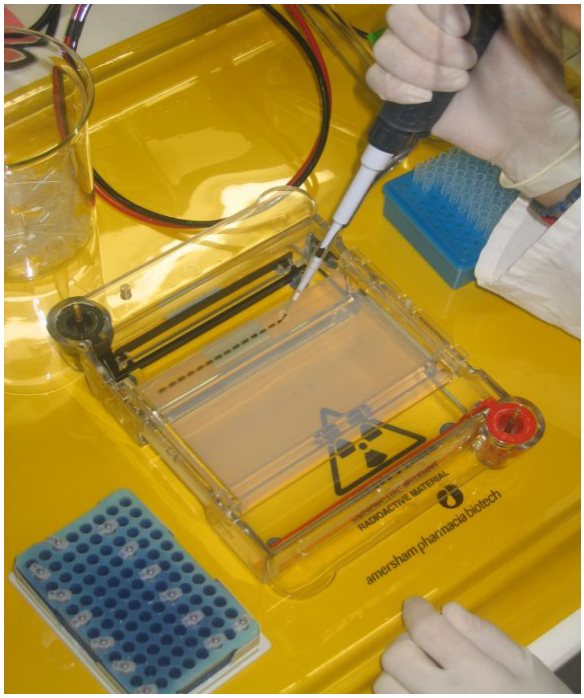
JRC Scientific and Technical Reports

Report on the Verification of the Performance of MON 89034 and NK 603 Event-specific Methods on the Maize Event MON 89034 x NK 603 Using Real-time PCR

Validation Report and Protocols

C. Savini, E. Grazioli, M. Mazzara, G. Van den Eede



EUR 24239 EN-2009

JRC EUROPEAN COMMISSION

ihcp 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/

JRC56613

EUR 24239 EN
ISBN 978-92-79-14985-6
ISSN 1018-5593
DOI 10.2788/5914

Luxembourg: Office for Official Publications of the European Communities

Printed in Italy

Report on the Verification of the Performance of MON89034 and NK603 Event-specific Methods on the Maize Event MON89034 x NK603 Using Real-Time PCR

9 September 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 a verification study to assess the performance of two quantitative event-specific methods on the maize event MON89034 x NK603 (unique identifier MON 89Ø34-3 x MON-ØØ6Ø3-6) which combines the MON89034 and NK603 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 NK603 maize (lot GLP-0701-17954-S), genomic DNA extracted from seeds of non-GM maize (lot GLP-0612-17871-S). The JRC prepared the verification samples (calibration samples and blind samples at different GM percentages).

The results of the 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 study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/.

Drafted by:
E. Grazioli

Report Verification Team:
1) L. Bonfini

2) G. Pinski

Scientific and technical approval:
M. Mazzara

Quality manager:
S. Cordeil

Authorisation to publish:
G. Van den Eede

Address of contact laboratory:
Community Reference Laboratory for GM Food and Feed
European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit
Via Fermi 2749, I-21027 Ispra (VA), Italy

Report on Steps 1-3 of the Validation Process

Monsanto Company submitted the detection methods and control samples of the maize event MON89034 x NK603 (unique identifier MON 89Ø34-3 x MON-ØØ6Ø3-6) 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 one request of complementary information was addressed to the applicant. Upon reception of the complementary information, the scientific assessment of the detection method for the MON89034 x NK603 maize was positively concluded in June 2007.

The event-specific detection methods for the two maize lines hosting the single events MON89034 and NK603 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 NK603 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 NK603 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% for MON89034 and 0.1% - 5% for NK603, on a DNA/DNA ratio. The experiments were performed in repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were 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 (below referred to as "the applicant") submitted the detection methods for MON89034 and NK603 and the control samples of the maize event MON89034 x NK603 (unique identifier MON 89Ø34-3 x MON-ØØ6Ø3-6) 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, established by Regulation (EC) 1829/2003, carried out a verification of the two event-specific methods for the detection and quantification of MON89034 and NK603 in the MON89034 x NK603 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 NK603 DNA to total maize DNA in the MON89034 x NK603 maize event. The procedures are simplex systems, in which the event MON89034 was quantified in reference to the maize *hmg* (high mobility group) endogenous gene and NK603 was quantified in reference to the maize *adh1* (alcohol dehydrogenase-1) 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 NK603 maize (lot GLP-0701-17954-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 NK603 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 NK603 events and available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

Table 1 shows the five GM contents of unknown samples used in the verification of the MON89034 and NK603 methods.

Table 1. MON89034 and NK603 GM contents in maize event MON89034 x NK603

MON89034 GM% (GM DNA / Non-GM DNA x 100)	**NK603 GM%** (GM DNA / Non-GM DNA x 100)
0.09	0.10
0.40	0.50
0.90	1.00
3.00	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 both the GM-specific system and the reference system (*hmg* or *adh1*). 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 NK603), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level. An Excel spreadsheet was used for determination of GM%.

4. Method

For specific detection of events MON89034 and NK603 in maize event MON89034 x NK603, two fragments of 77 bp and 108 bp respectively (covering the 3' 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 while at the 3′ end TAMRA and MGBNFQ are used as quencher dyes respectively for detection of NK603 and MON89034 events.

For relative quantification of event MON89034 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, was used.

For relative quantification of event NK603 DNA, a maize-specific reference system which amplifies a 70-bp fragment of the maize endogenous gene *adh1* (alcohol dehydrogenase-1), using two *adh1* gene-specific primers and an *adh1* gene-specific probe labelled with FAM and TAMRA, was used.

Standard curves are generated for each system (*hmg*, MON89034, and *adh1*, NK603), 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 standard curves are used to estimate the copy numbers in the unknown sample DNA 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

The values of the slopes of the standard curves, from which the PCR efficiency is 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 NK603 methods, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON89034 method on event MON89034 x NK603.

Run	MON89034			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.5	91	0.997	-3.2	107	0.997
2	-3.5	92	0.992	-3.3	100	0.998
3	-3.6	91	0.997	-3.3	101	0.998
4	-3.5	93	0.997	-3.2	104	0.996
5	-3.6	90	0.997	-3.2	104	0.998
6	-3.6	91	0.996	-3.3	102	0.998
7	-3.5	93	0.997	-3.2	104	0.999
8	-3.5	91	0.997	-3.3	103	0.997
Mean	-3.5	91	0.996	-3.3	103	0.998

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the NK603 method on event MON89034 x NK603.

Run	NK603			*adh1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.7	85	0.990	-3.2	105	0.991
2	-3.7	85	0.986	-3.1	109	0.992
3	-3.6	89	0.993	-3.2	105	0.990
4	-3.5	94	0.991	-3.3	103	0.992
5	-3.9	80	0.984	-3.1	109	0.991
6	-3.5	94	0.993	-3.2	106	0.992
7	-3.6	89	0.994	-3.1	109	0.991
8	-3.7	87	0.993	-3.1	108	0.993
Mean	-3.7	88	0.991	-3.2	107	0.991

The mean PCR efficiencies for the MON89034 and NK603 detection methods were 91% and 88% respectively for the MON89034 and NK603 specific systems. The linearity was 0.996 and 0.991

respectively for the MON89034 and NK603 specific systems. 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 NK603 maize samples.

6. Method performance requirements

The results of the verification study for the MON89034 and NK603 detection methods applied to event MON89034 x NK603 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 the CRL-GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm, see also Annex 1). In addition, Tables 4 and 5 report estimates 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 NK603 maize DNA.

MON89034					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.4**	**0.9**	**3.0**	**8.0**
Mean	0.11	0.44	1.01	2.97	7.77
SD	0.02	0.05	0.06	0.38	0.53
RSDr (%)	15	11	6.2	12.9	6.8
Bias (%)	23	10	13	-1.1	-2.8

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the NK603 method on event MON89034 x NK603 maize DNA

NK603					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.5**	**1.0**	**2.0**	**5.0**
Mean	0.12	0.52	1.19	1.89	5.66
SD	0.02	0.04	0.11	0.28	0.57
RSDr (%)	14	6.9	9.2	15	10.1
Bias (%)	23	3.7	19	-5.3	13

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 method performance between event MON89034 x NK603 and the single trait events

An indicative comparison of the two method performances on the maize event MON89034 x NK603 and the single trait events is shown in Tables 6 and 7. The performance of the methods on the single lines was previously assessed though international collaborative trials.

Table 6. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the MON89034 detection method on event MON89034 x NK603 and on event MON89034.

Trueness and repeatability of MON89034 quantification on MON89034 x NK603			**Trueness and repeatability of MON89034 quantification on single event MON89034***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.09	23	15	**0.09**	25	18
0.4	10	11	**0.4**	6.4	13
0.9	13	6.2	**0.9**	4.3	17
3.0	-1.1	12.9	**3.0**	-5.8	12
8.0	-2.8	6.8	**8.0**	-11	9.5

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

Table 7. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the NK603 detection method on event MON89034 x NK603 and on event NK603.

Trueness and repeatability of NK603 quantification on MON89034 x NK603			**Trueness and repeatability of NK603 quantification on single event NK603***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.10	23	14	**0.1**	83	24
0.5	3.7	6.9	**0.49**	73	15
1.0	19	9.2	**0.98**	47	17
2.0	-5.3	15	**1.96**	14	7.7
5.0	13	10	**4.91**	22	22

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

The MON89034 event-specific method (Table 6), when is applied to event MON89034 x NK603, shows higher bias (%) than the single trait line at GM contents of 0.4% and 0.9%, and lower bias at GM levels of 0.09%, 3% and 8%). The NK603 event-specific method (Table 7), when

applied to event MON89034 x NK603, shows lower bias (%) at all GM contents tested. In all cases, however, the trueness is within the acceptance level set by the ENGL (± 25%).

Regarding the relative repeatability standard deviation (RSDr %), the MON89034 event-specific method (Table 6) shows similar values when applied to the single event and to the MON89034 x NK603 hybrid, with the exception of a lower value at GM content of 0.9%. The NK603 method shows lower values of RSDr % on MON89034 x NK603 compared to the single line, except for the GM content of 2% (Table 7).

In all cases, the values of RSDr % are within the ENGL acceptance level established at maximum 25%.

Therefore, the method verification has demonstrated that the MON89034 and NK603 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 NK603.

8. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events MON89034 and NK603 combined in maize event MON89034 x NK603 have been evaluated with respect to the method acceptance criteria and the method performance requirements recommended by the ENGL (as detailed at 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 2 "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. International Standard (ISO) 5725:1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization.

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

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 $RSD_r \leq$ 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)
Biotechnology & GMOs Unit

Drafted by
C. Savini

Report Verification Team
1) M. Ermolli

2) M. Querci

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 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

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

Event-specific method for the quantitation of maize line NK603 using real-time PCR

Protocol

Method development:
Monsanto Biotechnology Regulatory Sciences
(only for the PCR part)

Method validation:
Joint Research Centre – European Commission
Biotechnology & GMOs Unit

Contents

Document Approval		
Name / *Function*	**Date**	**Signature**
Marco Mazzara *Sector Head*	10/01/2005	Signed
Stephane Cordeil *Quality Manager*	10/01/2005	Signed
Guy Van den Eede *B&GMOs Unit Head*	10/01/2005	Signed

Address of contact laboratory:

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 and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event NK603 DNA to total maize DNA in a sample. The procedure includes the following three modules:

a) DNA extraction: CTAB DNA extraction and purification protocol

b) Spectrophotometric quantitation of the amount of total DNA

c) Quantitative real-time PCR methodology specific for the NK603 event

The PCR assay has been optimised for use in an ABI Prism® 7700 sequence detection system. Other systems may be used, but thermal cycling conditions must be verified. The use of 200 ng of template DNA per reaction well is recommended.

DNA is extracted by means of a CTAB DNA extraction and purification protocol. For references, see Murray and Thompson (1980), Wagner *et al*. (1987) and Zimmermann *et al*. (1998). The protocol has been validated for soybeans (Anon, 1998), potato (Anon, 1996) and tomato (Anon, 1999). It has been tested for maize in a multi-laboratory pre-validation. The method was adopted from: Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Nucleic Acid Extraction. CEN/TC 275/WG11N0031. Draft November 2002.

Subsequently, purified DNA is quantified by means of spectrophotometry in order to determine the amount of DNA to be analysed by means of real-time PCR. The procedure "Basic ultraviolet spectrometric method" has been adopted from the Annex B "Methods for the quantification of the extracted DNA" of the prEN ISO 21571:2002. The method has been widely used and ring-tested (Anon. 2002).

For specific detection of event NK603 genomic DNA, a 108-bp fragment of the region that spans the 3′ insert-to-plant junction in maize event NK603 is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For relative quantitation of event NK603 DNA, a maize-specific reference system amplifies a 70-bp fragment of *adh*1, a maize endogenous gene, using a pair of *adh*1 gene-specific primers and an *adh*1 gene-specific probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantitation of the amount of

event NK603 DNA in a test sample, event NK603 and *adh*1 Ct values are determined for the sample. Standard curves are then used to calculate the relative content of event NK603 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for maize seeds, grain and flour containing mixtures of genetically modified NK603 and conventional maize.

The reproducibility and trueness of the method was tested through collaborative trial using samples of the CRM IRMM-415 series.

2.2 Collaborative trial

The method was validated in a collaborative trial by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 12 laboratories.

Each participant received ten unknown samples. The samples consist of five reference materials (CRM IRMM-415) of dried maize powder containing mixtures of genetically modified NK603 maize in conventional maize (w/w) between 0.1 % and 4.91 %.

For each unknown sample one DNA extraction has been carried out. Each test sample was analyzed by PCR in four repetitions. The study was designed as a blind duplicate collaborative trial. Each laboratory received each level of GM NK603 in two unknown samples, and the two replicates for each GM level were analyzed in two PCR plates.

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

2.3 Limit of detection

According the method developer, the relative LOD of the method is at least 0.05%. The relative LOD was not assessed in a collaborative trail. The lowest relative concentration of the target sequence included in collaborative trail was 0.1%.

2.4 Limit of quantitation

According the method developer, the relative LOQ of the method is 0.1%. The lowest relative concentration of the target sequence included in collaborative trail was 0.1%.

2.5 Molecular specificity

The method utilizes the unique DNA sequence at the junction of the insert and the genomic DNA flanking the insert. The sequence is specific to NK603 and thus imparts specificity to the detection method.

The specificity was experimentally tested against DNA extracted from plant materials containing the specific targets of GA21, MON863, MON810 maize, and from conventional corn, Roundup Ready® soybean, conventional soybean, Roundup Ready® canola, conventional canola and Roundup Ready® wheat. None of the materials yielded detectable amplification.

The target sequence is a single copy sequence in the haploid NK603 genome.

3. Procedures

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Maintain strictly separate working areas for DNA extraction, PCR set-up and amplification.
- All the equipment used must be sterilized prior to use and any residue of DNA has to be removed.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Use only powder-free gloves and change them frequently.
- Clean lab-benches and equipment periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.

3.2 DNA extraction

a. Moisten 200 mg of sample with 300 µl of sterile deionised water in a 1.5 ml tube.
b. Mix with a sterile loop until homogeneity is reached.
c. Add 700 µl of CTAB-buffer pre -warmed to 65°C; mix with a loop or a clean spatula
d. Add 10 µl of RNase solution; shake.
e. Incubate at 65° C for 30 min.
f. Add 10 µl of Proteinase K solution; mix smoothly.
g. Incubate at 65° C for 30 min.
h. Centrifuge for 10 min at 12000 g
i. Transfer supernatant to a 1.5 ml tube, containing 500 µl chloroform; shake for 30 sec.
j. Centrifuge for 15 min at 12000 g until phase separation occurs.
k. Transfer the aqueous upper phase into a new 1.5 ml tube containing 500 µl chloroform; shake.
l. Centrifuge for 5 min at 12000 g.
m. Transfer upper layer to a new 1.5 ml tube.
n. Add 2 volumes of CTAB precipitation solution, mix by pipetting.
o. Incubate for 60 min at room temperature.
p. Centrifuge for 5 min at 13000 rpm; discard the supernatant.
q. Dissolve precipitate in 350 µl NaCl (1.2 M).
r. Add 350 µl chloroform and shake for 30 sec.
s. Centrifuge for 10 min at 12000 g until phase separation occurs.
t. Transfer upper layer to a new reaction tube.
u. Add 0.6 volumes of isopropanol, mix smoothly by inversion. Incubate for 20 min at room temperature.
v. Centrifuge for 10 min at 12000 g. Discard the supernatant.
w. Add 500 µl of 70% ethanol solution and shake carefully.
x. Centrifuge for 10 min at 12000 g. Discard the supernatant.
ATTENTION: drain the supernatant carefully. DNA pellets may detach from the bottom of the tube at this stage.
y. Dry pellets and re-dissolve DNA in 100µl sterile, TE buffer.

z. **NOTE**: for thorough homogenisation of the DNA solution, it is recommended to re-suspend the sample by gentle agitation at +4°C for 24 h.

The DNA solution may be stored at ~ 4°C for a maximum of one week, or at -20°C for long-term storage.

3.3 Spectrophotometric measurement of DNA concentration

3.3.1 Measurement of a reference DNA solution

The correct calibration of the spectrometer can be verified as follows, with the use of a reference DNA solution:

a) For blank measurement only dilution buffer is used to fill the measurement vessel.

b) The reference DNA solution (Calf Thymus or Herring Testes DNA or Lambda DNA) is filled into the measurement vessel.

Absorption is measured for both blank and reference DNA solutions at λ= 260 nm and λ= 320 nm.

3.3.2 Measurement of a test DNA solution of unknown concentration

a) Blank measurement: mix the dilution buffer with a 2M sodium hydroxide solution, at the final NaOH concentration of 0.2M. This solution is used for the blank measurement.
b) Mix the DNA solutions with a 2M sodium hydroxide solution and, if needed, with dilution buffer, at the final NaOH concentration of 0.2M.
c) Measure the absorption after 1 min incubation time for both blank and reference DNA solution at λ = 260 nm and λ = 320 nm. The reading is stable for at least 1 h.

Example for blank measurement: Mix 90 µl dilution buffer and 10 µl of 2M sodium hydroxide solution and transfer to a 100 µl measurement vessel.

Example for the test DNA solution: Mix 80 µl of dilution buffer or water, 10 µl of 2M sodium hydroxide solution, 10 µl of DNA solution of unknown concentration and transfer to a 100 µl measurement vessel.

3.3.3 Evaluation

The absorption (OD) at 320 nm (background) is subtracted from the absorption at 260 nm resulting in the corrected absorption at 260 nm. If the corrected OD at 260 nm equals to 1, then the estimated DNA concentration is 38 µg/ml for single stranded DNA (denatured with sodium hydroxide).

Reliable measurements require OD values at λ=260 nm greater than 0.05.
The concentration of the double stranded test DNA solution is finally calculated taking into consideration the denaturation and the dilution factor applied.

3.4 Real-time PCR for quantitative analysis of NK603 maize

3.4.1 General

The PCR set-up for the taxon specific target sequence (*Adh*1) and for the GMO (NK603) 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 200 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 50 µl per reaction mixture with the reagents as listed in Table 1.

3.4.2 Calibration

Separate calibration curves with each primer/probe system are generated in the same analytical amplification run.

The calibration curves consist of five dilutions of DNA extracted from the 4.91 % CRM IRMM-415. A series of one to three dilution intervals (one to four for the last two dilutions) at a starting concentration of 110,092 maize genome copies may be used (corresponding to 300 ng of DNA with one maize genome assumed to correlate to 2.725 pg of haploid maize genomic DNA) (Arumuganathan & Earle, 1991).

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

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

3.4.3 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 NK603 system and one for the *adh*1 system) on ice, add the following components (Table 1) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixtures in the final volume/concentration per reaction well, for NK603/*adh*1 specific systems.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2X)	1x	25 µl
Primer NK603-F/*adh*1-F	150 nM	-
Primer NK603-R/*adh*1-R	150 nM	-
Probe NK603/*adh*1	50 nM	-
Nuclease free water		up to 50 µl
Template DNA (maximum 300 ng, see 3.4.1 and 3.4.2)		5 µl
Total reaction volume:		50 µl

3. Mix gently and centrifuge briefly.
4. Prepare two 1.5 ml reaction tubes (one for the NK603 and one for the *adh*1 master mix) 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. 45 x 3 = 135 µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 x 3 = 15 µl DNA for three PCR repetitions). Low-speed vortex each tubes at least three times for approx 30 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
6. Spin down the tubes in a micro-centrifuge. Aliquot 50 µl in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 2:

Table 2. Reaction conditions.

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG pre-PCR decontamination		50 °C	120″	No	1x
2	Activation of DNA polymerase and denaturation		95 °C	600″	No	1x
3		Denaturation	95 °C	15″	No	45x
4	Amplification	Annealing & Extension	60 °C	60″	Measure	

3.5 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. *adh*1) 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. NK603 system).

e) Save the settings and export all the data into an Excel file for further calculations.

3.6 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instruments software calculated the Ct-values for each reaction.

The standard curves are generated both for the *adh*1 and NK603 specific system 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 by interpolation from the standard curves.

For the determination of the amount of NK603 DNA in the unknown sample, the NK603 copy number is divided by the copy number of the maize reference gene (*adh*1) and multiplied by 100 to obtain the percentage value (GM% = NK603/*adh*1 * 100).

4. Materials

4.1 Equipment (equivalents may be substituted)

DNA extraction:

- Water bath or heating block
- Microcentrifuge
- Micropipettes
- Vortexer
- 1.5/2.0 ml tubes
- Tips and filter tips for micropipettes
- Rack for reaction tubes
- Vinyl or latex gloves
- Optional: vacuum dryer apt to dry DNA pellets

Spectrophotometry:

- UV spectrophotometer. Single beam, double beam or photodiode array instruments are suitable.
- Vortexer
- Measurement vessels. e.g. quartz cuvettes or plastic cuvettes suitable for UV detection at a wavelength of 260 nm. The size of the measurement vessels used determines the volume for measurement. This should be one of the following: half

micro cuvettes (1000 μl), micro cuvettes (400 μl), ultra micro cuvettes (100 μl) and quartz capillaries (3 μl to 5 μl). The optical path of standard cuvettes is usually 1 cm.

Real-time PCR:

- ABI Prism® 7700 Sequence Detection System. Applied Biosystems Part No 7700-01-200/208.
- ABI Prism® 7900HT Sequence Detection System. Applied Biosystems Part No 4329002 or 4329004.
- Software: Sequence Detection System version 1.7 (Applied Biosystems Part No 4311876) or equivalent versions.
- MicroAmp® optical 96-Well reaction plates. Applied Biosystems Part No N801-0560).
- MicroAmp® optical adhesive covers. Applied Biosystems Part No 4311971.
- MicroAmp Optical caps. Applied Biosystems Part No. No 801-0935.
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml tubes

4.2 Reagents

DNA extraction:

- CTAB: Cetyltrimethylammonium Bromide (Ultrapure grade)
- TRIS: Tris[hydroxymethyl] aminomethane hydrochloride (Molecular Biology grade)
- EDTA: Ethylenediaminetetraacetic acid, disodium salt (titration 99.9%)
- Ethanol (96% at least)
- Isopropanol (99.7% at least)
- Chloroform (99% at least)
- NaCl (99% at least)
- NaOH (98% at least, anhydrous)
- Distilled sterile water
- RNase A solution 10 mg/ml
- Proteinase K solution 20 mg/ml

Spectrophotometry:

- Dilution buffer: TRIS: Tris[hydroxymethyl] aminomethane hydrochloride (Molecular Biology grade). 10 mM, pH 9.0.
- NaOH (98% at least, anhydrous)

- Hydrochloric acid (HCl), φ (HCl) = 37 %
- Herring Testes DNA, Calf Thymus DNA, or Lambda DNA

Real-time PCR:

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

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
GMO target sequence	
NK603 primer F	ATGAATGACCTCGAGTAAGCTTGTTAA
NK603 primer R	AAGAGATAACAGGATCCACTCAAACACT
NK603 probe	6-FAM- TGGTACCACGCGACACACTTCCACTC-TAMRA
Reference gene target sequence	
*Adh*1 primer F	CCAGCCTCATGGCCAAAG
*Adh*1 primer R	CCTTCTTGGCGGCTTATCTG
*Adh*1 probe	6-FAM-CTTAGGGGCAGACTCCCGTGTTCCCT-TAMRA

5. Buffers and Solutions

The following describes the preparation, storage and stability of the buffers used in this procedure. Volume may be scaled as needed. Equivalent reagent may be substituted.

DNA extraction:

- **CTAB buffer (1 litre)**

  Weight and mix in an appropriate cylinder:

20 g/l CTAB	20 g
1.4 M NaCl	82 g
0.1 M Tris-HCl	15.75 g
20 mM Na_2EDTA	7.5 g

  a. Add 500 ml of sterile distilled water.
  b. Adjust pH to a value of 8.0 with 1M NaOH.
  c. Fill up to 1000 ml and autoclave.

  Store at 4° C for up to 6 months.

- **CTAB-precipitation solution (200 ml)**
  Weight and mix in an appropriate cylinder:

5 g/l CTAB	1 g
0.04 M NaCl	0.5 g

  a. Add 100 ml of distilled water.
  b. Adjust pH to a value of 8.0 with 1 M NaOH.
  c. Fill up to 200 ml and autoclave.

  Store at 4° C for up to 6 months.

- **NaCl 1.2 M (100 ml)**
  a. Dissolve 7 g of NaCl in 100 ml sterile distilled water in a cylinder.
  b. Autoclave

  Store at room temperature for up to 5 years

- **Ethanol-solution ~70 % (v/v) (100 ml)**
  a. Mix 70 ml of pure ethanol with 30 ml of sterile distilled water

  Store at room temperature or at -20° C for up to 5 years

- **NaOH 1M (50 ml)**
  a. Dissolve 2 g of NaOH in 50 ml of sterile water in a cylinder or a 50 ml conical tube.

  Store at room temperature for up to 6 months

- **TE buffer, pH 7.0 (Tris/HCl 10 mM, EDTA 1 mM, pH 7.0) (250 ml)**
  a. Mix 100 ml of nuclease-free water, 2.5 ml of 1M Tris, pH 8.0 and 0.5 ml of 0.5M EDTA
  b. Adjust pH to 7.0 with HCl
  c. Adjust final volume to 250 ml with nuclease-free water
  d. Filter sterilise

  Store at room temperature for up to 5 years

- **RNAse A 10 mg/ml**
  a. Dissolve the RNase A at a final concentration of 10 mg/ml in sterile water.
  b. If indicated by supplier: boil the RNase A solution at 95°C for 15’ to remove any residual nuclease activity.
  c. Aliquot solution as appropriate (thawing and re-freezing should be avoided)

Store aliquots at -20° C for up to 6 months

- **Proteinase K 20 mg/ml**
  a. Dissolve the Proteinase K at a final concentration of 20 mg/ml in sterile distilled water according to the supplier specifications.
  b. Aliquot solution as appropriate (thawing and re-freezing should be avoided)

  Store aliquots at -20° C for up to 6 months

Spectrophotometry:

- **Reference DNA solution**
  A DNA 10 mg/ml stock solution is prepared by dissolving 100 mg DNA (from Herring Testes or from Calf Thymus or Lambda DNA) in 10 ml dilution buffer (TRIS/HCl 10 mM, pH 9.0). At this concentrations DNA dissolves and homogenises slowly and the resulting solution is very viscous. The stock solution is further diluted with dilution buffer up to the desired working concentration (e.g. 25 μg/ml).

- **NaOH 2M (50 ml)**
  a. Dissolve 4 g of NaOH in 50 ml of sterile water in a cylinder or a 50 ml conical tube.

  Store at room temperature for up to 5 years

6. References

Anon. 1996. Untersuchung von Lebensmitteln: Nachweis einer gentechnischen Veränderung von Kartoffeln durch Amplifizierung der veränderten DNA-Sequenz mit Hilfe der PCR (Polymerase Chain Reaction) und Hybridisierung des PCR-Produktes mit einer DNA-Sonde. L 24.01-01, February 1996, revised in January 1997.. In: Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG; Verfahren zur Probenahme und Untersuchung von Lebensmitteln, Tabakerzeugnissen, kosmetischen Mitteln und Bedarfsgegenständen/ BgVV. Loseblattausgabe, Stand Jan 1997 Bd.1. Berlin, Köln. Beuth Verlag GmbH.

Anon 1998. Untersuchung von Lebensmitteln: Nachweis einer gentechnischen Veränderung von Sojabohnen durch Amplifizierung der veränderten DNA-Sequenz mit Hilfe der PCR (Polymerase Chain Reaction) und Hybridisierung des PCR-Produktes mit einer DNA-Sonde. L 23.01.22-1, March 1998. In: Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG; Verfahren zur Probenahme und Untersuchung von Lebensmitteln, Tabakerzeugnissen, kosmetischen Mitteln und Bedarfsgegenständen/ BgVV. Loseblattausgabe, Stand March 1998 Bd.1. Berlin, Köln. Beuth Verlag GmbH.

Anon. 1999. Untersuchung von Lebensmitteln: Nachweis einer gentechnischen Veränderung von Tomaten durch Amplifizierung der veränderten DNA-Sequenz mit Hilfe der PCR (Polymerase Chain Reaction) und Hybridisierung des PCR-Produktes mit einer DNA-Sonde L 25.03.01-1, November 1999. In: Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG; Verfahren zur Probenahme und Untersuchung von Lebensmitteln, Tabakerzeugnissen, kosmetischen Mitteln und Bedarfsgegenständen/ BgVV. Loseblattausgabe, Stand Nov 1999 Bd.1. Berlin, Köln. Beuth Verlag GmbH.

Anon. 2002. Swiss food manual, Chapter 52B, Section 1 to 5. Eidgenössische Drucksachen und Materialzentrale, CH-5005 Bern. Available on CD.

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

Murray, M.G. and Thompson, W.F. (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8, 4321–4325.

Wagner, D.B., Furnier, G.R., Saghay-Maroof, M.A., Williams, S.M., Dancik, B.P. and Allard, R.W. (1987). Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proceedings of the National Academy of Science USA 84, 2097–2100.

Zimmermann, A., Lüthy, J. and Pauli, U. (1998). Quantitative and qualitative evaluation of nine different extraction methods for nucleic acids on soya bean food samples. Zeitschrift für Lebensmittel-Untersuchung und -Forschung A 207, 81–90.

European Commission

EUR 24239 **EN – Joint Research Centre – Institute for Health and Consumer Protection**
Title: Report on the Verification of the Performance of MON89034 and NK603 Event-specific Methods on the Maize Event MON89034 x NK603 Using Real-Time PCR
Author(s): C. Savini, E. Grazioli, M. Mazzara, G. Van den Eede
Luxembourg: Office for Official Publications of the European Communities
2009 – 42 pp. – 21 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-14985-6
DOI 10.2788/5914

Abstract

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, has carried out a verification study to assess the performance of two quantitative event-specific methods on the maize event MON89034 x NK603 (unique identifier MON 89Ø34-3 x MON-ØØ6Ø3-6) which combines the MON89034 and NK603 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 NK603 maize (lot GLP-0701-17954-S), genomic DNA extracted from seeds of non-GM maize (lot GLP-0612-17871-S). The JRC prepared the verification samples (calibration samples and blind samples at different GM percentages).

The results of the 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 study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/.

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-24239-EN-C

Publications Office
Publications.europa.eu

ISBN 978-92-79-14985-6
9 789279 149856