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, MON 810, and NK603 Event-specific Methods on the Hybrid MON 863 x MON 810 x NK603 using Real-Time PCR

Validation Report

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

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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 three quantitative, event-specific methods, previously validated on the parental lines, to detect and quantify the MON 810, MON 863 and the NK603 transformation events on DNA from the hybrid maize line combining the three thereof traits (unique identifier MON-00863-5 x MON-00810-6 x MON-00603-6). The study was conducted according to internationally accepted guidelines.

Monsanto Company provided the method-specific samples (seeds MON 863 x MON 810 x NK603 and null), whereas the JRC extracted the DNA and 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 three 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 810, MON 863 and NK603 in the hybrid maize line combining the three traits derived through traditional breeding techniques. 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 from wild type and from the maize line MON 863 x MON 810 x NK603 was extracted following the methods enclosed in the validated protocols for events MON 810, MON 863 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 three event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of event MON 810, MON 863 and NK603 DNA to total maize DNA from the hybrid line. The MON 810 event was quantified in reference to a maize endogenous system obtained from a *hmg* gene (high mobility group). The MON 863 and the NK603 event 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 methods, the MON 863 x MON 810 x NK603 genomic DNA was extracted from maize seeds (lot number GLP-0405-15138-S) with background genetics of TPA461-HE, while the control DNA was extracted from non-GM maize seeds (lot number GLP-0402-14688-S) with background genetics of EXP258B.

Samples containing mixtures of 0% and 100% MON 863 x MON 810 x NK603 maize genomic DNA at different GMO concentrations were prepared by the JRC.

The protocols (reagents, concentrations, primer/probe sequences, amplification profile) used in the verification are those already published as validated methods for the MON 810, MON 863 and the NK603 events.

Table 1 shows the five levels of unknown samples used in the verification of the MON 810, MON 863 and NK603 methods on the hybrid DNA, MON 810 x MON 863 x NK603.

Table 1. GM contents in the unknown samples

MON 810 GM % (GM copy number/maize genome copy number *100)	MON 863 GM % (GM copy number/maize genome copy number *100)	NK603 GM % (GM copy number/maize genome copy number *100)
0.1	0.1	0.1
0.5	1.0	0.5
1.0	5.0	1.0
2.0	10.0	2.0
5.0		5.0

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. Five GM-levels were examined per run (from 5.00% down to 0.10%) in two replicate samples for methods MON 810 and NK603. Four GM levels were examined per run (from 10,00 down to 0,1%) in two replicate samples for method MON 863. Each sample was analyzed in triplicate. On the whole, for each method (MON 810, 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. Methods

4.1 Description of the operational steps

For specific detection of event MON 810 genomic DNA, a 92-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 event MON 810 DNA, a maize-specific reference system amplifies a 79-bp fragment of *Hmg* (high mobility group) a maize endogenous gene, using a pair of *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM and TAMRA.

For specific detection of event MON 863 genomic DNA, an 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 event MON 863 and 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.

The standard curves are generated both for the *hmg* and MON 810 system as well as for the *Adh1* and the MON 863 and the NK603 system 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 810 (MON 863 or NK603) DNA in the unknown sample, the MON 810 (MON 863 or NK603) copy number is divided by the copy number of the maize reference gene *hmg* (or *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 at http://gmo-crl.jrc.it/statusofdoss.htm

5. Deviations reported

No deviation from the protocol of the three 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, is summarized in Table 2, 3 and 4.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 810 method on hybrid MON 863 x MON 810 x NK603

Run	MON 810			Hmg		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.49	93.38	0.99	-3.22	95.34	1.00
2	-3.35	98.66	1.00	-3.17	93.34	0.99
3	-3.39	97.07	0.99	-3.19	94.24	0.99
4	-3.38	97.65	0.99	-3.13	91.52	1.00
5	-3.40	96.77	0.99	-3.45	95.02	0.99
Mean	-3.40	96.71	0.99	-3.23	93.89	0.99

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 863 method on hybrid MON 863 x MON 810 x NK603

Run	MON863			*Adh1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.49	93.36	1.00	-3.22	95.42	0.99
2	-3.48	93.85	1.00	-3.25	96.98	1.00
3	-3.86	81.54	1.00	-3.57	90.69	0.97
4	-3.48	93.71	1.00	-3.57	90.57	0.96
5	-3.53	92.11	1.00	-3.50	92.93	0.97
Mean	-3.57	90.91	1.00	-3.42	93.32	0.98

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the NK603 method on hybrid MON 863 x MON 810 x NK603

Run	NK603			*Adh1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.54	91.64	1.00	-3.23	96.14	0.99
2	-3.47	94.30	1.00	-2.99	84.24	0.99
3	-3.59	89.83	1.00	-3.41	96.62	1.00
4	-3.55	91.12	0.99	-3.24	96.64	099
5	-3.56	91.09	0.99	-3.13	91.21	1.00
Mean	-3.54	91.60	0.99	-3.20	92.97	1.00

Data reported in Table 2, 3 and 4 confirm the good performance characteristics of the tested methods.

In fact, the R^2 value of the regression line for the MON 810, MON 863 and NK603 method is above 0.99.
PCR efficiencies are constantly above 90%.

6.2. Method performance requirements

The results of the in-house verification for the MON 810, MON 863 and NK603 methods are reported in Table 5. These are evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by CRL.
In Table 5 estimates of both accuracy and precision for each GM-level and for both methods are reported.

Table 5. Estimates of accuracy and precision for the MON 810, MON 863 and for the NK603 systems on maize MON 863 x MON 810 x NK603

MON 810					
	Expected value (GMO %)				
Unknown sample GM%	**0.10**	**0.50**	**1,00**	**2,00**	**5,00**
Mean	**0.08**	**0.38**	**0.87**	**1.75**	**4.52**
SD	0.02	0.07	0.08	0.17	0.29
RSDr%	21.13	18.45	9.46	9.88	6.41
Bias%	-24.00	-24.38	-12.64	-12.54	-9.54
MON 863					
	Expected value (GMO %)				
Unknown sample GM%	**0.10**	**1.00**	**5.00**	**10.00**	
Mean	**0.11**	**1.22**	**5.70**	**11.91**	
SD	0.03	0.32	0.70	1.05	
RSDr%	26.65	26.11	12.33	8.79	
Bias%	11.80	22.27	14.05	19.07	
NK603					
	Expected value (GMO %)				
Unknown sample GM%	**0.10**	**0.50**	**1.00**	**2.00**	**5.00**
Mean	**0.11**	**0.46**	**0.87**	**1.91**	**4.13**
SD	0.02	0.07	0.16	0.31	0.46
RSDr%	18.70	15.34	18.24	16.30	11.21
Bias%	8.30	-7.96	-13.28	-4.56	-17.36

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. MON 810, MON 863 and NK603 methods fully satisfy this requirement over the whole dynamic range, the highest bias being around -24% for the 0.10% and 0.50% levels of the MON 810 method.

According to the ENGL acceptance criteria, the relative repeatability standard deviation (RSDr), which measures the intra-laboratory variability-, should lie within 25% at each GM-level.

This parameter is well within the limits set by the acceptance criteria for the three methods. Only a minor deviation can be detected at the 0.10 and 1.00% level of the MON 863 method where RSDr is around the 26%.

On the whole, the three methods, when tested on the hybrid maize MON863 x MON 810 x NK603 , fully 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 stack and parental lines

A synoptic comparison of the three method performances on the hybrid maize and parental lines respectively, is shown in Table 6, 7 and 8.

The MON 810, MON 863 and NK603 methods display similar performance characteristics on the hybrid product as on the parental line, as evaluated by checking both bias and RSDr. MON 810 method shows a greater bias on the hybrid than on the parental line, at the lowest GM-levels, which are however within the limits set by the ENGL minimum acceptance criteria.

Table 6. Comparison of accuracy and precision of MON810 method in the hybrid and parental line

Accuracy and precision of MON 810 quantitation in hybrid MON 863 x MON 810 x NK603			**Accuracy and precision of MON 810 quantitation in parental line MON 810***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
-	-	-	**<0.02**	>40.00	26.27
0.10	-24.00	21.13	**0.10**	2.30	35.60
0.50	-24.38	18.45	**0.50**	-7.74	20.82
1.00	-12.64	9.46	**1.00**	-16.73	16.51
2.00	-12.54	9.88	**2.00**	-10.93	15.93
5.00	-9.54	6.41	**5.00**	-9.69	28.65

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

Table 7. Comparison of accuracy and precision of MON863 method in the hybrid and parental line

Accuracy and precision of MON 863 quantitation in hybrid MON 863 x MON 810 x NK603			Accuracy and precision of MON863 quantitation in parental line MON 863*		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
			0.00	0.00	0.00
0.10	11.80	26.65	**0.10**	28.00	34.51
1.00	22.27	26.11	**1.00**	20.20	17.43
5.00	14.05	12.33	**5.00**	-0.12	10.13
10.00	19.07	8.79	**10.00**	-5.56	12.80

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

Table 8. Comparison of accuracy and precision of NK603 method in the hybrid and parental line

Accuracy and precision of NK603 quantitation in hybrid MON 863 x MON 810 x NK603			Accuracy and precision of NK603 quantitation in parental line NK603*		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.10	8.30	18.70	**0.10**	83.00	24.25
0.50	-7.96	15.34	**0.49**	72.86	15.24
1.00	-13.28	18.24	**0.98**	46.50	17.16
2.00	-4.56	16.30	**1.96**	14.03	7.69
5.00	-17.36	11.21	**4.91**	22.08	21.63

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

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 to 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 three 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.