

59122 maize system

❑ DAS-59122-7-rb1f (10 µM)	176 µl
❑ DAS-59122-7-rb1r (10 µM)	176 µl
❑ DAS-59122-7-rb1s probe (10 µM)	142 µl

Table 2 shows the GM contents of the unknown samples over the dynamic range.

Table 2. 59122 GM contents

59122 GM % **(GM copy number/maize genome copy number *100)**
0.10
0.40
0.90
2.00
4.50

4. Experimental design

Twenty unknown samples (ten for each of two plates), representing five GM levels, were used in the validation study. On each PCR plate, samples were analyzed in parallel with both the 59122 and Hmg gene specific system. Two plates in total were run, with two replicates for each GM level analysed on each run. The PCR analysis for triplicated 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).

5. Method

Description of the operational steps

For specific detection of event 59122 genomic DNA, an 86-bp fragment of the recombination region of parts of the construct inserted into the plant genome 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 59122 DNA, a maize-specific reference system amplifies a 79-bp fragment of Hmg (High mobility group), a maize endogenous gene, using a pair of Hmg gene-specific primers and an Hmg gene-specific probe labelled with FAM and TAMRA.
The standard curves are generated both for the Hmg and 59122 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 copy numbers in the unknown sample DNA by interpolation from the standard curves.

For the determination of the amount of 59122 DNA in the unknown sample, the 59122 copy number is divided by the copy number of the maize reference gene (Hmg) and multiplied by 100 to obtain the percentage value (GM% = 59122/Hmg * 100).

Calibration sample S1 was prepared by mixing the appropriate amount of 59122 DNA from the stock solution in control non-GM maize DNA to obtain 5% 59122 in a total of 200 ng maize DNA, corresponding the 3670 GM copies.
Samples S2-S4 were prepared by 1:5 serial dilutions of the S1 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 pg) (Arumuganathan & Earle, 1991).
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
Total amount (ng) of DNA in reaction	200	40	8	1.6
Maize genome copies	73394	14679	2936	587
59122 GM copies	3670	734	147	29

6. Deviations reported

Eight laboratories reported no deviations
One participating laboratory could not set the ramping rate at 1C/sec and could not define the single wells as independent samples.
Two laboratories inverted the positions of the reference and transgenic systems in one plate, but results were properly copied in the template results sheet.
One laboratory did not centrifuge the plates following the application of the adhesive cover.
One laboratory made a "120 seconds, 50 C" cycle precede the cycling program as set in the validation protocol.
One laboratory failed to save the settings in the sds-file on the ABI 7000 platform.
One laboratory carried out the validation with 20 µl instead of 25 µl per reaction well, re-calculating the volumes of the reaction mixes accordingly. Since that was considered as a major deviation affecting all the samples of both runs, this laboratory was excluded from further analysis.

7. Summary of results

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula ((10^(-1/slope))-1)*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 4.

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the reference gene (Hmg) and the GM specific (59122) systems. Data are reported as two-figure decimals.

		59122			Hmg		
Lab	Plate	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.54	91.54	1.00	-3.72	85.77	1.00
	B	-3.53	91.93	1.00	-3.67	87.24	1.00
2	A	-3.39	97.11	0.99	-3.40	97.01	1.00
	B	-3.28	98.10	0.99	-3.41	96.60	1.00
3	A	-3.69	86.63	0.99	-3.63	88.65	1.00
	B	-3.45	95.11	0.99	-3.51	92.75	1.00
4	A	-3.80	83.44	0.99	-3.59	90.00	1.00
	B	-3.32	99.76	0.99	-3.54	91.68	0.99
5	A	-3.32	99.93	0.99	-3.30	99.20	1.00
	B	-3.30	99.07	1.00	-3.17	93.05	0.99
6	A	-3.84	82.19	0.97	-3.38	97.69	1.00
	B	-3.14	91.89	0.97	-3.53	91.97	1.00
7	A	-4.17	73.75	0.98	-3.58	90.08	0.98
	B	-3.53	92.10	0.99	-3.47	94.28	0.99
8	A	-3.35	98.84	0.99	-3.52	92.28	1.00
	B	-3.52	92.37	1.00	-3.52	92.28	1.00
9	A	-3.77	84.27	1.00	-3.56	91.00	1.00
	B	-3.77	84.09	1.00	-3.70	86.28	1.00
10	A	-3.40	96.80	1.00	-3.47	94.09	1.00
	B	-3.52	92.30	1.00	-3.46	94.56	1.00
12	A	-3.10	90.01	0.96	-3.49	93.50	1.00
	B	-3.36	98.37	1.00	-3.34	99.40	0.99
13	A	-3.84	82.19	1.00	-3.77	84.29	1.00
	B	-3.81	83.11	0.99	-3.74	85.19	1.00
14	A	-3.30	99.08	0.98	-3.37	97.84	0.97
	B	-3.63	88.70	0.99	-3.29	98.54	0.99
Mean		**-3.53**	**91.26**	**0.99**	**-3.50**	**92.51**	**0.99**

Data reported in Table 4 confirm the performance characteristics of the method tested.
The mean PCR efficiency for the event-specific system was 91.26%, while that of the endogenous reference-system was 92.51%.

One run of a laboratory showed an important deviation in the slope of the 59122 system compared to the average. When data from said run were removed the efficiency of the event-specific system approached 92%, while that of the Hmg system remained unchanged.

The linearity of the method was equal to 0.99 for both the GM-specific 59122 and the Hmg system.

GMO quantitation

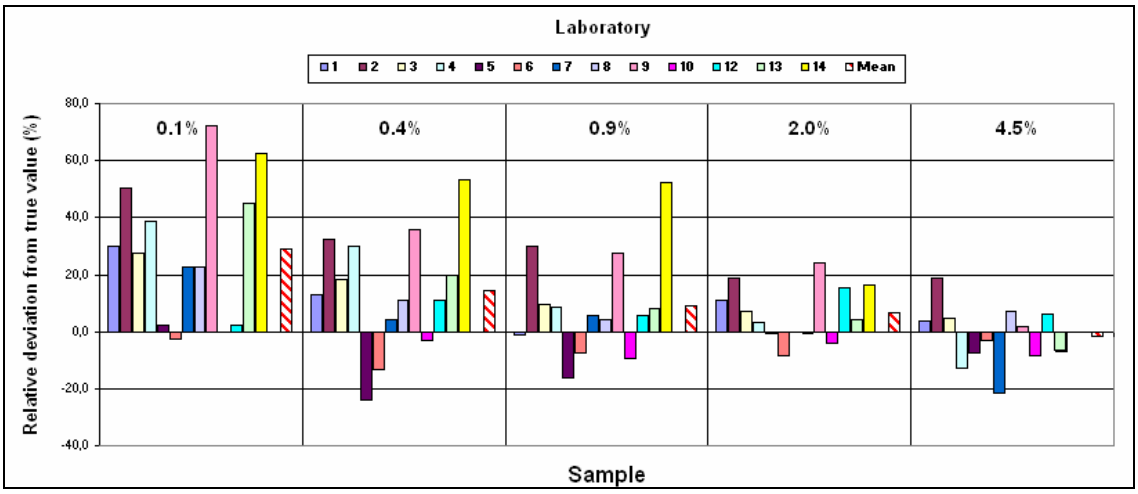
Table 5 shows the mean values of the four repetitions for each GM level as provided by all laboratories. Each mean value is the average of three PCR replicates.

Table 5. Replicates' mean value by laboratories and by all unknown samples.

	Sample GMO content (GM% = GM copy number/maize genome copy number *100)																			
LAB	0.1%				0.4%				0.9%				2.0%				4.5%			
	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	0.15	0.12	0.11	0.46	0.44	0.47	0.44	1.01	0.43	1.07	1.05	2.21	2.43	2.07	2.16	4.68	4.64	4.69	4.65
2	0.15	0.16	0.14	0.16	0.55	0.50	0.60	0.47	1.28	1.10	1.18	1.12	2.19	2.69	2.43	2.20	5.12	5.23	5.60	5.47
3	0.14	0.15	0.12	0.10	0.55	0.49	0.42	0.43	1.04	1.04	0.92	0.95	2.20	2.39	1.90	2.07	4.54	4.96	4.41	4.92
4	0.11	0.16	0.17	0.12	0.54	0.53	0.56	0.45	1.07	0.93	0.86	1.05	2.02	2.09	2.42	1.73	4.15	3.37	3.51	4.68
5	0.11	0.11	0.08	0.11	0.35	0.32	0.32	0.23	0.81	0.74	0.70	0.76	1.97	2.67	1.82	1.50	4.63	4.04	3.94	4.03
6	0.10	0.12	0.09	0.08	0.36	0.38	0.35	0.30	0.88	0.80	0.81	0.84	1.94	2.16	1.52	1.70	4.06	5.00	4.02	4.34
7	0.13	0.14	0.11	0.11	0.31	0.55	0.40	0.41	1.00	1.01	0.91	0.88	1.77	2.25	2.14	1.83	2.77	4.07	4.19	3.06
8	0.11	0.10	0.14	0.14	0.46	0.42	0.40	0.50	0.88	0.93	0.92	1.02	1.55	2.28	1.96	2.17	4.61	5.17	4.42	5.08
9	0.15	0.15	0.15	0.24	0.48	0.63	0.53	0.53	1.43	0.76	1.10	1.31	2.11	2.56	2.86	2.41	4.29	4.44	5.15	4.42
10	0.10	0.09	0.11	0.10	0.36	0.39	0.42	0.38	0.84	0.86	0.80	0.77	2.01	1.91	1.80	1.95	4.19	4.11	4.03	4.15
12	0.08	0.11	0.10	0.12	0.37	0.41	0.58	0.42	0.91	0.73	1.01	1.15	1.94	2.52	2.40	2.37	4.93	4.49	4.82	4.89
13	0.16	0.14	0.16	0.12	0.48	0.48	0.54	0.42	0.92	0.90	1.10	0.97	2.08	2.18	2.02	2.04	4.48	3.97	4.30	4.09
14	0.11	0.20	0.17	0.17	0.69	0.66	0.45	0.65	1.61	1.29	1.06	1.53	2.14	2.53	2.87	1.75	4.92	8.24	4.28	6.51

In Figure 1 the relative deviation from the true value for each GM level and per laboratory is shown. The coloured bars represent the relative GM quantification obtained in the participating laboratories; the red dashed bar represents the overall mean. In this computation, several laboratories overestimated the true value at the lowest GM levels of the dynamic range (0.10%-0.40%). The mean overall bias of the GM quantitation, however, was well within the acceptance criteria for method performance at each GM-level, with the exception of a modest bias at 0.1% level, indicating overall a good correlation between estimated and true value.

Figure 1. Relative deviation (%) from the true value of 59122 for all laboratories (coloured bars) and the overall mean (red dashed bar)



8. Method performance requirements

The results of the collaborative trial are reported in table 6. 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 6 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 6. 59122 validation data.

Unknown sample GM%	Expected value (GMO %)				
	0.10	**0.40**	**0.90**	**2.00**	**4.50**
Laboratories having returned results	14	14	14	14	14
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	0	0	1
Reason for exclusion	-	-	-	-	1C
Mean value	**0.13**	**0.46**	**0.98**	**2.13**	**4.43**
Repeatability relative standard deviation (%)	18.16	13.89	15.84	13.59	8.45
Repeatability standard deviation	0.02	0.06	0.16	0.29	0.37
Reproducibility relative standard deviation (%)	24.59	21.80	21.77	14.94	13.15
Reproducibility standard deviation	0.03	0.10	0.21	0.32	0.58
Bias (absolute value)	0.03	0.06	0.08	0.13	0.07
Bias (%)	29	15	9	7	-1

C. test = Cochran's 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 6, the method fully satisfies this requirement at all GM level tested. In fact, the highest value of RSD_R (%) is 24.59 at the 0.10% level, well within the acceptance criterion.

In the same table the *relative repeatability standard deviation (RSD_r)* values are also reported, as 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 25%, as indicated by ENGL. As it can be observed from the values reported in table 6, the method satisfies this requirement throughout the whole dynamic range tested.

In table 6 measures of method bias, which allows 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 = 29%); however, this small deviation is not seen as sufficient to consider the method unsatisfactory, also considering that at the same level the values of repeatability and reproducibility standard deviation fall within the accepted limit.

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 to 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. The method bias although satisfying performance requirements at and above GM-level of 0.40% shows a bias modestly exceeding performance requirements at 0.10% GM concentration. While applying the present method it is suggested to keep into consideration this slight overestimation.

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

10. References

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