European Commission

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS

Event-specific Method for the Quantification of Maize Line T25 Using Real-time PCR v. 1.01

Validation Report and Validated Method

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Joint Research Centre

EURL European Union Reference Laboratory for GM Food & Feed

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Validation Report:
Page 8 §1:
For relative quantification of event T25 DNA, a maize-specific reference system amplifies a 136-bp fragment changed by
*For relative quantification of event T25 DNA, a maize-specific reference system amplifies a **135**-bp fragment*
Page 14 §10:
Update References
Validated method Page 8 §1:
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Note:
Since 01/12/2009 the term "Community Reference Laboratory (CRL) " is changed into "European Union Reference Laboratory (EURL)".
Since 01/03/2009 the JRC-unit that hosts the EU-RL GMFF is named "Unit for Molecular Biology and Genomics" instead of "Biotechnology and GMO Unit".

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Unit for Molecular Biology and Genomics

Event-specific method for the quantitation of Maize line T25 using real-time PCR

Validation Report

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

14 June 2005
Corrected version 1- 28/08/2013 (see page 2)

Executive Summary

The JRC as European Union Reference Laboratory (EURL) 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 European Union 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.

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

The EURL GMFF is also ISO 17043:2010 accredited (Proficiency test provider) and apply the corresponding procedures and processes for the management of ring trials during the method validation.

The EURL GMFF conducts its activities under the certification ISO 9001:2008 of the IHCP Institute provided by CERMET

Correction from the previous versions:

Corrected version 1 - 28/08/2013

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1. Introduction

The Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute of Health and Consumer Protection) as European Union 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 135-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

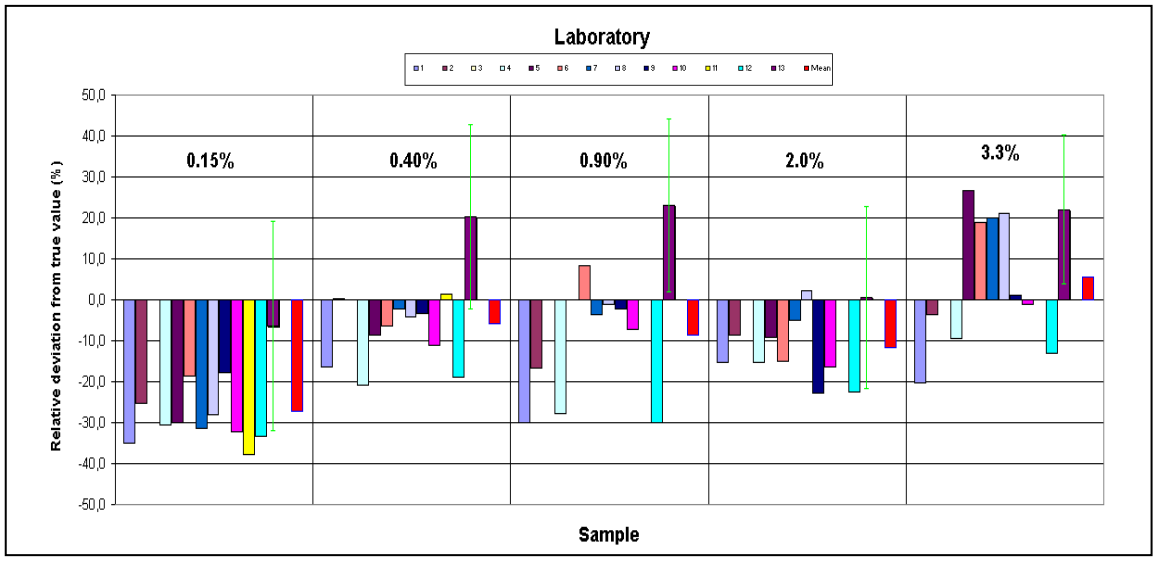
Table 5 shows the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

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 EURL. 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 EURL 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

Arumuganathan, K., Earle, E.D. (1991). Nuclear content of some important plant species. Plant Mol Biol Reporter 9, 208-218.

Horwitz, W. (1995) Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67, 331-343.

Annex 1: Event-specific method for the quantitation of Maize line T25 Using real-time PCR

Validated Method

14/06/2005

Corrected version 1- 30/11/2011 (see page 2)

Method development:

Bayer CropScience

Method validation:

Joint Research Centre – European Commission
Unit for Molecular Biology and Genomics

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Contents

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event T25 DNA to total maize DNA in a sample.

The PCR assay was optimised for the use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in this method.

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

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 quantitation of event T25 DNA, a maize-specific reference system amplifies a 135-bp fragment of *Adh*1 (Alcohol dehydrogenase-1) gene, 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 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 quantitation of the amount of event T25 DNA in a test sample, the normalized ΔCt values of the calibration samples are used to calculate by linear regression a reference curve ΔCt-formula. The normalized ΔCt values of the unknown samples are measured and, by means of the reference ΔCt-formula, the relative amount of T25 event DNA is estimated.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for ground maize seed and grain material, containing mixtures of genetically modified T25 and conventional maize.

The reproducibility and trueness of the method was tested through collaborative trial using samples at different GMO contents.

2.2 Collaborative trial

The method was validated in a collaborative trial by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 13 laboratories.

Each participant received twenty unknown samples containing T25 maize genomic DNA at five GM contents, between 0.15 % and 3.3 %.

Each test sample was analyzed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM T25 in four unknown samples. Two replicates of each GM level were analyzed on the same PCR plate.

A detailed validation report can be found under http://gmo-crl.jrc.it/statusofdoss.htm

2.3 Limit of detection

According to the method developer, the relative LOD of the method is at least 0.045% GM T25 in 200 ng of total maize DNA. The relative LOD was not assessed in a collaborative trial. The lowest relative GM content of the target sequence included in collaborative trail was 0.15%.

2.4 Limit of quantitation

According to the method developer, the relative LOQ of the method is ≤ 0.09% GM T25 in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.15%.

2.5 Molecular specificity

The method utilizes a unique DNA sequence of the recombination region of parts of the construct inserted into the plant genome. The sequence is specific to T25 and thus imparts event-specificity to the detection method.

The specificity was experimentally tested against DNA extracted from plant materials containing the specific targets of LL Rice62, OSR Ms1, Ms8, Rf1, Fr2, Rf3, Topas19/2, T45, Soybean A2704/12 and Cotton LL25, Maize Mon810, Bt11, Bt176, GA21, NK603, CBH351 and Roundup Ready Soybean. None of the materials yielded detectable amplification.

3. Procedures

3.1 General instructions and precautions

- All handling of reagents and controls should occur in an ISO 9001:2000 or ISO 17025 environment or equivalent.
- The procedures require experience of working under sterile conditions.
- Laboratory organization, e.g. "flow direction" during PCR-setup, should follow the guidelines given by relevant authorities like e.g. ISO, CEN, Codex alimentarius commission.
- PCR-reagents shall be stored and handled in a separate room and freezer and in equipment 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 used must be sterilized 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 shall not adsorb protein or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Use only powder-free gloves and change them frequently.
- Clean lab-benches and equipment periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - shall 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 T25 maize

3.2.1 General

The PCR set-up for the taxon specific target sequence (*Adh*1) and for the GMO (T25) target sequence should be 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 25 µl per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curve consists of five samples containing fixed percentages of T25 DNA in a total amount of 200 ng maize DNA (corresponding to 73,394 maize genome copies with one maize genome assumed to correlate to 2.725 pg of haploid maize genomic DNA) (Arumuganathan & Earle, 1991). The GM content of the standard samples ranges from 3.6% to 0.09%.

A calibration curve is produced by plotting the ∆Ct-values of calibration samples against the logarithm of the respective GM % contents; the slope (a) and the intercept (b) of the standard curve (y = ax + b) are then used to calculate the mean % GM content of the blind samples based on their normalised ΔCt values.

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. In two reaction tubes (one for T25 system and one for the *Adh*1 system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the reference *Adh*1 specific system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM182 primer (10 µM)	200 nM	0.5
KVM183 primer (10 µM)	200 nM	0.5
TM014 probe (10 µM)	200 nM	0.5
Nuclease free water	#	6
Template DNA (max 200 ng, see 3.2.1 and 3.2.2)	#	5
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for T25 specific system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5 µl
MLD143 primer (10 µM)	400 nM	1
MDB551 primer (10 µM)	400 nM	1
TM016 Probe (10 µM)	200 nM	0.5
Nuclease free water	#	5
Template DNA (max 200 ng, see 3.2.1 and 3.2.2)	#	5
Total reaction volume:		25 µl

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the T25 and one for the *Adh*1 master mix) for each DNA sample to be tested (reference curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of master mix (e.g. 20 x 3 = 60 µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA

(e.g. 5 x 3 = 15 µl DNA for three PCR repetitions). Low-speed vortex each tubes at least three times for approx 30 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.

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 250 x *g* for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Cycling program for maize T25-*Adh*1 systems

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG		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″	Measure	

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. T25) 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. Press the update button to ensure changes affect Ct values. 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. *Adh*1 system).

e) Save the settings and export all the data into an Excel 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 Reference ΔCt-curve is generated by plotting the ΔCt-values measured for the calibration points against the logarithm of the GM% content, and by fitting a linear regression line into these data.

Thereafter, the reference ΔCt-curve formula is used to estimate the relative amount (%) of T25 event in the unknown samples of DNA.

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for evaluating data after standard curve method (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml tubes

4.2 Reagents

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

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
T25 target sequence	
MLD143	5'-ACA AGC GTG TCG TGC TCC AC-3'
MDB551	5'-GAC ATG ATA CTC CTT CCA CCG-3'
TM016 (Probe)	FAM-5'-TCA TTG AGT CGT TCC GCC ATT GTC G-3'-TAMRA
Reference gene Adh1 target sequence	
KVM182	5'-CGT CGT TTC CCA TCT CTT CCT CCT-3'
KVM183	5'-CCA CTC CGA GAC CCT CAG TC-3'
TM014 (Probe)	FAM-5'-AAT CAG GGC TCA TTT TCT CGC TCC TCA-3'-TAMRA

5. References

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Abstract

The JRC as European Union Reference Laboratory (EURL) 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-ZM00З-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 ENGLs performance requirements and the scientific understanding about satisfactory method performance. Therefore, the JRC as European Union 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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