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 T25 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 T25 transformation event in maize flour (unique identifier ACS-ZMØØ3-2). The collaborative trial was conducted according to internationally accepted guidelines.

Bayer CropScience provided the method-specific samples (genomic DNA extracted from the 0% and 100% event T25 maize), whereas the JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage). The trial involved thirteen laboratories from eleven Countries of the European Union.

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.

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 T25 maize. The study involved thirteen 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 February-March 2005.
Following the evaluation of the data and the results of the laboratory tests, the ring trial was organized and took place in April 2005.

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 T25 DNA to total maize DNA. The procedure is a simplex system, in which a maize *Adh*1 (*Alcohol dehydrogenase*-1) endogenous assay (reference gene) and the target assay (T25) 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 thirteen 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 maize T25.

Laboratory	Country
Agricultural Biotechnology Centre	Hungary
AGES-Institute for Food Control Vienna (CC Biochemistry)	Austria
Centro Nacional de Alimentación – Agencia Española de Seguridad Alimentaria	Spain
General Chemical State Laboratory, Food Division	Greece
Institute of Chemical Technology Prague	Czech R.
Institute of Public Health	Belgium
Istituto Superiore di Sanita', ISS	Italy
Laboratoire de la DGCCRF	France
Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen Amtliche Lebensmittelüberwachung	Germany
LVGA Saarbrucken	Germany
RIKILT Institute of Food Safety	Netherlands
Scottish Agricultural Science Agency	UK
The Food and Consumer Product Safety Authority	Netherlands

3. Materials

For the validation of the quantitative event-specific method, a T25 DNA stock solution, extracted from T25 line (Bayer BioScience, Lot Number 32RRMM0034), was provided by the applicant, while the control DNA stock solution was extracted from a non-GM near-isogenic line (Bayer BioScience, Lot Number 32RRMM0033).
Samples containing mixtures of 0% and 100% T25 maize genomic DNA at different GMO concentrations were prepared by the JRC in a constant amount of total maize DNA.

The participants received the following materials:

- ✓ Five calibration samples (200 µl of DNA solution each) labelled from S1 to S5.
- ✓ 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, primers and probes for the *Adh*1 reference gene and for the T25 specific systems as follows:
- ✓
  - ❑ Universal PCR Master Mix 2X, 2 bottles: 5 ml each
  - ❑ Distilled sterile water: 4 ml

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

  *Adh*1 system
  - ❑ KVM182 primer (10 µM): 160 µl
  - ❑ KVM183 primer (10 µM): 160 µl
  - ❑ TM014 TaqMan® probe (10 µM): 160 µl

  T25 maize system
  - ❑ MLD143 primer (10 µM): 320 µl
  - ❑ MDB551 primer (10 µM): 320 µl
  - ❑ TM016 TaqMan® probe (10 µM): 160 µl

Table 2 shows the GM contents of the unknown samples distributed to the participants.

Table 2. T25 GM contents

T25 GM % **(GM copy number/maize genome copy number *100)**
0.15
0.40
0.90
2.00
3.30

4. Experimental design

Twenty unknown samples, representing five GM levels, were used in the validation study. On each PCR plate, samples were analyzed in parallel with both the T25 and *Adh*1 specific system. Two plates in total were run, with two replicates for each GM level analysed on each run. The PCR analysis was 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 T25 genomic DNA, a 102-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 T25 DNA, a maize-specific reference system amplifies a 136-bp fragment of *Adh*1 (alcohol dehydrogenase-1) a maize endogenous gene (Hernandez *et al* 2004), using a pair of *Adh*1 gene-specific primers and an *Adh*1 gene-specific probe labelled with FAM and TAMRA.

For quantification of the amount of event T25 DNA in a test sample, the normalized ∆Ct values of calibration samples are used to calculate, by linear regression, a reference curve (plotting ∆Ct values against the logarithm of the amount of T25 event DNA). The normalized ∆Ct values of the unknown samples are measured and, by means of the regression formula, the relative amount of T25 event DNA is estimated.

Calibration samples from S1 to S5 were prepared by mixing the appropriate amount of T25 DNA from the stock solution in control non-GM maize DNA to obtain the following relative contents of T25: 3.6%, 1.8%, 0.9%, 0.45% and 0.09%. The total DNA amount was 200 ng, when 5 µl per reaction/well are used (40 ng/µl).

GM contents of the calibration samples and total DNA quantity used in PCR are provided in Table 3 (% GM calculated considering the 1C value for maize genomes, 2.725 pg [Arumuganathan & Earle, 1991]).

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	200	200	200	200
% GM (DNA/DNA)	3.6	1.8	0.9	0.45	0.09

6. Deviations reported

Eight laboratories reported no deviations from the protocol.

One laboratory did not perform the tests and was therefore unable to return the results concerning its participation to the validation trial.

One laboratory did not centrifuge the reaction plate but all samples were very carefully loaded into the bottom of the reaction tubes and no drop was present on the sides of the reaction tubes.

One laboratory performed the setting of baseline and threshold according to Bio-Rad iCycler IQ 3.1 instructions.

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)

LAB	PLATE	Slope	PCR Efficiency (%)	Linearity (R^2)
1	A	-3.60	90	0.97
	B	-3.51	93	0.98
2	A	-3.58	90	0.97
	B	-3.57	91	0.97
4	A	-3.47	95	0.98
	B	-3.50	93	0.96
5	A	-3.22	96	0.98
	B	-3.24	96	0.97
6	A	-3.41	96	0.96
	B	-3.58	90	0.97
7	A	-3.24	96	0.98
	B	-3.43	96	0.99
8	A	-3.47	94	0.97
	B	-3.30	99	0.98
9	A	-3.52	93	0.99
	B	-3.69	87	0.99
10	A	-3.48	94	0.99
	B	-3.30	99	0.99
11	A	-3.26	97	0.93
	B	-2.65	62	0.87
12	A	-3.45	95	0.99
	B	-3.61	89	0.99
13	A	-3.64	88	1.00
	B	-3.71	86	1.00
	Mean	**-3.44**	**92**	**0.97**

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

In fact, the PCR efficiency was on average 92%. Laboratory number 11 reported a value of efficiency for one of the two runs greatly deviating from the mean of the other laboratories.

The linearity of the method was on average equal to 0.97. However, also in this case the same laboratory reported a low value of linearity for the same run. If this value is excluded, the mean value for R^2 raises to 0.98.

GMO quantitation

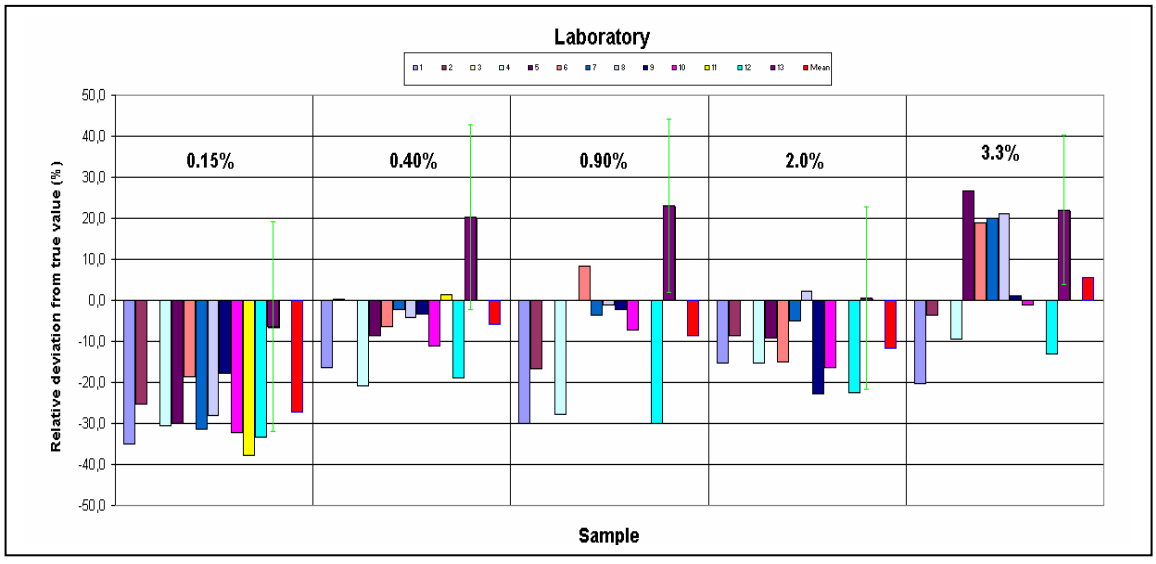
Table 5 shows the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

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

	Sample GMO content (GM% = GM copy number/maize genome copy number *100)																			
LAB	0.15				0.40				0.9				2.0				3.30			
	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	0.08	0.10	0.11	0.32	0.34	0.42	0.26	0.69	0.75	0.55	0.53	1.96	2.20	1.22	1.41	2.46	2.87	2.65	2.57
2	0.09	0.11	0.10	0.15	0.33	0.34	0.52	0.41	0.72	0.73	0.82	0.73	2.15	2.15	1.60	1.41	3.02	3.38	3.06	3.26
4	0.10	0.08	0.10	0.13	0.27	0.31	0.38	0.31	0.64	0.65	0.63	0.68	2.04	2.13	1.11	1.48	2.63	3.25	2.64	3.43
5	0.17	0.07	0.11	0.06	0.45	0.43	0.26	0.33	2.12	1.37	0.83	0.90	1.25	1.18	2.29	2.55	4.59	4.64	3.97	3.51
6	0.12	0.1	0.18	0.08	0.37	0.43	0.22	0.48	1.01	0.97	0.97	0.95	1.12	1.12	2.21	2.34	4.58	3.69	3.54	3.89
7	0.11	0.09	0.10	0.11	0.33	0.40	0.41	0.43	0.94	0.86	0.82	0.86	2.01	1.84	1.84	1.92	4.05	4.50	3.61	3.68
8	0.11	0.12	0.10	0.10	0.39	0.37	0.39	0.39	0.76	0.91	0.94	0.95	1.90	2.25	1.85	2.18	3.33	4.24	4.00	4.43
9	0.09	0.14	0.11	0.15	0.34	0.34	0.36	0.51	0.71	0.90	1.00	0.92	1.69	1.26	1.58	1.66	3.27	3.45	2.95	3.66
10	0.10	0.11	0.12	0.08	0.35	0.38	0.35	0.34	0.83	0.95	0.81	0.75	1.62	1.42	1.91	1.75	3.06	2.83	3.74	3.41
11	0.16	0.09	0.07	0.05	0.46	0.60	0.35	0.21	1.09	0.90	1.09	2.11	3.36	2.45	2.83	3.67	6.52	5.02	7.78	5.85
12	0.09	0.10	0.11	0.11	0.28	0.31	0.39	0.31	0.60	0.53	0.74	0.65	1.65	1.75	1.37	1.44	2.65	3.02	3.14	2.66
13	0.15	0.12	0.14	0.15	0.39	0.51	0.42	0.61	0.99	1.27	1.10	1.07	1.85	1.97	2.02	2.20	4.22	4.35	3.63	3.90

In Figure 1 the deviation from the true value for each GM level tested is shown for each laboratory. As it can be observed, all laboratories underestimated the true value of T25 content at 0.15% GM content, although the mean value (represented by the red bar) is placed just around the lower limit of the trueness acceptance level (bias = 25%). The average relative deviation at all the other GM levels is definitely modest. No overall overestimation/underestimation trend can be observed.

Figure 1. Relative deviation (%) from the true value of T25 for all laboratories



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. T25 validation data.

	Expected value (GMO %)				
Unknown sample GM%	**0.15**	**0.40**	**0.90**	**2.00**	**3.30**
Laboratories having returned results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	2	1	1
Reason for exclusion	-	-	2 C. test	1 G. test	1 C. test
Mean value	**0.109**	**0.377**	**0.822**	**1.769**	**3.486**
Repeatability relative standard deviation (%)	25.56	22.18	10.09	22.27	10.77
Repeatability standard deviation	0.03	0.08	0.08	0.39	0.38
Reproducibility relative standard deviation (%)	25.56	22.50	21.09	22.27	18.19
Reproducibility standard deviation	0.03	0.08	0.17	0.39	0.63
Bias (absolute value)	- 0.041	- 0.023	- 0.078	- 0.231	0.186
Bias (%)	-27	- 6	- 9	- 12	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 6, the method fully satisfies this requirement at all GM level tested. In fact, the highest value of RSD_R (%) is 25.56 at the 0.15% 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 be 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, with a minor deviation at 0.15% T25 level.

In table 6 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 throughout the whole dynamic range

tested, with the only exception of a minor deviation from the requirement for the 0.15% level (bias=27%); however, this small deviation is not 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 complying with the current labeling requirements in Europe.

10. References

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