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

Verification of Performances of MON 863 and NK603 Event-specific Methods on the Hybrid MON 863 x NK603 using Real-Time PCR

Validation Report

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

10 March 2006

Executive Summary

The JRC as Community Reference Laboratory (CRL) for the GM Food and Feed (see Regulation EC 1829/2003), has carried out an in-house verification study to assess the performance of two quantitative, event-specific methods, previously validated on the parental lines, to detect and quantify the MON 863 and the NK603 transformation events on seeds from the hybrid maize line combining the two thereof traits (unique identifier MON-00863-5xMON-00603-6). The study was conducted according to internationally accepted guidelines.

Monsanto Company provided the method-specific samples (seeds MON863xNK603 and null), whereas the JRC prepared the verification samples (calibration samples and blind samples at unknown GM percentage).

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 the validation results for the two parental lines (http://gmo-crl.jrc.it/statusofdoss.htm).

The results of in-house verification are publicly available under http://gmo-crl.jrc.it/.

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Document Approval		
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1. Introduction

The Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for the GM Food and Feed (see Regulation EC 1829/2003) carried out an in-house verification of the event-specific methods for the detection and quantification of MON 863 and NK603 in the hybrid maize line combining the two traits derived through traditional breeding techniques between progeny of MON 863 and NK603 maize. The single methods had been previously validated further to collaborative trial on the single parental lines (http://gmo-crl.jrc.it/statusofdoss.htm).

Upon reception of methods, samples and related data, the JRC carried out the scientific evaluation of documentation and the in-house testing of the methods, according to the requirements of Regulation (EC) 641/2004 and following its operational procedures.
The CRL method verification was carried out between September and October 2005.

Genomic DNA was extracted from wild type and MON863xNK603 maize seeds following the methods enclosed in the validated protocols for events MON863 and NK603 (http://gmo-crl.jrc.it/).

The operational procedure of the in-house verification comprised 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 event MON 863 and NK603 DNA to total maize DNA from the hybrid line. The MON 863 and NK603 events were quantified in reference to the maize endogenous system from gene *Adh1* (*Alcohol dehydrogenase-1*). The procedure is a simplex system.

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

- ✓ ISO 5725 (1994).
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995).

2. Materials

For the validation of the quantitative event-specific method, the MON863xNK603 genomic DNA was extracted from maize seeds, line DKC57-81 (GLP-0402-14658S), while the control DNA was extracted from non-GM maize seeds, line EXP258B (GLP-0402-14688-S).

Samples containing mixtures of 0% and 100% MON863xNK603 maize genomic DNA at different GMO concentrations were prepared by the JRC.

The protocols (reagents, concentrations, primer/probe sequences, amplification profile) used in the in-house verification are those already published as validated methods for the MON 863 and the NK603 event.

Table 1 shows the levels of unknown samples used in the verification of the MON 863 and NK603 methods on the hybrid DNA of MON863xNK603.

Table 1. GM contents in the unknown samples

MON 863 GM % (GM copy number/maize genome copy number *100)	NK603 GM % (GM copy number/maize genome copy number *100)
0.10	0.10
1.00	0.50
5.00	1.00
10.00	2.00
	5.00

3. Experimental design

Five runs for each method were carried out. In each run, samples were analyzed in parallel with both the GM-specific system and the reference system. Four GM levels in two replicate samples were examined per run for the MON 863 system: from 10,00% down to 0.10%; five GM levels in two replicate samples were tested for the NK603 system: from 5,00% to 0,10%. Each sample was analyzed in triplicate. On the whole, for each method (MON 863 and NK603), quantification of the GM levels was performed as an average of ten replicate samples/GM-level, each resulting from an average of three repetitions.
An internally validated Excel spreadsheet was used for the calculations of the GM% of all the samples.

4. Method

4.1 Description of the operational steps

For specific detection of event MON 863 genomic DNA, a 84-bp fragment of the region that spans the 5' insert-to-plant junction in maize event MON 863 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 specific detection of event NK603 genomic DNA, a 108-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5-'end and TAMRA as a quencher dye at its 3'-end.

For relative quantification of events MON 863 and NK603 DNA, a maize-specific reference system amplifies a 70-bp fragment of *Adh1* (alcohol dehydrogenase) a

maize endogenous gene, using a pair of *Adh1* gene-specific primers and an *Adh1* gene-specific probe labelled with FAM and TAMRA.

The standard curves are generated for the *Adh1* and the NK603 and MON 863 systems respectively, 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 MON 863 (or NK603) DNA in the unknown sample, the MON 863 (or 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% = GM-specific system/maize reference system * 100).

For detailed information on the preparation of standard curve calibration samples refer to the protocols of validated methods under http://gmo-crl.jrc.it/summaries/NK603-WEB-Protocol%20Validation.pdf and http://gmo-crl.jrc.it/summaries/MON863-Val-report_mm.pdf

5. Deviations reported

No deviation from the protocol of the two validated methods was introduced.

6. Summary of results

6.1. PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula ((10^(-1/slope))-1)*100] of the standard curves and of the R^2 (expressing the linearity of the regression) reported for both PCR systems in the five runs, are summarised in Tables 2 and 3.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 863 method on hybrid MON863xNK603

Run	MON 863			*Adh1*		
	Slope	PCR Efficiency (%)	Linearity (R2)	Slope	PCR Efficiency (%)	Linearity (R2)
1	-3.68	87.02	0.99	-3.22	95.78	0.97
2	-3.66	87.75	0.99	-3.42	96.21	0.98
3	-3.26	97.49	1.00	-3.27	97.77	0.97
4	-3.48	93.66	1.00	-3.24	96.42	0.97
5	-3.58	90.39	1.00	-3.34	99.22	0.97
Mean	**-3.53**	**91.26**	**0.99**	**-3.30**	**97.08**	**0.97**

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the NK603 method on hybrid MON863xNK603

Run	NK603			*Adh1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.64	88.09	0.99	-3.08	88.91	0.98
2	-3.66	87.48	1.00	-3.06	87.55	0.99
3	-3.97	78.71	1.00	-3.28	98.43	1.00
4	-3.87	81.24	1.00	-3.35	98.77	0.99
5	-3.82	82.69	0.99	-3.06	87.84	1.00
Mean	**-3.79**	**83.64**	**0.99**	**-3.17**	**92.30**	**0.99**

Data reported in Table 2 and 3 confirm the good performance characteristics of the method tested.

In fact, the R^2 value of the regression line for the MON 863 and NK603 method is above 0.99 and slightly lower (0.97) for the reference system of the MON863 method.

PCR efficiencies are above 90%, with the exception of the NK603 specific system (83.49%).

6.2. Method performance requirements

The results of the in-house verification for the MON 863 and for the NK603 methods are reported in Table 4. These are evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by CRL.

In table 4 estimates of both accuracy and precision for each GM level and for both methods are reported.

Table 4. Estimates of accuracy and precision for the MON863 and for the NK603 systems on maize MON863xNK603

MON863					
Unknown sample GM%	**Expected value (GMO %)**				
	0.10	**1.00**	**5.00**	**10.00**	
Mean	**0.11**	**1.25**	**5.04**	**11.59**	
SD	0.03	0.09	0.59	0.96	
RSDr%	29.51	7.48	11.70	8.32	
Bias%	6.30	25.22	0.81	15.91	
NK603					
Unknown sample GM%	**Expected value (GMO %)**				
	0.10	**0.50**	**1.00**	**2.00**	**5.00**
Mean	**0.15**	**0.49**	**1.25**	**1.84**	**5.51**
SD	0.02	0.07	0.08	0.22	0.27
RSDr%	13.74	13.29	6.25	11.98	4.81
Bias%	46.50	-1.14	25.34	-7.98	10.26

According to the ENGL acceptance criteria, the accuracy of the quantification, measured as bias from the accepted value, should be within 25% over the whole dynamic range, and the relative repeatability standard deviation, which measures the intra-laboratory variability, should lie within 25% at each GM-level.

The accuracy of the MON 863 method satisfies the ENGL requirements over the whole dynamic range, a border line accuracy (25.22%) being detected at the 1.00% GM-level; RSDr is acceptable at all GM-concentrations, with the minor exception of an RSDr (29.51%) slightly above the limit at the 0.1% level.

The accuracy of the NK603 method is acceptable over the dynamic range with the exception of a high bias (46.5%) at the lowest GM concentration and a border line bias (25.34%) at the 1.00% GM-level. The relative repeatability standard deviation (RSDr) is well within the limits set by the acceptance criteria in the NK603 method. On the whole, the two methods satisfy the acceptance criteria for CRL verification of GMO detection and quantification methods previously validated through collaborative trial on the parental maize lines.

6.3. Comparison of method performance between hybrid and parental lines

A synoptic comparison of the two method performances in the hybrid maize and parental lines respectively, is shown in Table 5 and 6.

The MON 863 method has similar performance characteristics in the stacked product as in the parental line, as evaluated by checking both accuracy and precision of the method in respect of the ENGL minimum acceptance criteria.

Table 5. Comparison of accuracy and precision of MON 863 method in the hybrid and parental line

Accuracy and precision of MON863 quantitation in hybrid MON863xNK603			**Accuracy and precision of MON863* quantitation in parental line MON863**		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
-	-	-	**0.00**	0.00	0.00
0.10	6,30	29.51	**0.10**	28.00	34.51
1.00	25.22	7.48	**1.00**	20.20	17.43
5.00	0.81	11.70	**5.00**	-0.12	10.13
10.00	15.91	8.32	**10.00**	-5.56	12.80

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

Table 6. Comparison of accuracy and precision of NK603 method in the hybrid and parental line

Accuracy and precision of NK603 quantitation in hybrid MON863xNK603			Accuracy and precision of NK603 quantitation in parental line NK603*		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.10	46.50	13.74	**0.10**	83.00	24.25
0.50	-1.14	13.29	**0.49**	72.86	15.24
1.00	25.34	6.25	**0.98**	46.50	17.16
2.00	-7.98	11.98	**1.96**	14.03	7.69
5.00	10.26	4.81	**4.91**	22.08	21.63

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

The NK603 method shows better performances when tested on the hybrid DNA as compared to the performances displayed on the parental line in terms of accuracy of quantitation, and shows comparable relative repeatability standard deviations at all GM-levels. Therefore, the in-house method verification has demonstrated that the MON 863 and the NK603 methods can be equally applied in quantitation of the respective events in the hybrid maize product.

7. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (available under http://gmo-crl.jrc.it). The method acceptance criteria were reported by the applicant and used to evaluate the method prior the in-house verification.

The results obtained during the present study indicate that the methods validated on the parental GM-lines show a comparable performance when applied to the material combining the two traits.

8. References

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

International Standard (ISO) 5725. 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Swizerland.