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Report on the Verification of the Performance of 59122 and NK603 Event-specific Methods on the Hybrid Maize Line 59122xNK603 Using Real-Time PCR

Validation Report and Protocol

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28 January 2008

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GeneScan Analytics GmbH

Method validation:

Joint Research Centre – European Commission
Biotechnology & GMOs Unit
Community Reference Laboratory for GM Food and Feed

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Biotechnology & GMOs 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 hybrid maize line 59122xNK603 (unique identifier DAS-59122-7xMON-ØØ6Ø3-6) which combines the 59122 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, Pioneer Hi-Bred Intl. Inc. provided the detection methods and the control samples (59122xNK603 ground maize flour, conventional ground maize flour and non genetically modified control DNA). 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.it/doc/Method%20requirements.pdf>) and to the validation results on the individual parental events (<http://gmo-crl.jrc.it/statusofdoss.htm>).

The results of this CRL-GMFF in-house verification studies are made publicly available at <http://gmo-crl.jrc.it/>.

Drafted by:

C. Delobel

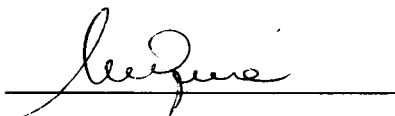


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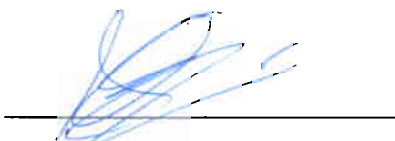
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Report on Steps 1-3 of the Validation Process

Pioneer Hi-Bred submitted the detection methods and control samples of the hybrid maize line 59122xNK603 (unique identifier DAS-59122-7xMON-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 to 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.it/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.it/doc/Method%20requirements.pdf>) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, three scientific assessments were performed and two requests of complementary information were addressed to the applicant. Upon reception of the last complementary information, the scientific assessment of the detection method for the 59122xNK603 maize was positively concluded in June 2006.

The event-specific detection methods for the two maize lines hosting the single events 59122 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.it/statusofdoss.htm>). Hence, the detection methods applied on the hybrid maize line 59122xNK603 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 hybrid 59122xNK603 combining the two traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In May 2007, 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 0.1%-5%, on a DNA copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, treuness 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.

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1. Introduction

Pioneer Hi-Bred submitted the detection methods (for 59122 and NK603) and the control samples of the hybrid maize line 59122xNK603 (unique identifier DAS-59122-7xMON=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, Biotechnology and GMOs 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 59122 and NK603 in the 59122xNK603 hybrid maize line combining the two traits derived through traditional breeding techniques between progeny of the two genetically modified lines. The single methods had been previously validated by international collaborative studies on the single-trait maize events (<http://gmo-crl.jrc.it/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 May 2007.

The validated methods of DNA extraction from single-trait maize seeds were used by the CRL-GMFF to extract DNA from the control samples received. The protocols for DNA extraction are available at <http://gmo-crl.jrc.it/>.

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 59122 and NK603 DNA to total maize DNA in the 59122xNK603 hybrid maize line. The procedures are simplex systems, in which the event 59122 was quantified in reference to the maize *hmg* (high mobility group) endogenous gene; the NK603 event 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 quantitative event-specific methods, control samples consisting of ground flour of hybrid 59122xNK603 (lot HW05056216HWC03GR300), non genetically modified ground flour (36B08 lot PIW3CON11012-P5) and DNA of conventional maize (36B08 Lot PIT3SZA11087-00) were provided by the applicant. The genomic DNA was extracted from ground flour control samples.

Samples containing mixtures of 100% 59122xNK603 and non-GM maize genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the control samples provided, in a constant amount of total maize DNA.

The protocols (reagents, concentrations, primer/probe sequences) followed in the in-house verification are those already published as validated methods for the individual 59122 and NK603 events and available at <http://gmo-crl.jrc.it/statusofdoss.htm>.

Table 1 shows the five GM levels of unknown samples used in the verification of the 59122 and NK603 methods.

Table 1. 59122 and NK603 GM contents in hybrid 59122xNK603

59122 GM % (GM copy number/maize genome copy number *100)	NK603 GM % (GM copy number/maize genome copy number *100)
0.1	0.1
0.4	0.5
0.9	1.0
2.0	2.0
4.5	5.0

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 (59122 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

Description of the operational steps

For specific detection of event 59122 and NK603 genomic DNA, an 86-bp and 108-bp fragment of the integration region of the construct inserted into the plant genome is amplified using two specific primers.

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

For the relative quantification of event 59122 DNA, a maize-specific reference system which amplifies a 79-bp fragment of *hmg* (high mobility group) gene, using a pair of *hmg* gene-specific primers, and an *hmg* gene-specific probe labelled with FAM and TAMRA, were used. For relative quantification of event NK603 DNA, a maize-specific reference system which amplifies a 70-bp fragment of *adh1* (alcohol dehydrogenase 1) gene, using a pair of *adh1* gene-specific primers and an *adh1* gene-specific probe labelled with FAM and TAMRA, were used.

Standard curves are generated for each GM specific system (59122 or NK603) and its respective reference gene (*hmg* or *adh1*), by plotting the Ct-values measured for the calibration samples against the logarithm of the DNA copy number, 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 59122 or NK603 DNA in the unknown sample, the 59122 or NK603 copy number is divided by the copy number of the maize reference gene *hmg* (59122) or *adh1* (NK603) and multiplied by 100 to obtain the percentage value (GM% = GM-specific system/maize reference system * 100).

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.it/statusofdoss.htm>.

5. Deviations reported

No deviations from the protocols of the two previously validated methods were introduced.

6. Summary of results

PCR efficiency and linearity

The values of the slopes of the standard curves, from which the PCR efficiency is calculated using the formula $[10^{(-1/\text{slope})}-1]*100$, 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 59122 and NK603 methods, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 59122 method (59122 assay and endogenous *hmg* assay) on hybrid 59122xNK603

Run	59122			<i>hmg</i>		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.20	105	1.00	-3.07	112	1.00
2	-3.43	96	1.00	-3.21	105	1.00
3	-3.42	96	0.99	-3.12	109	1.00
4	-3.49	93	0.99	-3.20	105	1.00
5	-3.34	99	1.00	-3.13	109	1.00
6	-3.45	95	0.99	-3.26	103	1.00
7	-3.39	97	1.00	-3.11	110	1.00
8	-3.52	92	1.00	-3.27	102	1.00
Mean	-3.41	97	1.00	-3.17	107	1.00

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the NK603 method (NK603 assay and endogenous *Adh1* assay) on hybrid 59122xNK603

Run	NK603			<i>Adh1</i>		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.53	92	0.99	-3.43	96	0.99
2	-3.54	92	1.00	-3.40	97	0.99
3	-3.57	90	0.99	-3.11	110	0.99
4	-3.69	86	0.99	-3.34	99	0.99
5	-3.59	90	0.99	-3.35	99	0.99
6	-3.47	94	1.00	-3.30	101	0.99
7	-3.58	90	0.99	-3.26	103	0.99
8	-3.58	90	1.00	-3.20	105	0.99
Mean	-3.57	91	0.99	-3.30	101	0.99

The mean PCR efficiencies of the GM specific systems were 97% and 91% respectively for the 59122 and NK603 specific systems; the efficiency of the *adh1* endogenous assay was close to

100%, while the mean efficiency of the *hmg* endogenous assay was 107%. The linearity of all methods (R^2 value) was above 0.99. Overall, data reported in Table 2 and 3 confirm the appropriate performance characteristics of the two methods tested on 59122xNK603 hybrid maize samples in terms of PCR efficiency and linearity.

7. Method performance requirements

The results of the in-house verification study for the 59122 and NK603 detection methods applied to hybrid 59122xNK603 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.it/guidancedocs.htm>, see also Annex 1). In addition, Tables 4 and 5 report estimates of trueness and repeatability standard deviation for each GM level and for all methods.

Table 4. Estimates of trueness (expressed as bias %) and repeatability standard deviation (RSDr %) of the 59122 method on hybrid 59122xNK603 maize DNA

59122					
Unknown sample GM%	Expected value (GMO %)				
	0.1	0.4	0.9	2.0	4.5
Mean	0.10	0.36	0.90	1.84	3.96
SD	0.02	0.03	0.06	0.13	0.47
RSDr (%)	15.8	9.6	6.8	6.8	11.9
Bias%	3.4	-10.6	-0.1	-7.9	-12.6

Table 5. Estimates of trueness (expressed as bias %) and repeatability standard deviation of the NK603 method on hybrid 59122xNK603 maize DNA

NK603					
Unknown sample GM%	Expected value (GMO %)				
	0.1	0.5	1.0	2.0	5.0
Mean	0.13	0.55	1.23	2.14	6.23
SD	0.02	0.08	0.15	0.29	0.63
RSDr (%)	16.6	13.8	12.4	13.6	10.1
Bias%	31.1	9.1	23.1	7.1	24.5

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 $\pm 25\%$ across the entire dynamic range. As shown in Tables 4 and 5, both methods satisfy the above requirement throughout their respective dynamic ranges, except the NK603 detection method at 0.1% (bias of 31%).

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 be below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" [<http://gmo-crl.jrc.it/guidancedocs.htm>]).

As it can be observed from the values reported in Tables 4 and 5, the two methods satisfy this requirement throughout their respective dynamic ranges.

8. Comparison of method performance between hybrid 59122xNK603 and the single trait events

A synoptic comparison of the two method performances on the maize hybrid 59122xNK603 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 repeatability standard deviation (RSD_r %) of the 59122 detection method on hybrid 59122xNK603 and on line 59122 maize DNA.

Trueness and repeatability of 59122 quantification on 59122xNK603			Accuracy and precision of 59122 quantification on single line 59122*		
GM%	Bias (%)	RSD _r (%)	GM%	Bias (%)	RSD _r (%)
0.1	3.4	15.8	0.1	29	25
0.4	-10.6	9.6	0.4	15	22
0.9	-0.1	6.8	0.9	9.0	22
2.0	-7.9	6.8	2.0	7.0	15
4.5	-12.6	11.9	4.5	-1.0	13

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

Table 7. Trueness (bias %) and repeatability standard deviation (RSD_r %) of the NK603 detection method on hybrid 59122xNK603 and on line NK603 maize DNA.

Trueness and repeatability of NK603 quantification on 59122xNK603			Trueness and repeatability of NK603 quantification on single line NK603*		
GM%	Bias (%)	RSD _r (%)	GM%	Bias (%)	RSD _r (%)
0.1	31.1	16.6	0.10	83	24
0.5	9.1	13.8	0.49	73	15
1.0	23.1	12.4	0.98	47	17
2.0	7.1	13.6	1.96	14	7.7
5.0	24.5	10.1	4.91	22	22

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

The 59122 event-specific method, when applied to the hybrid line, shows a lower bias (%) at GM level 0.1% (3.4 compared to 29 on the single line) and a higher bias at GM level 4.5% (12.6), but in all cases the bias is well within the acceptance level set by the ENGL (maximum bias 25%).

The 59122 method also shows an RSDr (%) generally lower when applied to the hybrid maize line compared to single line. In all cases the value of the RSDr (%) is within the acceptance level set by the ENGL (maximum 25%).

The NK603 event-specific method, when applied to the hybrid line, shows a lower bias (%) at all GM levels with the exception of level 5% where the bias (%) is comparable. The bias (%) at GM level 0.1% (31) is slightly higher than the acceptance level set by the ENGL (maximum bias 25%), but much lower in comparison to the corresponding value resulting from the validation study on the single line (83%).

The NK603 method also shows an RSDr (%) generally lower when applied to the hybrid maize line compared to single line, with the exception of GM level 2% where the RSDr (%) is slightly higher. In all cases the value of the RSDr (%) is within the acceptance level set by the ENGL (maximum 25%).

Therefore, the in-house method verification has demonstrated that the 59122 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 the hybrid line 59122xNK603.

9. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events 59122 and NK603 combined in the maize hybrid 59122xNK603 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.it/guidancedocs.htm>), and to the validation results obtained for the single trait events (<http://gmo-crl.jrc.it/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, with the single exception of NK603 detection method for which the bias obtained at 0.1% was 31% which remains below the bias obtained on the single-trait event (83%). 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.

10. 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)].

11. References

1. Horwitz, W. (1995) Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67, 331-343.
2. International Standard (ISO) 5725:1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization.

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

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

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

Method Acceptance Criteria

Applicability

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

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

Practicability

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

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

Specificity

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

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

Dynamic Range

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

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

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

Acceptance Criterion: The accuracy should be within $\pm 25\%$ 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 = $[10^{-(1/\text{slope})}] - 1$

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of $(- 3.1 \geq \text{slope} \geq - 3.6)$

R² Coefficient

Definition: The R² coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R² 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 1/10th 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 1/20th of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq 5\%$ 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.



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Event-specific method for the quantitation of maize 59122 using real-time PCR

Protocol

Method development:

Pioneer Hi-Bred International
GeneScan Analytics GmbH

Method validation:

Joint Research Centre – European Commission
Biotechnology & GMOs Unit
Community Reference Laboratory for GM Food and Feed

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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 DAS-59122-7 DNA to total maize DNA in a sample.

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

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

For specific detection of event DAS-59122-7 genomic DNA, an 86-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantitation of event DAS-59122-7 DNA, a maize-specific reference system amplifies a 79-bp fragment of the High Mobility Group (Hmg) gene, a maize endogenous gene, using a pair of Hmg gene-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 quantitation of the amount of event DAS-59122-7 DNA in a test sample, event DAS-59122-7 and Hmg Ct values are determined for the sample. Standard curves are then used to calculate the relative content of event DAS-59122-7 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for DNA extracted from seeds, containing mixtures of genetically modified and conventional maize.

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

2.2 Collaborative trial

The method was validated in a collaborative trial by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 14 laboratories.

Each participant received twenty unknown samples containing DAS-59122-7 maize genomic DNA at five concentration levels, between 0.10 % and 4.5 %.

Each test sample was analyzed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM DAS-59122-7 in four unknown samples. Two replicates of each GM level were analyzed on the same PCR plate.

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

2.3 Limit of detection

According to the method developer, the relative LOD of the method is 0.045%. The relative LOD was not assessed in a collaborative trial. The lowest relative concentration of the target sequence included in collaborative trial was 0.10%.

2.4 Limit of quantitation

According to the method developer, the relative LOQ of the method is 0.09%. The lowest relative concentration of the target sequence included in collaborative trial was 0.10%.

2.5 Molecular specificity

The method utilizes a unique DNA sequence of the recombination region of parts of the construct inserted into the plant genome. The sequence is specific to DAS-59122-7 and thus imparts event-specificity to the detection method.

The specificity was assessed by Blastsearch at the National Center for Biotechnology Information (NCBI) with the “Standard nucleotide-nucleotide BLAST [blastn]” (www.ncbi.nlm.nih.gov/blast/Blast.cgi) on the amplicon resulting from the event-specific amplification of the transition region of the sugar beet genomic DNA into the specific event.

No 100% match with other plant GMO sequences was found.

The specificity was experimentally tested against DNA extracted from plant materials containing the specific targets (at least 1000 genomic copies/reaction) of the T25 maize, 1507 maize, Bt176 maize, Bt11 maize, NK603 maize, GA21 maize, MON810 maize, RR

soy, RR rape, potato “new leaf”. In addition DNA extracted from non GM-wheat and -rice was also tested.

None of the materials yielded detectable amplification, apart from the event DAS-59122-7.

3. Procedures

3.1 General instructions and precautions

- All handling of reagents and controls should occur in an ISO 9001:2000 or ISO 17025 environment or equivalent.
- The procedures require experience of working under sterile conditions.
- Laboratory organization, e.g. “flow direction” during PCR-setup, should follow the guidelines given by relevant authorities like e.g. ISO, CEN, Codex alimentarius commission.
- PCR-reagents shall be stored and handled in a separate room and freezer and in equipment where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment – especially pipettes.
- All the equipment used must be sterilized prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and shall not adsorb protein or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Use only powder-free gloves and change them frequently.
- Clean lab-benches and equipment periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - shall be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles, aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of DAS-59122-7 maize

3.2.1 General

The PCR set-up for the taxon specific target sequence (Hmg) and for the GMO (DAS-59122-7) 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 of 200 ng of template DNA per reaction well is recommended

The method is developed for a total volume of 25 µl per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

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

The calibration curves consist of four samples. The first point of the calibration curves is a 5% DAS-59122-7 in non-GM maize DNA for a total of 200 ng of DNA (corresponding to 73.394 maize genome copies with one genome assumed to correlate to 2.725 pg of haploid maize genomic DNA) (Arumuganathan & Earle, 1991).

A series of 1:5 dilutions down to 1.6 ng of total maize DNA/sample (S4) starting from S1 may be used.

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.

3.2.3 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 DAS-59122-7 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 reference Hmg specific system.

Component	Final concentration	µl/reaction
PCR buffer II 10x	1x	2.5
Rox Reference Dye (50x)	0.7x	0.35
Tween-20 1%	0.01%	0.25
Glycerol 20%	0.8%	1
dATPs (10 mM)	200 µM	0.5
dCTPs (10 mM)	200 µM	0.5
dGTPs (10 mM)	200 µM	0.5
dUTPs (20 mM)	400 µM	0.5
MgCl ₂ (100 mM)	5.5 mM	1.375
MaiJ-F2 primer (10 µM)	400 nM	1
mhmg-rev primer (10 µM)	400 nM	1
mhmg-probe (10 µM)	150 nM	0.375
Ampli Taq Gold (5U/µl)	0.04 U/µl	0.2
Nuclease free water	#	9.95
Template DNA (see 3.2.1 and 3.2.2)		(5)
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for DAS-59122-7 specific system.

Component	Final concentration	µl/reaction
PCR buffer II 10x	1x	2.5
Rox Reference Dye (50x)	0.7x	0.35
Tween-20 1%	0.01%	0.25
Glycerol 20%	0.8%	1
dATPs (10 mM)	200 µM	0.5
dCTPs (10 mM)	200 µM	0.5
dGTPs (10 mM)	200 µM	0.5
dUTPs (20 mM)	400 µM	0.5
MgCl ₂ (100 mM)	5.0 mM	1.25
DAS-59122-7-rb1f primer (10 µM)	250 nM	0.625
DAS-59122-7-rb1r primer (10 µM)	250 nM	0.625
DAS-59122-7-rb1s probe (10 µM)	200 nM	0.5
Ampli Taq Gold (5U/µl)	0.04 U/µl	0.2
Nuclease free water	#	10.7
Template DNA (see 3.2.1 and 3.2.2)		(5)
Total reaction volume:		25

- Mix gently and centrifuge briefly.
- Prepare two reaction tubes (one for the DAS-59122-7 and one for the Hmg master mix) for each DNA sample to be tested (reference curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of master mix (e.g. 20 x 3 = 60 µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 x 3 = 15 µl DNA for three PCR repetitions). Low-speed vortex each tubes at least three times for approx 30 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
- Spin down the tubes in a micro-centrifuge. Aliquot 25 µl in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g.

approximately 250 x *g* for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.

7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Reaction conditions.

Step	Stage	T °C	Time (sec)	Acquisition	Cycles
1	Initial denaturation	95 °C	600"	No	1x
2a	Denaturation	95 °C	15"	No	
2b	Amplification Annealing & Extension	60 °C	60"	Measure	50x

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. DAS-59122-7) 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 into an Excel file for further calculations.

3.4 Calculation of results

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

The standard curves are generated both for the Hmg and DAS-59122-7 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 DAS-59122-7 DNA in the unknown sample, the DAS-59122-7 copy number is divided by the copy number of the maize reference gene (Hmg) and multiplied by 100 to obtain the percentage value ($GM\% = \text{DAS-59122-7/Hmg} * 100$).

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 evaluating data after standard curve method (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml tubes

4.2 Reagents

(equivalents may be substituted)

- PCR buffer II 10x (Applied Biosystems Part No. N808-0019)
- $MgCl_2$ for molecular biology (SIGMA, Part No. M1028-1 ML)

- Rox (Invitrogen Part No 12223-012)
- Tween20 for molecular biology (SIGMA Part No P9416-50 ML)
- Glycerol for molecular biology (minimum 99%) (SIGMA Part No G5516-100 ML)
- dATP (Amersham-Pharmacia Part No 27-2050-02)
- dCTP (Amersham-Pharmacia Part No 27-2060-02)
- dGTP (Amersham-Pharmacia Part No 27-2070-02)
- dUTP (Amersham-Pharmacia Part No 27-2040-01)
- AmpliTaq Gold polymerase (Applied Biosystems Part No N8080240)
- TE-Buffer pH=8.0 (10/1 mM) (Appllichem Part No A2575,1000)
- Primers and probes (Metabion)

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
<i>DAS-59122-7 target sequence</i>	
DAS-59122-7-rb1f	5'- GGG ATA AGC AAG TAA AAG CGC TC -3'
DAS-59122-7-rb1r	5'- CCT TAA TTC TCC GCT CAT GAT CAG -3'
DAS-59122-7-rb1s Probe	6-FAM- TTT AAA CTG AAG GCG GGA AAC GAC AA-TAMRA-3'
<i>Reference gene Hmg target sequence</i>	
MaiJ-F2	5'- TTG GAC TAG AAA TCT CGT GCT GA -3'
mhmj-Rev	5'- GCT ACA TAG GGA GCC TTG TCC T -3'
Mhmj probe	6-FAM- CAA TCC ACA CAA ACG CAC GCG TA -TAMRA

5. References

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



EUROPEAN COMMISSION
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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

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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 *adh1*, a maize endogenous gene, using a pair of *adh1* gene-specific primers and an *adh1* 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 *adh1* 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 trial. 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 $\lambda = 260$ nm and $\lambda = 320$ 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 $\lambda = 260$ nm and $\lambda = 320$ nm. The reading is stable for at least 1 h.

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

Example for the test DNA solution: Mix 80 μ l of dilution buffer or water, 10 μ l of 2M sodium hydroxide solution, 10 μ l of DNA solution of unknown concentration and transfer to a 100 μ l 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 μ g/ml for single stranded DNA (denatured with sodium hydroxide).

Reliable measurements require OD values at $\lambda=260$ 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 (*Adh1*) 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 μ l 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.**

- In two reaction tubes (one for NK603 system and one for the *adh1* 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/*adh1* specific systems.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2X)	1x	25 µl
Primer NK603-F/ <i>adh1</i> -F	150 nM	-
Primer NK603-R/ <i>adh1</i> -R	150 nM	-
Probe NK603/ <i>adh1</i>	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

- Mix gently and centrifuge briefly.
- Prepare two 1.5 ml reaction tubes (one for the NK603 and one for the *adh1* master mix) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- 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.
- 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.
- Place the plate into the instrument.
- 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. *adh1*) 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 *adh1* 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 (*adh1*) and multiplied by 100 to obtain the percentage value ($GM\% = NK603/adh1 * 100$).

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 μ l), micro cuvettes (400 μ l), ultra micro cuvettes (100 μ l) and quartz capillaries (3 μ l to 5 μ l). 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')
<i>GMO target sequence</i>	
NK603 primer F	ATGAATGACCTCGAGTAAGCTTGTTAA
NK603 primer R	AAGAGATAACAGGATCCACTCAAACACT
NK603 probe	6-FAM- TGGTACCACGCGACACACTTCCACTC-TAMRA
<i>Reference gene target sequence</i>	
<i>Adh1</i> primer F	CCAGCCTCATGGCCAAAG
<i>Adh1</i> primer R	CCTTCTTGGCGGCTTATCTG
<i>Adh1</i> probe	6-FAM-CTTAGGGGCAGACTCCCCTGTTCCCT-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 ₂ EDTA	7.5 g

- Add 500 ml of sterile distilled water.
- Adjust pH to a value of 8.0 with 1M NaOH.
- 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

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

Store at 4° C for up to 6 months.

- **NaCl 1.2 M (100 ml)**

- Dissolve 7 g of NaCl in 100 ml sterile distilled water in a cylinder.
- Autoclave

Store at room temperature for up to 5 years

- **Ethanol-solution ~70 % (v/v) (100 ml)**

- 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)**

- 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)**

- Mix 100 ml of nuclease-free water, 2.5 ml of 1M Tris, pH 8.0 and 0.5 ml of 0.5M EDTA
- Adjust pH to 7.0 with HCl
- Adjust final volume to 250 ml with nuclease-free water
- Filter sterilise

Store at room temperature for up to 5 years

- **RNase A 10 mg/ml**

- Dissolve the RNase A at a final concentration of 10 mg/ml in sterile water.
- If indicated by supplier: boil the RNase A solution at 95°C for 15' to remove any residual nuclease activity.
- 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

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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 hybrid maize line 59122xNK603 (unique identifier DAS-59122-7xMON-ØØ6Ø3-6) which combines the 59122 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, Pioneer Hi-Bred Intl. Inc. provided the detection methods and the control samples (59122xNK603 ground maize flour, conventional ground maize flour and non genetically modified control DNA). 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.it/doc/Method%20requirements.pdf>) and to the validation results on the individual parental events (<http://gmo-crl.jrc.it/statusofdoss.htm>).

The results of this CRL-GMFF in-house verification studies are made publicly available at <http://gmo-crl.jrc.it/>.



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