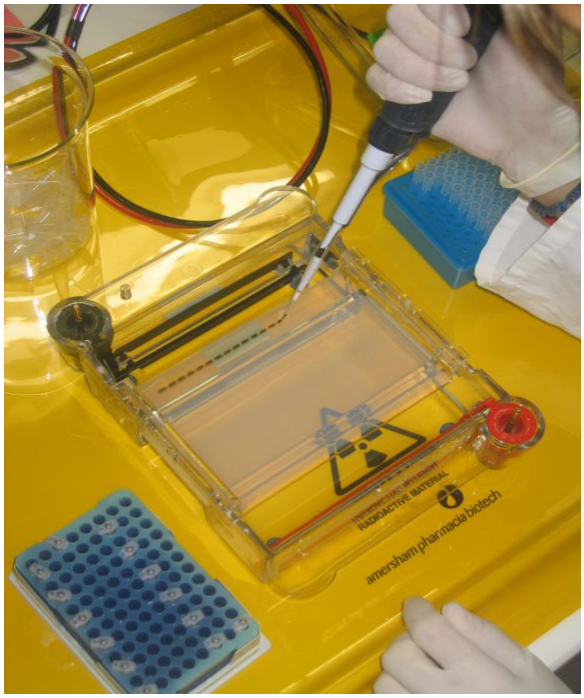
JRC Scientific and Technical Reports

Report on the Verification of the Performance of MON 88017 and MON 810 Event-specific Methods on the Maize Event MON 88017 x MON 810 Using Real-time PCR

Validation Report and Protocols

C. Delobel, G. Pinski, M. Mazzara, G. Van den Eede



EUR 24241 EN-2009

JRC EUROPEAN COMMISSION

Institute for Health and Consumer Protection

Community Reference Laboratory CRL GM Food and Feed

The mission of the JRC-IHCP is to protect the interests and health of the consumer in the framework of EU legislation on chemicals, food, and consumer products by providing scientific and technical support including risk-benefit assessment and analysis of traceability.

European Commission
Joint Research Centre
Institute for Health and Consumer Protection

Contact information
Address: Molecular Biology and Genomics Unit
E-mail: JRC-BGMO@ec.europa.eu
Tel.: +39 0332 789379
Fax: +39 0332 786159

http://ihcp.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

JRC56612

EUR 24241 EN
ISBN 978-92-79-14987-0
ISSN 1018-5593
DOI 10.2788/60224

Luxembourg: Office for Official Publications of the European Communities

Printed in Italy

Report on the Verification of the Performance of MON 88017 and MON 810 Event-specific Methods on the Maize Event MON 88017 x MON 810 Using Real-Time PCR

15 July 2009

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

Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, has carried out an in-house verification study to assess the performance of two quantitative event-specific methods on the maize event MON 88017 x MON 810 (unique identifier MON-88Ø17-3 x MON-ØØ810-6) which combines the MON 88017 and MON 810 transformation events. The two methods have been previously validated individually on single-trait event, to detect and quantify each event in maize samples; a validation report for each method is available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm. This study was conducted according to internationally accepted guidelines [1, 2].

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto Europe S.A. provided the detection methods and the control samples: whole seeds of MON 88017 x MON 810 maize (TPX151-DT, GLP-0409-15526-S) and whole conventional maize seeds of EXP258 (GLP-0409-15528-S). The JRC prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and to the validation results on the individual parental events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results of this CRL-GMFF in-house verification studies are made publicly available at http://gmo-crl.jrc.ec.europa.eu/.

Drafted by:
C. Delobel

Report Verification Team:
1) M. Ermolli

2) G. Pinski

Scientific and technical approval:
M. Mazzara

Compliance with CRL Quality System:
S. Cordeil

Authorisation to publish:
G. Van den Eede

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit – Community Reference Laboratory for Genetically Modified Food and Feed
Via Fermi 2749, I-21027 Ispra (VA), Italy

Report on Steps 1-3 of the Validation Process

Monsanto Europe S.A. submitted the detection methods and control samples of the maize event MON 88017 x MON 810 (unique identifier MON-88Ø17-3 x MON-ØØ810-6) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Community Reference Laboratory for GM Food and Feed (CRL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the CRL-GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/doc/Method%20requirements.pdf) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed and requests of complementary information were addressed to the applicant. Upon reception of the complementary information, the scientific assessment of the detection method for the MON 88017 x MON 810 maize was concluded in March 2006.

The event-specific detection methods for the two maize lines hosting the single event MON 88017 and MON 810 were validated by the CRL-GMFF following the conclusion of the respective international collaborative studies and the publication of the validation reports (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). Hence, the detection methods applied on the maize event MON 88017 x MON 810 did not undergo a full validation process. The CRL-GMFF performed an in-house verification of the detection methods to verify that they exhibit a comparable performance on samples of event MON 88017 x MON 810 combining the two traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In February 2008, the CRL-GMFF concluded the experimental verification of the method characteristics (step 3, experimental testing of the samples and methods) by quantifying, with each specific method, five blind GM-levels within the range 0.09%-8%, on a DNA/DNA ratio. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were within the limits established by the European Network of GMO Laboratories (ENGL).

A Technical Report summarising the results of tests carried out by the CRL-GMFF (step 3) is available on request.

Content

1. Introduction

The applicant Monsanto Europe S.A. submitted the detection methods for MON 88017 and MON 810 and the control samples of the maize event MON 88017 x MON 810 (unique identifier MON-88Ø17-3 x MON-ØØ810-6) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Joint Research Centre (JRC, Molecular Biology and Genomics Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) 1829/2003, carried out an in-house verification of the two event-specific methods for the detection and quantification of MON 88017 and MON 810 in the MON 88017 x MON 810 maize event combining the two traits. The single methods had been previously validated by international collaborative studies on the single-trait maize events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Upon reception of methods, samples and related data (step 1), the CRL-GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the methods (step 3) according to the requirements of Regulation (EC) 641/2004 and following CRL-GMFF operational procedures. The CRL-GMFF method verification was concluded in February 2008.

A method for DNA extraction from maize seeds, submitted by the applicant, was evaluated by the CRL-GMFF in order to confirm its performance characteristics. The protocol for DNA extraction is available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

The operational procedure of the in-house verification included the following module:

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology consists of two event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of events MON 88017 and MON 810 DNA to total maize DNA in the MON 88017 x MON 810 maize event. The procedures are simplex systems, in which the events MON 88017 and MON 810 are quantified in reference to the maize *hmg* (high mobility group) endogenous gene.

The study was carried out in accordance to the following internationally accepted guidelines:

- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method performance studies" (1).
- ✓ ISO 5725:1994 (2).

2. Materials

For the verification of the quantitative event-specific methods, control samples consisting of:

- seeds of MON 88017 x MON 810 maize (TPX151-DT, GLP-0409-15526-S),
- seeds of conventional maize EXP258 (GLP-0409-15528-S),

were provided by the applicant, in accordance to the provisions of Regulation (EC) No 1829/2003, Art 2.11 ["control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)"].

Samples containing mixtures of 100% MON 88017 x MON 810 and conventional maize genomic DNA at different GMO concentrations were prepared by the CRL-GMFF in a constant amount of total maize DNA, using the control samples provided.

The protocols (reagents, concentrations, primer/probe sequences) followed in the in-house verification are those already published as validated methods for the individual MON 88017 and MON 810 events and available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

Table 1 shows the GM% used in the verification of the MON 88017 and MON 810 methods.

Table 1. MON 88017 and MON 810 GM contents in maize event MON 88017 x MON 810.

MON 88017 GM% (GM DNA / Non-GM DNA x 100)	**MON 810 GM%** (GM DNA / Non-GM DNA x 100)
0.09	0.10
0.50	0.50
0.90	1.00
5.00	2.00
8.00	5.00

3. Experimental design

Eight runs for each event-specific method were carried out. In each run, samples were analysed in parallel with the GM-specific system and the reference system (*hmg*). Five GM levels per run were examined and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. In total, for each method (MON 88017 and MON 810), the quantification of the five GM levels was calculated as average of sixteen replicates per GM level.

4. Method

Description of the operational steps

For specific detection of events MON 88017 and MON 810 in maize event MON 88017 x MON 810, two specific fragments of the integration regions of the constructs inserted into the plant genome, of 95 bp and 92 bp respectively, are amplified using specific primers.

PCR products are measured during each cycle (real-time) by means of target-specific oligonucleotide probes labelled with two fluorescent dyes: 6-FAM is used for MON 88017 and MON 810 as reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For relative quantification of events MON 88017 and MON 810 DNA, a maize-specific reference system which amplifies a 79 bp fragment of the maize endogenous gene *hmg* (high mobility group), using two *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM and TAMRA, was used.

Standard curves are generated for each GM specific system (MON 88017 or MON 810), by plotting Ct values of the calibration standards against the logarithm of the DNA copy numbers MON 88017 or MON 810 DNA, and fitting a linear regression into these data. Thereafter, the Ct values of the blind samples are measured and, by means of the regression formula, the relative amount of event MON 88017 or MON 810 DNA is estimated respectively.

For detailed information on the preparation of the standard curve calibration samples please refer to the protocols of the validated methods at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

5. Deviations reported

No deviations were introduced.

6. Summary of results

PCR efficiency and linearity

The values of the slopes of the standard curves, from which the PCR efficiency is calculated using the formula [10^(-1/slope)-1] x 100, and of the R^2 (expressing the linearity of the regression) reported for all PCR systems in the eight runs, are presented in Table 2 and 3 for MON 88017 and MON 810 methods, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 88017 method on event MON 88017 x MON 810.

Run	MON 88017			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.48	94	0.99	-3.11	110	1.00
2	-3.41	97	1.00	-3.07	112	1.00
3	-3.66	87	0.99	-3.11	110	1.00
4	-3.44	95	0.99	-3.01	115	1.00
5	-3.39	97	1.00	-2.98	116	0.99
6	-3.63	89	0.99	-3.27	102	1.00
7	-3.63	89	1.00	-3.15	108	1.00
8	-3.70	86	0.99	-3.17	107	1.00
Mean	-3.54	92	0.99	-3.11	110	1.00

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 810 method on event MON 88017 x MON 810.

Run	MON 810			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.40	97	0.99	-3.44	95	1.00
2	-3.58	90	0.98	-3.54	92	1.00
3	-3.77	84	0.99	-3.58	90	1.00
4	-3.65	88	0.97	-3.53	92	1.00
5	-3.64	88	0.98	-3.43	96	0.99
6	-3.44	95	0.99	-3.48	94	0.99
7	-3.66	88	0.98	-3.48	94	1.00
8	-3.55	91	0.97	-3.57	91	1.00
Mean	-3.58	90	0.98	-3.51	93	1.00

The mean PCR efficiencies of the GM specific systems are 92% and 90% respectively for the MON 88017 and MON 810 specific systems. The linearity of the methods is equal to or above 0.98 for both systems. Overall, data reported in Table 2 and 3 confirm the appropriate performance characteristics of the two methods tested on MON 88017 x MON 810 maize samples in terms of PCR efficiency and linearity.

7. Method performance requirements

The results of the in-house verification study for the MON 88017 and MON 810 detection methods applied to event MON 88017 x MON 810 maize DNA are reported in Tables 4 and 5, respectively. Results were evaluated with respect to the method acceptance criteria and

performance requirements, as established by ENGL and adopted by the CRL-GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm, see also Annex 1). In addition, Tables 4 and 5 report estimates of the trueness and repeatability standard deviation for each GM level and for both methods.

Table 4. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 88017 method on event MON 88017 x MON 810 maize DNA.

MON 88017					
Blind sample GM%	**Expected value (GMO%)**				
	0.09	**0.5**	**0.9**	**5.0**	**8.0**
Mean	0.09	0.44	0.99	4.60	7.97
SD	0.02	0.08	0.12	0.48	0.68
RSD_r (%)	24.9	18.3	11.6	10.5	8.5
Bias (%)	2.6	-11.4	10.0	-8.0	-0.3

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the MON 810 method on event MON 88017 x MON 810 maize DNA.

MON 810					
Blind sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**1.0**	**2.0**	**5.0**
Mean	0.11	0.48	1.09	1.92	5.25
SD	0.02	0.07	0.07	0.23	0.60
RSD_r (%)	18.8	15.5	6.3	12.2	11.5
Bias (%)	12.0	-3.9	9.4	-4.1	5.0

The *trueness* of the method is estimated using the measures of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method, measured as bias from the accepted value, should be within ± 25% across the entire dynamic range. As shown in Tables 4 and 5, both methods satisfy the above requirement throughout their respective dynamic ranges.

Tables 4 and 5 further document the relative repeatability standard deviation (RSD_r) as estimated for each GM level. In order to accept methods for collaborative trial, the CRL-GMFF requires that RSD_r values are below 25%, as indicated by ENGL ("Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm]). As it can be observed from the values reported in Tables 4 and 5, the two methods satisfy this requirement throughout their respective dynamic ranges.

8. Comparison of method performance between event MON 88017 x MON 810 and the single trait events

An indicative comparison of the two method performances on the maize event MON 88017 x MON 810 and the single trait events is shown in Tables 6 and 7. The performance of the methods on the single lines was previously assessed through international collaborative trials.

Table 6. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 88017 detection method on event MON 88017 x MON 810 and on event MON 88017.

Trueness and repeatability of MON 88017 quantification on MON 88017 x MON 810			Trueness and repeatability of MON 88017 quantification on single event MON 88017*		
GM %	Bias (%)	RSD_r (%)	GM %	Bias (%)	RSD_r (%)
0.09	2.6	24.9	0.09	-2.6	28
0.5	-11.4	18.3	0.5	2.9	13
0.9	10.0	11.6	0.9	-9.6	19
5.0	-8.0	10.5	5.0	-4.8	19
8	-0.3	8.5	8	-7.6	18

*method validated (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 7. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 810 detection method on event MON 88017 x MON 810 and on event MON 810.

Trueness and repeatability of MON 810 quantification on MON 88017 x MON 810			Trueness and repeatability of MON 810 quantification on single event MON 810*		
GM %	Bias (%)	RSD_r (%)	GM %	Bias (%)	RSD_r (%)
0.1	12.0	18.8	0.1	-14	19.1
0.5	-3.9	15.5	0.5	-9	22.2
1.0	9.4	6.3	1.0	-5	7.5
2.0	-4.1	12.2	2.0	-7	10.7
5.0	5.0	11.5	5.0	-8	11.1

*method validated (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

For trueness, the MON 88017 even-specific method (Table 6), when applied to event MON 88017 x MON 810 and compared to the single line, shows a higher absolute bias (%) at 0.5% and 5% and a lower bias at 8%. The MON 810 event-specific method (Table 7), when applied to event MON 88017 x MON 810, shows a lower absolute bias (%) at all levels of GM, except for 1% level. The trueness is within the acceptance value set by ENGL (± 25%) over the whole dynamic range.

With the exception of the 0.5% GM level of the MON 88017 method and the 2.0% of the MON 810 method, both the MON 88017 and MON 810 (Table 6 and 7) event-specific methods show lower or equal values for relative repeatability standard deviation (RSD_r %) when applied to the hybrid and compared to the single events. The results are in all cases within the ENGL acceptance level established at maximum 25%.

Therefore, the in-house method verification has demonstrated that the MON 88017 and MON 810 detection methods developed to detect and quantify the single events can be equally applied for the quantification of the respective events combined in event MON 88017 x MON 810.

9. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events MON 88017 and MON 810 combined in maize event MON 88017 x MON 810 has been evaluated with respect to the method acceptance criteria and the method performance requirements recommended by the ENGL (as detailed under http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), and to the validation results obtained for the single trait events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results obtained in this verification study indicate that the analytical modules of the methods submitted by the applicant comply with ENGL performance criteria. The methods are therefore applicable to the control samples provided (see section 2 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32231) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)].

11. References

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

2. International Standard (ISO) 5725:1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization.

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: The description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: The practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative

requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: The closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: The accuracy should be within ± 25% of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: The rate of amplification that leads to a theoretical slope of –3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $[10^{(-1/slope)}] - 1$.

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of (- 3.1 ≥ slope ≥ - 3.6).

R^2 Coefficient

Definition: The R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R^2 should be ≥ 0.98.

Repeatability Standard Deviation (RSD_r)

Definition: The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: The relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

Limit of Quantitation (LOQ)

Definition: The limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than $1/10^{th}$ of the value of the target concentration with an RSD_r ≤ 25%. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

Acceptance Criterion: LOD should be less than 1/20th of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring ≤ 5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance Criterion: The response of an assay with respect to these small variations should not deviate more than ± 30%. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements

Dynamic Range

Definition: In the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD_R)

Definition: The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An RSD_R < 50% is acceptable for concentrations below 0.2%.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within ± 25% of the accepted reference value over the whole dynamic range.

Event-specific Method for the Quantification of Maize Line MON 88017 Using Real-time PCR

Protocol

13 October 2008

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

Method development:

Monsanto Company

Collaborative trial:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Biotechnology & GMOs Unit

Drafted by
A. Bogni

Report Verification Team
1) M. Querci

2) M. Ermolli

Scientific and technical approval
M. Mazzara

Compliance to CRL Quality System
S. Cordeil

Authorisation to publish
G. Van den Eede

Address of contact laboratory:

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 2749, 21027 Ispra (VA) - Italy

Content

1. General information and summary of the method

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event MON 88017 DNA to total maize DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels.

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

For the specific detection of maize event MON 88017 DNA, a 94-bp fragment of the integration region of the construct inserted into the plant genome (located at the 5' flanking DNA region) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye 6-FAM as a reporter at its 5′ end and with TAMRA as a quencher dye at its 3' end. The 5' nuclease activity of the Taq DNA polymerase results in the specific cleavage of the probe, leading to increased fluorescence, which is then monitored.

For the relative quantification of maize event MON 88017 DNA, a maize-specific reference system amplifies a 79-bp fragment of the maize endogenous *hmg* gene (*high mobility group*), using two specific primers and a *hmg* gene-specific probe labelled with 6-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 quantification of the amount of event MON 88017 DNA in a test sample, MON 88017 and *hmg* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of maize event MON 88017 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from maize seeds and grains containing mixtures of genetically modified and conventional maize.

The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GMO contents (%).

2.2 Collaborative trial

The method was validated in an international collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve laboratories in July 2007.

Each participant received twenty blind samples containing MON 88017 genomic DNA at five GM contents, ranging from 0.09% to 8.0%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM MON 88017 in four unknown samples. Four replicates of each GM level were analysed on the same PCR plate.

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

2.3 Limit of detection (LOD)

According to the data provided by the applicant, the relative LOD of the method is at least 0.045% in 200 ng of total maize DNA. The relative LOD was not assessed in collaborative study.

2.4 Limit of quantification (LOQ)

According to the data provided by the applicant, the relative LOQ of the method is at least 0.09% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in the international collaborative study was 0.09%.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to event MON 88017 and thus imparts event-specificity to the method.

The specificity of MON 88017 assay (forward/reverse primers and probe) was experimentally tested in real-time PCR by the applicant against DNA extracted from samples containing Roundup Ready® maize MON 88017 (positive control), Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® Corn Borer maize (MON810), YieldGard® Rootworm maize (MON863), Lysine maize (LY038), conventional maize, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard® cotton (MON 757), BollgardII® cotton (MON 15985), Roundup Ready® Flex cotton (MON 88913), conventional cotton, Roundup Ready® soybean (40-3-2), conventional soybean, Roundup Ready® wheat (MON 71800), conventional wheat, lentil, sunflower, buckwheat, rye berries and peanut.

According the applicant, none of the GM lines tested, except the positive control MON 88017, produced amplification signals.
The specificity of the maize reference assay *hmg* was experimentally tested by the applicant against DNA extracted from samples containing Roundup Ready® corn MON 88017, Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® Corn Borer maize (MON810), YieldGard® Rootworm maize (MON863), Lysine maize (LY038), conventional maize, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard® cotton (MON 757), BollgardII® cotton (MON 15985), Roundup Ready® Flex cotton (MON 88913), conventional cotton, Roundup Ready® soybean (40-3-2), conventional soybean, Roundup Ready® wheat (MON 71800), conventional wheat, lentil, sunflower, buckwheat, rye berries and peanut.

Only the positive control maize line MON 88017 and Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® Corn Borer maize (MON810), YieldGard® Rootworm maize (MON863), Lysine maize (LY038) and conventional maize produced amplification signals.

3. Procedure

3.1 General instructions and precautions

- The procedures require sterile conditions working experience.
- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.
- PCR-reagents should be stored and handled in a separate room 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.
- Equipment used should be sterilised prior to use and any residue of DNA should be removed. All material used (e.g. vials, containers, pipette tips, etc.) should be suitable for PCR and molecular biology applications; it should be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.

- All handling steps - unless specified otherwise - should 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 MON 88017 maize

3.2.1 General

The PCR set-up for the taxon specific target sequence (*hmg*) and for the GMO (event MON 88017) 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 MON 88017, in a total volume of 50 µL per reaction mixture with the reagents as listed in Table 1 and, for *hmg*, in a total volume of 25 µL per reaction mixture with the reagents as listed in and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% MON 88017 in non-GM maize DNA for a total of 200 ng of DNA (corresponding to approximately 73394 maize genome copies with one genome assumed to correspond to 2.725 pg of haploid maize genomic DNA) [(1)].

A calibration curve is produced by plotting the Ct values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

The copy number measured for the unknown sample DNA is obtained by interpolation from the standard curves.

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 the MON 88017 system and one for the *hmg* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (<u>except DNA</u>) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 88017 specific system.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
MON 88017 AF (10 µM)	150 nM	0.75
MON 88017 AR (10 µM)	150 nM	0.75
MON 88017 AP (5 µM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* reference system.

Component	Final concentration	µL / reaction
Buffer A (10x)	1x	2.5
Hmg F (10 µM)	300 nM	0.75
hmg R (10 µM)	300 nM	0.75
hmg P (5 µM)	160 nM	0.80
$MgCl_2$ (25 mM)	6.5 mM	6.5
dNTPs mix (10 mM)	200 µM	0.5
Nuclease free water	#	8.95
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the MON 88017 and one for the *hmg* master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of master mix (e.g. 46 x 3 = 138 µL master mix for three PCR repetitions for MON 88017 and 21 x 3 = 63 µL master mix for three PCR repetitions for *hmg*). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 µL DNA for three PCR repetitions). Vortex each tubes for approx. 10 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 microcentrifuge. Aliquot 50 µL in each well for MON 88017 and 25 µL for *hmg*. 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 MON 88017 and maize *hmg* system

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	Yes	

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. MON 88017) 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. *hmg* system).

e) Save the settings and export all the data to a text 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 standard curves are generated both for the *hmg* and the MON 88017 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.

For the determination of the amount of event MON 88017 DNA in the unknown sample, the MON 88017 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value (GM% = MON 88017/*hmg* x 100).

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)
- Analysis software
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No. 4304437
- 0.5 M EDTA. Sigma Cat. No. E-7647-01-0
- PCR Nucleotide Mix (10mM dNTPs). Promega Cat. No. C114G
- TaqMan® 1000X Rxn Gold/Buffer A Pack. Applied Biosystems Cat. No. 4304441
- AmplTaq Gold Polymerase. Applied Biosystems Cat. No. N808-0244
- Nuclease-free water. Sigma Cat. No. W-4502
- 1 M Tris-HCl, pH 8.0. Sigma Cat. No. T-3038

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
MON 88017 target sequence	
MON 88017 AF	5' – GAG CAG GAC CTG CAG AAG CT – 3'
MON 88017 AR	5' – TCC GGA GTT GAC CAT CCA – 3'
MON 88017 AP (Probe)	6-FAM-TCC CGC CTT CAG TTT AAA CAG AGT CGG GT-TAMRA
Reference gene hmg target sequence	
Hmg F	5' – TTG GAC TAG AAA TCT CGT GCT GA– 3'
Hmg R	5' – GCT ACA TAG GGA GCC TTG TCC T– 3'
hmg P (Probe)	6-FAM- CAA TCC ACA CAA ACG CAC GCG TA -TAMRA

5. References

1. Arumuganathan K., Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218.

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

CRL assessment on the validation of an event specific method for the relative quantitation of maize line MON 810 DNA using real-time PCR as carried out by Federal Institute for Risk Assessment (BfR)

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

10 March 2006

Executive Summary

An event-specific method for the quantitation of maize MON 810 by means of real-time PCR has been validated in a collaborative trial by the Federal Institute for Risk Assessment (BfR) in collaboration with the American Association of Cereal Chemists (AACC), Joint Research Centre (JRC) of the European Commission (EC), Institute for Reference Material and Measurement (IRMM), the Institute for Health and Consumer Protection (IHCP) and GeneScan, Berlin.

The trial involved fifteen laboratories and was conducted according to internationally accepted guidelines.

The method is annexed to the standard ISO 21570:2005, "Foodstuffs -- Methods of analysis for the detection of genetically modified organisms and derived products -- Quantitative nucleic acid based methods".

Contents

Document Approval		
Name / *Function*	**Date**	**Signature**
Marco Mazzara *Sector Head*	**10/03/2006**	**Signed**
Stephane Cordeil *Quality Manager*	**10/03/2006**	**Signed**
Guy Van den Eede *B&GMOs Unit Head*	**10/03/2006**	**Signed**

Address of contact laboratories:

Federal Institute for Risk Assessment
Bundesinstitut fuer Risikobewertung (BfR)
Dahlem
FG 213,
Thielallee 88-92,
D-14195 Berlin

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory
Via Fermi 1, 21020 Ispra (VA) - Italy

1. General information

This protocol describes an event-specific detection and quantitative TaqMan® PCR procedure for the relative determination of event MON 810 maize in total maize. The real time PCR was optimized for block thermal cycler. Template DNA extracted should be tested for quality and quantity prior to PCR assay.

For specific detection of event MON 810 maize a 92 bp fragment of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from CaMV (35S promoter) as a result of *in vitro* recombination present in the genetically modified insect-protected MON 810 ("YieldGuard") maize (Monsanto) is amplified in TaqMan® PCR.

For relative quantitation of MON 810 maize, a 79 bp fragment of the taxon specific maize (*Zea mays*) high mobility group protein gene (hmg) gene using a gene specific combination of primers and probe is amplified.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called "Ct-value". For quantitation of the amount of event MON 810 maize in a test sample, event MON 810 and hmg Ct values are determined for the sample. A standard curve procedure is then used to calculate the relative number of MON 810 specific genome copies to total maize genome copies.

2. Validation status

The method was optimized for ground maize seeds (certified reference materials [CRM IRMM-413]), containing mixtures of genetically modified MON 810 and conventional maize.

The reproducibility and accuracy of the method was tested through a collaborative study using samples at different GMO contents.

The method was originally developed for the ABI PRISM® 7700 Sequence Detection System (SDS).

This method has been validated in a collaborative study conducted by the Federal Institute for Risk assessment (BfR) in collaboration with The American Association of Cereal Chemists (AACC), Joint Research Centre (JRC) of the European Commission (EC), Institute for Reference Material and Measurement (IRMM) and Institute for Health and Consumer Protection (IHCP) and GeneScan.

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

- DNA extraction: GENESpin DNA extraction system (GeneScan)

- Quantitative real-time PCR (Polymerase Chain Reaction): for detection of event MON 810 maize a 92 bp fragment of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from CaMV (35S promoter) as a result of *in vitro* recombination present in the genetically modified insect-protected MON 810 ("YieldGuard") maize (Monsanto) was amplified in TaqMan® PCR

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).

The study was undertaken with 15 laboratories using either the ABI PRISM® 7700, ABI PRISM® 7900 (Applied Biosystems Inc) or the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories).

Fourteen laboratories from countries all over the world reported results.

For each unknown sample one DNA extraction has been carried out. Each test sample was analyzed by real time PCR in 3 repetitions.
Each participant received 12 unknown samples. The samples consisted of 6 certified reference materials (CRM IRMM-413) between <0,02 % and 5 % GM MON 810 in conventional maize (w/w).

Each laboratory received each level of GM MON 810 CRM in two separate unknown samples.
Details of the results of the collaborative study performed in 2003/2004 are shown in table 1.

Table 1: Statistics of the collaborative study for the real time PCR procedure to quantify MON 810 specific material.

Sample	**Sample 1 <0.02 %**	**Sample 2 0.1 %**	**Sample 3 0.5 %**	**Sample 4 1 %**	**Sample 5 2%**	**Sample 6 5 %**
Number of laboratories reported	11	14	14	14	14	14
Number of outliers	1	1	0	2	0	0
Number of laboratories retained after eliminating outliers	10	13	14	12	14	14
Mean value (%)	0.028	0.1023	0.4613	0.8327	1.7814	4.5154
Repeatability standard deviation s_r	0.00736	0.03641	0.9606	0.13744	0.28385	1.29374
Repeatability relative standard deviation RSD_r (%)	26.27	35.60	20.82	16.51	15.93	28.65
Repeatability limit r (r = 2,8 x s_r)	0.0206	0.1019	0.269	0.3848	0.7948	3.6225
Reproducibility standard deviation s_R	0.02326	0.04646	0.20068	0.26534	0.56609	1.65451
Reproducibility relative standard deviation RSD_R (%)	83.03	45.43	43.5	31.86	31.78	36.64
Reproducibility limit R (R=2,8 x s_R)	0.0651	0.1301	0.5619	0.743	1.5851	4.6326
Bias (%)	-	2.3	- 7.74	- 16.73	- 10.93	- 9.69

[a] Outliers were identified with the Grubbs and Cochran tests

These results 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 1, 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.

Method bias, which allows estimating trueness, is reported for each GM level in table 1. 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. The method satisfies such requirement for all GM values tested.

The relative reproducibility standard deviation (RSDR), 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 1, the method satisfies this requirement at the target concentration (1%) and at GM level of 0.1% and 2%; a minor deviation can be seen at 5% (36.64), while the RSDR at GM level 0.5% is 43.5

3. Specificity

Specificity tests prior to the study showed no cross reactivity of the detection systems to the following non-target species/samples: soybean DNA.

No cross reactivity has been occurred with the following genetically modified maize: Event176, Bt11, T25, GA21 and GTS 40-3-2 soybean.

4. Limit of detection (LOD)

According to the method developer, the absolute LOD has been determined to be 5 copies of the target sequence

According to the method developer, the relative LOD has been demonstrated to be at least 0.1 % (w/w).

5. Limit of quantitation (LOQ)

According to the method developer, the absolute limit of quantitation has been determined to be 10 copies of the target sequence.

According to the method developer the relative limit of quantitation has been determined to be at least 0.1% (equal to the lowest concentration point of the calibration curve used; [w/w]).

6. Procedure

All handling of reagents and controls should occur in an ISO 17025 environment or equivalent.

Further appropriate ISO/EN Norms dealing with the detection and quantitation of GMO derived material should be taken into consideration.

7. Primer/probe systems

The following primers and TaqMan® probes were used in the collaborative study (table 2).

Table 2 : Primer and probe sequences

Name	Oligonucleotide DNA sequence	Final conc. in PCR
Reference gene target sequence		
ZM1-F	5´-TTg gAC TAg AAA TCT CgT gCT gA-3‘	300 nmol/l
ZM1-R	5´-gCT ACA TAg ggA gCC TTg TCC T-3‘	300 nmol/l
Probe ZM1	5´-FAM—CAA TCC ACA CAA ACg CAC gCg TA-TAMRA-3‘	160 nmol/l
GMO target sequence		
Mail-F1	5´-TCg AAg gAC gAA ggA CTC TAA CgT-3´	300 nmol/l
Mail-R1	5´-gCC ACC TTC CTT TTC CAC TAT CTT-3´	300 nmol/l
Probe Mail-S2	5´-FAM-AAC ATC CTT TgC CAT TgC CCA gC-TAMRA P-3´	180 nmol/l
FAM: 6-carboxylfluorecein, TAMRA: 6-carboxytetramethylrhodamine		

8. Sample extraction

For DNA extraction the GENESpin DNA extraction system (GeneScan) was used according to the manufacturer’s instruction.

9. PCR set-up

The PCR set-up for the taxon specific target sequence and for the GMO 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 method is described for a total volume of 25 µl per reaction mixture with the reagents listed in Table 3.

Table 3: Amplification reaction mixture in the final volume/concentration per reaction vial

Total reaction volume		25 µl
Template DNA added (2,3 ng to 150 ng maize DNA)		5 µl
DNA polymerase	AmpliTaq Gold® (Applied Biosystems Inc)	1.25 U
Decontamination system	dUTP AmpErase uracil N-glycosylase	400 µmol/l 0.5 U
Reaction buffer	TaqMan™ buffer A	1 fold
	$MgCl_2$	6.5 mmol/l
Primers	see Table D.6	see Table 2
DNTP	dATP. dCTP. dGTP	200 µmol/l each
Probe	see Table 2	see Table 2

As a positive control and as calibrant reference material, certified reference materials of MON810 (material containing <0.02 % to 5 % of genetically modified maize) produced by IRMM, Geel, Belgium (IRMM-413 series) may be used.

A series of 1:4 dilution steps of DNA from 5 % CRM is used to establish the standard curves for the MON 810 specific and hmg specific PCR, respectively.

10. Temperature-time-programme

The temperature-time-programme as outlined in Table 4 was optimised for the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems Inc). In the validation study it was used in combination with the AmpliTaq Gold® DNA polymerase. Table 4 describes the reaction conditions.

Table 4: Procedure - Reaction conditions

		Time (s)	Temperature (°C)
Pre-PCR – decontamination (optional)		120	50
Pre-PCR – activation of DNA polymerase and denaturation of template DNA		600	95
PCR (45 cycles)			
	Denaturation	15	95
	Annealing Elongation	60	60

11. Data analysis

The baseline range is usually set to cycles 3 to 15. If amplifications do not appear before cycle 20, the baseline stop can be extended to cycle 20.

After defining a threshold value within the logarithmic phase of amplification (e.g. 0.01 to 0.1 normalized reporter dye fluorescence [Rn]) the instruments software calculates the Ct values for each reaction. The Ct values measured for the calibration points in the taxon specific maize or MON 810 specific PCR system, respectively, are plotted against the natural logarithm

of the DNA copy numbers introduced into PCR. The copy numbers measured for the unknown sample DNA are obtained by interpolation from the standard curves.

A calibration curve is produced by plotting Ct values against the logarithm of the target copy number for the calibration points.

For the determination of the amount of MON 810 DNA in the test sample, the MON 810 copy number is divided by the number of maize genome equivalents and multiplied by 100 to get the percentage value.

12. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (available at http://gmo-crl.jrc.it).

The results obtained during the collaborative trial indicate that the method can be considered as fit for enforcement purposes with respect to its trueness and inter-laboratory variability, taking into account the observations on RSD_R reported above.

13. References

ISO 21570:2005, "Foodstuffs -- Methods of analysis for the detection of genetically modified organisms and derived products -- Quantitative nucleic acid based methods".

European Commission

EUR 24241 **EN – Joint Research Centre – Institute for Health and Consumer Protection**
Title: Report on the Verification of the Performance of MON 88017 and MON 810 Event-specific Methods on the Maize Event MON 88017 x MON 810 Using Real-time PCR
Author(s): C. Delobel, G. Pinski, M. Mazzara, G. Van den Eede
Luxembourg: Office for Official Publications of the European Communities
2009 – 34 pp. – 21 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-14987-0
DOI 10.2788/60224

Abstract

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, has carried out an in-house verification study to assess the performance of two quantitative event-specific methods on the maize event MON 88017 x MON 810 (unique identifier MON-88Ø17-3 x MON-ØØ810-6) which combines the MON 88017 and MON 810 transformation events. The two methods have been previously validated individually on single-trait event, to detect and quantify each event in maize samples; a validation report for each method is available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm. This study was conducted according to internationally accepted guidelines [1, 2].

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto Europe S.A. provided the detection methods and the control samples: whole seeds of MON 88017 x MON 810 maize (TPX151-DT, GLP-0409-15526-S) and whole conventional maize seeds of EXP258 (GLP-0409-15528-S). The JRC prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and to the validation results on the individual parental events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results of this CRL-GMFF in-house verification studies are made publicly available at http://gmo-crl.jrc.ec.europa.eu/.

How to obtain EU publications

Our priced publications are available from EU Bookshop (http://bookshop.europa.eu), where you can place an order with the sales agent of your choice.

The Publications Office has a worldwide network of sales agents. You can obtain their contact details by sending a fax to (352) 29 29-42758.

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

JRC
EUROPEAN COMMISSION

LB-NA-24241-EN-C

ISBN 978-92-79-14987-0

9 789279 149870

Publications Office
Publications.europa.eu