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COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Event-specific method for the quantitation of maize line TC1507 using real-time PCR

Validation Report

Biotechnology & GMOs Unit
Institute for Health and Consumer Protection
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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 TC1507 transformation event in maize flour (also known as Herculex^{TM} I, unique identifier DAS-Ø15Ø7-1). The collaborative trial was conducted according to internationally accepted guidelines.

Pioneer Hi-Bred International provided the method-specific reagents and samples (genomic DNA extracted from the 0% and 100% event TC1507 maize), whereas the JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage). The trial involved fourteen laboratories from ten European Countries and Switzerland.

The results of the collaborative trial 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.

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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 TC1507 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 July-August 2004.
Following the evaluation of the data and the results of the laboratory tests, the ring trial was organized and took place in November 2004.

A method for DNA extraction from maize grain/seed, 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 TC1507 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 (TC1507) are performed in separate wells. The PCR assay has been optimised for use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in the method.

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.

Table 1. ENGL laboratories participating in the validation study of TC1507.

Laboratory	Country
Behoerde fuer Umwelt und Gesundheit	Germany
Biogeves	France
Central Science Laboratory	United Kingdom
CRA-W, Département Qualité' des Productions Agricoles	Belgium
Danish Institute for Food and Veterinary Research	Denmark
Department of Plant Genetics and Breeding	Belgium
Ente Nazionale Sementi Elette, Laboratorio Analisi Sementi	Italy
General Chemical State Laboratory, Food Division	Greece
Istituto Zooprofilattico Sperimentale Lazio e Toscana	Italy
LGC	United Kingdom
National Food Administration	Sweden
National Institute of Biology	Slovenia
Swiss Federal Research Station for Animal Production and Dairy Products	Switzerland
Umweltbundesamt GmbH	Austria

3. Materials

For the validation of the quantitative event-specific method, DNA was extracted by the applicant from non-GM maize kernels (Pioneer Brand Number X1083A NULL) and from 100% TC1507 hybrid kernels (Pioneer Brand Number 38P04-DAS-01507-1).
Samples containing mixtures of 0% and 100% TC1507 maize genomic DNA at different GMO concentrations were prepared by the JRC.

The participants received the following materials:

- ✓ Four calibration samples (200 µl of DNA solution each) labelled from S1 to S4.
- ✓ Twelve unknown DNA samples (100 µl of DNA solution each), labelled from U1 to U12.
- ✓ Negative DNA target control (labelled C1): Bt176 maize DNA (200 µl @20ng/µl).
- ✓ Negative DNA target control (labelled C2): 0% TC1507 maize DNA (200 µl @35ng/µl). In addition to the negative DNA target controls, amplification reagent control was used on each PCR plate.
- ✓ Reaction reagents, primers and probes for the HMG reference gene and for the TC1507 specific systems as follows:
  - ❑ 10X Buffer, including Rox: 1.3 ml
  - ❑ dNTP-Mix (10/20 mM): 260 µl
  - ❑ $MgCl_2$ (25 mM): 2.6 ml
  - ❑ Distilled sterile water: 6 ml
  - ❑ AmpliTaq Gold Polymerase (5U/µl): 100 µl

- ✓ Primers and probes (1 tube each) as follows:
  - ❑ MaiY-F1 primer (100 µM): 20 µl (TC1507 system)
  - ❑ MaiY-R3 primer (100 µM): 20 µl
  - ❑ MaiY-S1 probe (100 µM): 10 µl

  - ❑ MaiJ-F2 primer (100 µM): 20 µl (HMG reference system)
  - ❑ mhmg-rev primer (100 µM): 20 µl
  - ❑ mhmg probe (100 µM): 12 µl

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

Table 2. TC1507 GM contents

TC1507 GM % **(GM copy number/maize genome copy number *100)**
0.00
0.10
0.50
0.90
2.00
5.00

4. Experimental design

Twelve unknown samples representing six GM levels, were used in the validation study. The two replicates for each GM level were analyzed on the same PCR plate. Two plates in total were run. The PCR analysis was triplicated for all samples. Thus, each unknown sample was quantified based on three repetitions. Each participating laboratory 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 TC1507 genomic DNA, a 58-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 TC1507 DNA, a maize-specific reference system amplifies a 79-bp fragment of HMG (High Mobility Group) gene (Krech *et al.*, 1999), a maize endogenous gene, using a pair of HMG gene-specific primers and an HMG 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 TC1507 DNA in a test sample, the Ct-values of a certain sample for maize event TC1507 and HMG are determined. Standard curves are then used to calculate relative content of event TC1507 DNA to total maize DNA.

The first standard curve point S1 was derived from a 10% TC1507 sample. This corresponds to 7,339 GM copies in 200 ng of DNA when 5 µl per reaction/well are used (40 ng/µl). Standard curve points S2 – S4 were obtained by serial dilution of the 10% standard S1.

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) (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
Maize genome copies	73,394	14,679	2,936	587
TC1507 GM copies	7,339	1,468	294	59

6. Deviations reported

Eleven laboratories reported no deviations from the protocol.

One laboratory added in the cycling program a 2' at 50°C step, once before the initial denaturation.

One laboratory mistakenly used the upper half of the plate for the amplification of the maize reference system, and the lower half of the plate for the amplification of the GM specific TC1507. Calculations of the % GM were corrected accordingly by the participant.

One laboratory repeated the run for the plate B due to an incorrect use of wells sealing system.

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 (TC1507) systems

		HMG			TC1507		
LAB	PLATE	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	A	- 3.47	94	0.998	- 3.37	98	0.997
	B	- 3.47	94	0.999	- 3.34	99	0.998
2	A	- 3.45	95	0.998	- 3.25	97	0.998
	B	- 3.43	96	0.998	- 3.35	99	0.999
3	A	- 3.39	97	0.999	- 3.26	97	0.996
	B	- 3.20	95	0.998	- 3.38	98	0.998
4	A	- 3.40	97	0.997	- 3.08	89	0.994
	B	- 3.47	94	0.997	- 3.07	88	0.986
5	A	- 3.62	89	0.998	- 3.29	99	0.993
	B	- 3.61	89	0.998	- 3.22	96	0.992
6	A	- 3.51	93	0.997	- 3.32	100	0.997
	B	- 3.52	92	0.999	- 3.39	97	0.998
7	A	- 3.39	97	0.995	- 3.33	100	0.993
	B	- 3.36	98	0.998	- 3.30	99	0.994
8	A	- 3.35	99	0.999	- 3.31	99	0.991
	B	- 3.39	97	0.999	- 3.29	99	0.998
9	A	- 3.58	90	0.999	- 3.46	95	0.998
	B	- 3.47	94	0.999	- 3.34	99	0.998
10	A	- 3.59	90	0.998	- 3.41	96	0.992
	B	- 3.37	98	0.998	- 3.39	97	0.995
11	A	- 3.38	98	0.999	- 3.24	96	0.996
	B	- 3.46	95	0.999	- 3.42	96	0.995
12	A	- 3.29	99	0.997	- 3.16	93	0.995
	B	- 3.44	95	0.998	- 3.16	93	0.996
13	A	- 3.51	93	0.999	- 3.32	100	0.996
	B	- 3.47	94	0.999	- 3.52	92	0.998
14	A	- 3.40	97	0.983	- 3.31	99	0.982
	B	- 3.20	95	0.990	- 3.23	96	0.993
	Mean	**- 3.43**	**95**	**0.997**	**- 3.30**	**97**	**0.995**

Data reported in Table 4 confirm the excellent performance characteristics of the method tested.

In fact, the PCR efficiency of the HMG reference system was on average 95%, while the overall efficiency of the TC1507 system was 97%.

The linearity of reference gene and GM specific systems was on average equal to 0.997 and 0.995, respectively.

GMO quantitation

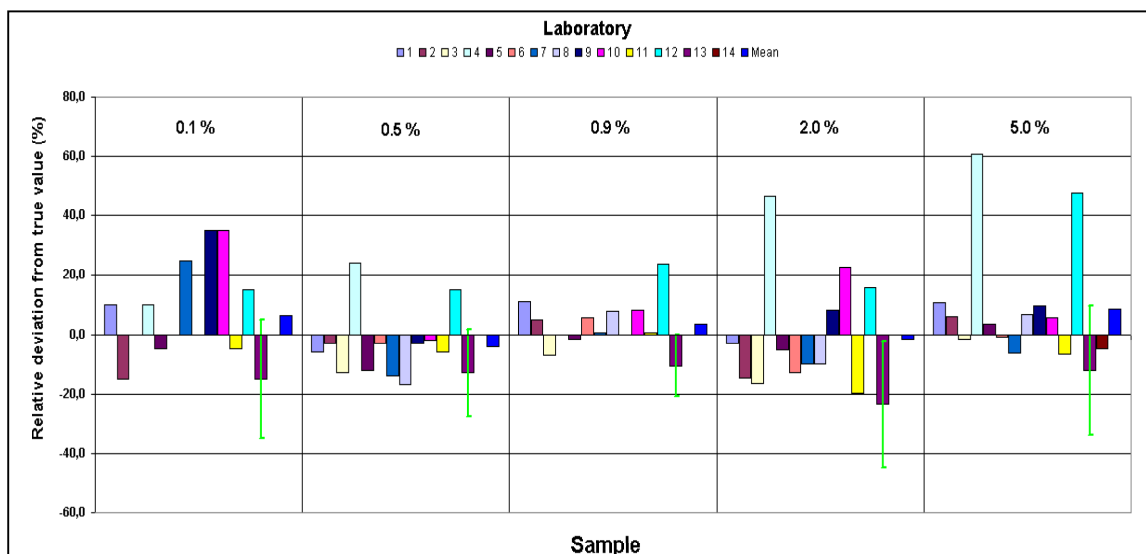
Table 5 shows the mean values of both replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

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

	Unknown sample GMO concentration (GM% = GMO copy number/maize genome copy number * 100)											
LAB	**0.00**		**0.10**		**0.50**		**0.90**		**2.00**		**5.00**	
	REP 1	**REP 2**	**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	0.10	0.12	0.41	0.53	1.02	0.98	1.93	1.95	5.42	5.64
2	0.00	0.00	0.08	0.09	0.49	0.48	0.95	0.94	1.74	1.67	5.21	5.38
3	0.00	0.00	0.09	0.11	0.43	0.44	0.88	0.79	1.69	1.65	5.19	4.64
4	0.00	0.00	0.11	0.11	0.53	0.71	1.28	1.53	3.13	2.73	8.28	7.80
5	0.00	0.00	0.12	0.07	0.40	0.48	0.94	0.83	2.07	1.72	6.02	4.31
6	0.00	0.00	0.11	0.09	0.47	0.50	1.05	0.85	1.79	1.70	5.25	4.65
7	0.00	0.00	0.12	0.13	0.46	0.40	0.94	0.87	1.93	1.67	4.40	4.97
8	0.00	0.00	0.10	0.10	0.45	0.38	1.01	0.93	1.74	1.86	4.85	5.83
9	0.00	0.00	0.13	0.14	0.47	0.50	0.94	0.86	2.27	2.06	5.57	5.40
10	0.00	0.00	0.11	0.16	0.48	0.50	0.89	1.06	2.71	2.20	5.00	5.57
11	0.00	0.00	0.09	0.10	0.47	0.47	0.96	0.85	1.61	1.60	4.97	4.35
12	0.00	0.00	0.09	0.14	0.51	0.64	1.12	1.11	2.23	2.40	7.91	6.86
13	0.00	0.00	0.07	0.10	0.42	0.45	0.78	0.83	1.58	1.48	4.42	4.37
14	0.00	0.00	0.09	0.11	0.17	0.22	0.43	1.23	2.12	3.26	3.17	6.34

In Figure 1 the deviation from the true value for each GM level tested is shown for each laboratory. As it can be observed, at all GM levels the relative deviation from the true value is limited, with the exception of two laboratories that overestimated by over 40% from the true value at 5% GM level; one of these two laboratories clearly overestimated also at 2% GM level. No overall overestimation/underestimation trend can be observed.

Figure 1. Relative deviation (%) from the true value for all laboratories and TC1507 levels



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. TC1507 validation data.

Unknown sample GM%	Expected value (GMO %)					
	0.00	**0.10**	**0.50**	**0.90**	**2.00**	**5.00**
Laboratories having returned results	14	14	14	14	14	14
Samples per laboratory	2	2	2	2	2	2
Number of outliers	0	0	1	2	1	0
Reason for exclusion	-	-	G. test	G. test; C. test	C. test	-
Mean value	**0.000**	**0.106**	**0.480**	**0.933**	**1.966**	**5.420**
Repeatability relative standard deviation (%)	0.00	18.11	11.70	7.68	8.48	14.41
Repeatability standard deviation	0.00	0.02	0.06	0.07	0.17	0.78
Reproducibility relative standard deviation (%)	0.00	19.91	14.78	10.24	21.19	21.65
Reproducibility standard deviation	0.00	0.02	0.07	0.10	0.42	1.17
Bias (absolute value)	0.00	0.006	- 0.02	0.033	- 0.034	0.42
Bias (%)	0.00	6.00	- 4.00	3.70	- 1.70	8.40

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 level tested. In fact, the highest value of RSD_R (%) is 21.65, well within the acceptance criterion.

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.

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 excellently satisfies such requirement throughout the whole dynamic range tested. Remarkably, the highest value of bias is 8.40%, obtained for the sample containing 5% of TC1507.

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 complying with the current labeling requirements in Europe.

10. References

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