JRC Scientific and Technical Reports

Report on the Verification of the Performance of MON89034 and MON88017 Event-specific Methods on the Maize Event MON89034 x MON88017 Using Real-time PCR

Validation Report and Protocols

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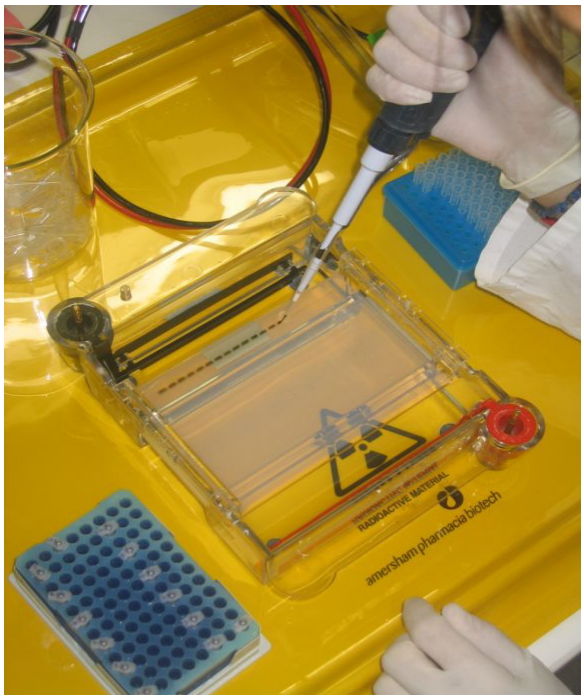

EUR 24276 EN-2009

JRC EUROPEAN COMMISSION

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Community Reference Laboratory CRL GM Food and Feed

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JRC57222

EUR 24276 EN
ISBN 978-92-79-15221-4
ISSN 1018-5593
DOI 10.2788/71542

Luxembourg: Office for Official Publications of the European Communities

Printed in Italy

Report on the Verification of the Performance of MON89034 and MON88017 Event-specific Methods on the Maize Event MON89034 x MON88017 Using Real-Time PCR

22 January 2010

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 MON89034 x MON88017 (unique identifier MON-89Ø34-3 x MON-88Ø17-3) which combines the MON89034 and MON88017 transformation events. The two methods have been validated individually on single-trait events, to detect and quantify each event in maize samples. 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 Company provided the detection methods and the control samples: genomic DNA extracted from seeds of MON89034 x MON88017 maize, genomic DNA extracted from seeds of non-GM maize, seeds of MON89034 x MON88017 and seed of conventional maize. 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 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/.

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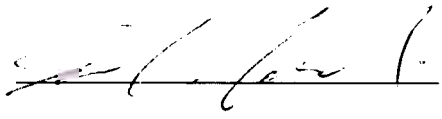

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Report on Steps 1-3 of the Validation Process

Monsanto Company submitted the detection methods and control samples of the maize event MON89034 x MON88017 (unique identifier MON-89Ø34-3 x MON-88Ø17-3) 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 two requests of complementary information were addressed to the applicant. Upon reception of the complementary information, the scientific assessment of the detection method for the MON89034 x MON88017 maize was positively concluded in June 2007.

The event-specific detection methods for the two maize lines hosting the single events MON89034 and MON88017 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 MON89034 x MON88017 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 MON89034 x MON88017 combining the two traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In January 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 mostly within the limits established by the ENGL.

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

Content

1. Introduction

Monsanto Company submitted the detection methods for MON89034 and MON88017 and the control samples of the maize event MON89034 x MON88017 (unique identifier MON 89Ø34-3 x MON-88Ø17-3) 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, established by Regulation (EC) 1829/2003, carried out an in-house verification of the two event-specific methods for the detection and quantification of MON89034 and MON88017 in the MON89034 x MON88017 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 January 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 protocols for DNA extraction are available at http://gmo-crl.jrc.ec.europa.eu/.

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 MON89034 and MON88017 DNA to total maize DNA in the MON89034 x MON88017 maize event. The procedures are simplex systems, in which the events MON89034 and MON88017 were 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:

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

2. Materials

For the verification of the methods, control samples consisting of:

- genomic DNA extracted from homogenised seeds of MON89034 x MON88017 maize (lot GLP-0701-17957-S),
- genomic DNA extracted from homogenised seeds of non-GM maize (lot GLP-0612-17871-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% MON89034 x MON88017 and non GM maize genomic DNA at different GMO contents 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 MON89034 and MON88017 events and available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

Table 1 shows the five GM levels of unknown samples used in the verification of the MON89034 and MON88017 methods.

Table 1. MON89034 and MON88017 GM contents in maize event MON89034 x MON88017

MON89034 GM% (GM DNA / Non-GM DNA x 100)	MON88017 GM% (GM DNA / Non-GM DNA x 100)
0.09	0.09
0.40	0.50
0.90	0.90
3.00	5.00
8.00	8.00

3. Experimental design

Eight runs for each event-specific method were carried out. In each run, samples were analysed in parallel with both 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 (MON89034 and MON88017), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level.

4. Method

Description of the operational steps

For detection of events MON89034 and MON88017 in maize event MON89034 x MON88017, two fragments of 77 bp and 95 bp respectively, covering the 3′ and the 5′ insert-to-plant junctions), 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: FAM is used as reporter dye at its 5′ end and TAMRA (for MON88017) or MGBNFQ (for MON89034) as a quencher dye at its 3′ end.

For relative quantification of events MON89034 and MON88017 DNA, a maize-specific reference system which amplifies a 79 bp fragment of the maize endogenous gene *hmg* (high mobility group, accession number AJ131373), using two *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM and TAMRA, is used.

Standard curves are generated for each system (*hmg*, MON89034 and MON88017), by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the copy numbers in the unknown sample DNA are estimated by interpolation from the standard curves.

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. Summary of results

PCR efficiency and linearity

The values of the slopes of the standard curves, from which the PCR efficiency was calculated using the formula [10^(-1/slope)-1]*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 MON89034 and MON88017 methods, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for MON89034 and *hmg* methods on event MON89034 x MON88017.

Run	MON89034			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.5	92	0.991	-3.1	111	0.996
2	-3.5	92	0.994	-3.3	102	0.998
3	-3.7	88	0.994	-3.2	107	0.996
4	-3.4	95	0.996	-3.1	109	0.990
5	-3.6	91	0.993	-3.2	106	0.993
6	-3.4	95	0.996	-3.1	109	0.993
7	-3.4	95	0.996	-3.1	110	0.995
8	-3.4	98	0.998	-3.1	112	0.990
Mean	-3.5	93	0.995	-3.1	108	0.994

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for MON88017 and *hmg* methods on event MON89034 x MON88017.

Run	MON88017			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.3	102	0.994	-3.1	111	0.992
2	-3.4	96	0.997	-3.1	108	0.998
3	-3.5	94	0.995	-3.1	110	0.992
4	-3.5	93	0.995	-3.2	107	0.995
5	-3.5	94	0.993	-3.4	98	0.998
6	-3.6	90	0.998	-3.3	101	0.998
7	-3.5	94	0.993	-3.2	104	0.996
8	-3.4	97	0.991	-3.3	101	0.997
Mean	-3.4	95	0.994	-3.2	105	0.996

The mean PCR efficiencies of the MON89034 and MON88017 detection methods on event MON89034 x MON88017 were 93% and 95% respectively. The linearity was 0.995 and 0.994 for the MON89034 and MON88017 specific systems. The mean PCR efficiency of the *hmg* reference system was 108% and 105% when tested in conjunction with the MON89034 and the MON88017 specific systems, respectively; likewise, the linearity of the *hmg* was 0.994 and 0.996.

Overall, data reported in Table 2 and 3 confirm the appropriate performance characteristics in terms of PCR efficiency and linearity of the two methods tested on MON89034 x MON88017 maize samples.

6. Method performance requirements

The results of the verification study for the MON89034 and MON88017 detection methods applied to event MON89034 x MON88017 maize DNA are reported in Tables 4 and 5, respectively. Results were evaluated with respect to the method acceptance criteria, 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 the 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 (RSDr%) of the MON89034 method on event MON89034 x MON88017 maize DNA.

MON89034					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.4**	**0.9**	**3.0**	**8.0**
Mean	0.10	0.41	0.92	2.97	7.92
SD	0.01	0.06	0.08	0.25	0.64
RSDr (%)	14	15	8.4	8.5	8.1
Bias (%)	10	3.4	2.6	-1.0	-0.9

Table 5. Estimates of trueness (expressed as bias%) and relative repeatability standard deviation (RSDr%) of the MON88017 method on event MON89034 x MON88017 maize DNA

MON88017					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.5**	**0.9**	**5.0**	**8.0**
Mean	0.10	0.52	0.93	4.97	8.03
SD	0.02	0.10	0.12	0.72	0.71
RSDr (%)	18	19	13	14	8.9
Bias (%)	12	3.6	3.8	-0.5	0.4

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 ± 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 evaluation, 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.

7. Comparison of methods performance between event MON89034 x MON88017 and the single trait events

An indicative comparison of the performances of the two methods on the maize event MON89034 x MON88017 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 (RSDr%) of the MON89034 detection method on event MON89034 x MON88017 and on event MON89034.

Trueness and repeatability of MON89034 quantification on MON89034 x MON88017			**Trueness and repeatability of MON89034 quantification on single event MON89034***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.09	10	14	**0.09**	25	18
0.4	3.4	15	**0.4**	6.4	13
0.9	2.6	8.4	**0.9**	4.3	17
3.0	-1.0	8.5	**3.0**	-5.8	12
8.0	-0.9	8.1	**8.0**	-11	9.5

*method validated in collaborative trail (http://gmo-crl.jrc.it/statusofdoss.htm)

Table 7. Trueness (bias%) and relative repeatability standard deviation (RSDr%) of the MON88017 detection method on event MON89034 x MON88017 and on event MON88017.

Trueness and repeatability of MON88017 quantification on MON89034 x MON88017			**Trueness and repeatability of MON88017 quantification on single event MON88017***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.09	12	18	**0.09**	-2.6	28
0.5	3.6	19	**0.5**	2.9	13
1.0	3.8	13	**1.0**	-9.6	19
5.0	-0.5	14	**5.0**	-4.8	19
8.0	0.4	8.9	**8.0**	-7.6	18

*method validated (http://gmo-crl.jrc.it/statusofdoss.htm)

The MON89034 event-specific method (Table 6), when is applied to event MON89034 x MON88017 shows lower bias than the single line at all GM contents. The MON88017 event-specific method (Table 7), when applied to event MON89034 x MON88017, shows a higher bias at GM content of 0.09% and comparable or lower values at higher GM contents. Moreover, in three cases (0.09%, 1.0% and 8.0%), the bias observed on MON89034 x MON88017 was positive while it was originally negative on the single line.

Regarding the relative repeatability standard deviation (RSDr%), the MON89034 method shows slightly lower or similar values when applied to MON89034 x MON88017 hybrid, with the exception of a reduction by half of the RSDr% at GM content of 0.9% (Table 6). The MON88017 method shows lower values of RSDr% on MON89034 x MON88017 event compared to the single line, with the exception of a higher RSDr% at GM content of 0.5%.

In all cases, the values of trueness and RSDr% are within the ENGL acceptance levels established at a maximum of 25%.

Therefore, the method verification has demonstrated that the MON89034 and MON88017 detection methods developed to detect and quantify the single events can be equally applied for the quantification of the respective events combined in event MON89034 x MON88017.

8. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events MON89034 and MON88017 combined in maize event MON89034 x MON88017 have 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 during the present 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 paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

9. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32232) 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)].

10. 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.

11. 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 $1/20^{th}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq$ 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 $\pm$ 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 $\pm$ 25% of the accepted reference value over the whole dynamic range.

Event-specific Method for the Quantification of Maize Line MON 89034 Using Real-time PCR

Protocol

21 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)
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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 maize event MON 89034 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 89034 DNA, a 77-bp fragment of the integration region of the construct inserted into the plant genome (located at the 3' insert-to-plant junction) 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 the fluorescent dye FAM as a reporter at its 5′ end and with the non-fluorescent quencher MGBNFQ (minor groove binding non-fluorescent quencher) at its 3' end.

For the relative quantification of maize event MON 89034 DNA, a maize specific reference system amplifies a 79-bp fragment of the maize endogenous *hmg* gene (high mobility group), using a two specific primers and an *hmg* 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 quantification of the amount of event MON 89034 DNA in a test sample, MON 89034 and *hmg* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of maize event MON 89034 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from maize leaves, 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 participating laboratories in November 2007.

Each participant received twenty blind samples containing maize MON 89034 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 maize MON 89034 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 method developer, the relative LOD of the method is at least 0.04% in 200 ng of total maize DNA. The relative LOD was not assessed in a collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.085% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.09 %.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to maize event MON 89034 and thus imparts event-specificity to the method.

The specificity of event-specific assay was experimentally tested in real-time PCR by the applicant against DNA extracted from plant materials containing the specific targets of Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® corn borer maize (MON 810), YieldGard® Rootworm/Roundup Ready® maize (MON 88017) , YieldGard® Rootworm maize (MON 863), lysine maize (LY038), MON 89034 maize, conventional maize, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard® cotton (MON 757), BollgardII® cotton (MON 15985), MON 88913 cotton, conventional cotton, Roundup Ready® soybean 40-3-2, MON 89788 soybean, conventional soybean, Roundup Ready® wheat (MON71800), conventional wheat, lentil, quinoa, sunflower nuts, buckwheat, pinenuts, rye berries, millet, peanut (shelled).
None of the GM-lines tested, except the positive control maize line MON 89034, produced detectable amplification signals.

The specificity of the maize reference assay *hmg* was experimentally tested by the applicant against DNA extracted from plant materials containing Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® corn borer maize (MON 810), YieldGard® Rootworm/Roundup Ready® maize (MON 88017), YieldGard® rootworm maize (MON863), lysine maize (LY038), MON 89034 maize, conventional maize, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard® cotton (MON 757), BollgardII® cotton (MON 15985), MON 88913 cotton, conventional cotton, Roundup Ready® soybean 40-3-2, MON 89788 soybean, conventional soybean, Roundup Ready® wheat (MON71800), conventional wheat, lentil, sunflower, buckwheat, rye berries, peanut.
None of the samples tested, except the control maize lines GA21, NK603, MON 810, MON 863, NON 88017, LY038, MON 89034 and conventional maize, produced detectable amplification signals.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow the guidelines given by relevant authorities, 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.
- All the equipment used should be sterilised 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 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 maize event MON 89034

3.2.1 General

The PCR set-up for the taxon specific target sequence (*hmg*) and for the GMO (event MON 89034) 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 50 µL for the MON 89034 reaction and of 25 µL for the *hmg* reaction mixture with the reagents as listed in Table 1 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 89034 maize DNA 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 89034 system and one for the *hmg* 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 MON 89034 specific system.

Component	Final concentration	µL/reaction
TaqMan® 2x PCR Master Mix	1x	25
MON 89034 primer 1 (10 µM)	450 nM	2.25
MON 89034 primer 2 (10 µM)	450 nM	2.25
MON 89034 probe (5 µM)	100 nM	1.00
Nuclease free water	#	15.5
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
Nuclease free water	#	8.95
TaqMan® buffer A (10x)	1x	2.5
$MgCl_2$ (25 mM)	6.5 mM	6.5
dNTP mix (10 mM each)	200 µM each	0.5
AmpliTaq Gold polymerase (5 U/µl)	1.25 U	0.25
hmg primer 1 (10 µM)	300 nM	0.75
hmg primer 2 (10 µM)	300 nM	0.75
hmg probe (5 µM)	160 nM	0.8
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 89034 event 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 the MON 89034 reactions and 21 x 3 = 63 µL for *hmg* reactions). 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 (or 25 depending on the reaction mixture) 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 MON 89034 and *hmg* 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	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 89034) 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 89034 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 MON 89034 maize DNA in the unknown sample, the copy number of the GMO is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value (GM% = MON 89034/*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)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® 2X PCR Master Mix, Applied Biosystems Part No 4304437
- TaqMan® 1000X Rxn Gold/Buffer A Pack (10x) Applied Biosystems Part No 4304441

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
MON 89034 target sequence	
MON 89034 primer 1	5' – TTC TCC ATA TTG ACC ATC ATA CTC ATT– 3'
MON 89034 primer 2	5' – CGG TAT CTA TAA TAC CGT GGT TTT TAA A– 3'
MON 89034 (Probe)	6-FAM 5' – ATC CCC GGA AAT TAT GTT – 3' MGBNFQ
Reference gene hmg target sequence	
hmg primer 1	5' – TTG GAC TAG AAA TCT CGT GCT GA– 3'
hmg primer 2	5' – GCT ACA TAG GGA GCC TTG TCC T – 3'
hmg (Probe)	6-FAM 5' – CAA TCC ACA CAA ACG CAC GCG TA – 3' TAMRA

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)
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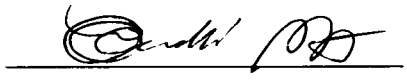

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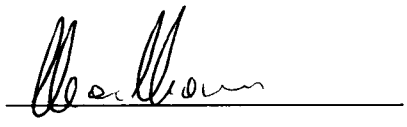

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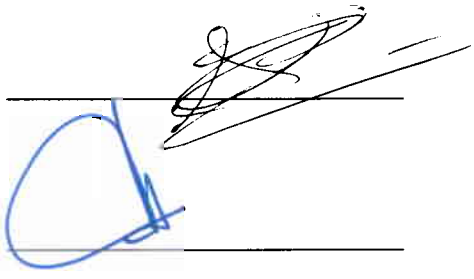

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Content

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 (except DNA) 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

EUR 24276 **EN – Joint Research Centre – Institute for Health and Consumer Protection**
Title: Report on the Verification of the Performance of MON89034 and MON88017 Event-specific Methods on the Maize Event MON89034 x MON88017 Using Real-Time PCR
Author(s): C. Delobel, E. Grazioli, M. Mazzara, G. Van den Eede
Luxembourg: Office for Official Publications of the European Communities
2009 – 37 pp. – 21 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-15221-4
DOI 10.2788/71542

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 MON89034 x MON88017 (unique identifier MON-89Ø34-3 x MON-88Ø17-3) which combines the MON89034 and MON88017 transformation events. The two methods have been validated individually on single-trait events, to detect and quantify each event in maize samples. 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 Company provided the detection methods and the control samples: genomic DNA extracted from seeds of MON89034 xMON88017 maize, genomic DNA extracted from seeds of non-GM maize, seeds of MON89034 xMON88017 and seed of conventional maize. 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 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/.

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JRC
EUROPEAN COMMISSION

LB-NA-24276-EN-C

ISBN 978-92-79-15221-4

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Publications.europa.eu