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COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Verification of Performances of MON 863 and MON8 10 Event-specific Methods on the Hybrid MON 863 x MON 810 using Real-Time PCR

Validation Report

Biotechnology & GMOs Unit
Institute for Health and Consumer Protection
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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 two quantitative, event-specific methods, previously validated on the parental lines, to detect and quantify the MON863 and the MON810 transformation events on seeds from the hybrid maize line combining the two thereof traits (unique identifier MON-00863-5xMON-00810-6). The study was conducted according to internationally accepted guidelines.

Monsanto Company provided the method-specific samples (seeds MON863xMON810 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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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 MON 810 in the hybrid maize line combining the two traits derived through traditional breeding techniques between progeny of MON863 and MON810 maize. The single methods had been previously validated through collaborative trial on the parental lines of the hybrid (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 January 2005 and January 2006.

Genomic DNA was extracted from wild type and MON863xMON810 maize seeds following the methods enclosed in the validated protocols for events MON863 and MON810 (http://gmo-crl.jrc.it/statusofdoss.htm).

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 MON 810 DNA to total maize DNA from the stacked line. The MON 863 event was quantified in reference to the maize endogenous system from gene *Adh1* (*Alcohol dehydrogenase-1*). The MON810 event was quantified in reference to a maize endogenous system obtained from a *hmg* gene (high mobility group). 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 MON863xMON810 genomic DNA was extracted from maize seeds, line DKC60-14 (GLP-0401-14535S), while the control DNA was extracted from non-GM maize seeds, line EXP258B (GLP-0402-14688-S) and line EXP258B (GLP-0307-14210S-S) and used for standards and "unknown" samples of MON 863 and MON 810 method, respectively.
Samples containing mixtures of 0% and 100% MON810xNK603 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 810 and the NK 603 event.

Table 1 shows the levels of unknown samples used in the verification of the MON 863 and MON 810 methods on the hybrid DNA, MON863xMON810

Table 1. GM contents in the unknown samples

MON 863 GM % **(GM copy number/maize genome copy number *100)**	MON 810 GM % **(GM copy number/maize genome copy number *100)**
0.1	0.1
1.0	0.5
5.0	1.0
10.0	2.0
	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. 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 MON 810 system: from 5.00% to 0.10%. Each sample was analyzed in triplicate. On the whole, for each method (MON 863 and MON 810), 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, 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 relative quantification of event MON 863 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.

For specific detection of event MON810 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.

The standard curves are generated both for the *Adh1* and the MON 863 system as well as for the *hmg* and MON810 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 863 (or MON 810) DNA in the unknown sample, the MON 863 (or MON 810) copy number is divided by the copy number of the maize reference gene *Adh1* (or *Hmg*) 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 at http://gmo-crl.jrc.it/statusofdoss.htm

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, is summarised in Table 2 and 3.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 863 method on hybrid MON863xMON810

Run	MON 863			*Adh1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.36	98.57	1.00	-3.19	94.24	0.99
2	-3.40	96.97	1.00	-3.07	88.17	0.99
3	-3.41	96.45	1.00	-3.12	90.94	0.99
4	-3.47	94.30	1.00	-3.05	87.28	0.99
5	-3.57	90.54	1.00	-3.17	93.22	1.00
Mean	**-3.44**	**95.36**	**1.00**	**-3.12**	**90.77**	**0.99**

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 810 method on hybrid MON863xMON810

Run	MON810			*Hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.42	96.11	0.99	-3.18	93.85	1.00
2	-3.04	86.98	0.99	-2.99	83.74	0.99
3	-3.16	92.68	1.00	-3.08	88.58	1.00
4	-3.31	99.40	0.99	-3.14	91.95	1.00
5	-3.41	96.46	0.99	-3.14	92.02	1.00
Mean	**-3.27**	**94.33**	**0.99**	**-3.11**	**90.03**	**1.00**

Data reported in Tables 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 the MON 810 methods is above 0.99 and PCR efficiencies are constantly above 90%.

6.2. Method performance requirements

The results of the in-house verification for the MON 863 and for the MON 810 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 MON 863 and for the MON 810 systems on maize hybrid MON863xMON810

MON 863				
Unknown sample GM%	**Expected value (GMO %)**			
	0.10	**1.00**	**5.00**	**10.00**
Mean	**0.12**	**1.14**	**5.86**	**9.88**
SD	0.018	0.189	0.407	0.991
RSDr%	14.61	16.63	6.94	10.03
Bias%	20.25	13.85	17.23	-1.18

MON 810					
Unknown sample GM%	**Expected value (GMO %)**				
	0.10	**0.50**	**1.00**	**2.00**	**5.00**
Mean	**0.09**	**0.42**	**0.83**	**1.63**	**4.29**
SD	0.02	0.08	0.10	0.15	0.16
RSDr%	27.99	18.28	11.60	9.40	3.73
Bias%	-14.60	-15.06	-17.13	-18.32	-14.30

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 be within 25% at each GM-level.

Both methods fully satisfy this requirement over the whole dynamic range, showing bias values within the set limits.

The relative repeatability standard deviation (RSDr) is definitely within the limits set by the acceptance criteria for both the MON 810 and in the MON 863 methods, with the minor exception of an RSDr of 27.99% for the MON 810 method at 0.1% GM-content.

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.

When tested on the hybrid line, the MON 863 method shows performances comparable to those displayed on the parental line in terms of accuracy and precision of quantitation. The MON 810 method has similar performance characteristics on the hybrid product as on the parental line, as evaluated by checking both accuracy and precision of the method with respect of the ENGL minimum acceptance criteria.

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

Accuracy and precision of MON863 quantitation in MON863xMON810			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	20.25	14.61	**0.10**	28.00	34.51
1.00	13.85	16.63	**1.00**	20.20	17.43
5.00	17.23	6.94	**5.00**	-0.12	10.13
10.00	-1.18	10.03	10.00	-5.56	12.80

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

Table 6. Comparison of accuracy and precision of MON 810 method on the hybrid and on parental line

Accuracy and precision of MON 810 quantitation in stack MON863xMON810			Accuracy and precision of MON 810 quantitation in parental line MON810*		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
-	-	-	**<0,02**	>40,00	26.27
0.10	-14.60	27.99	**0.10**	2.30	35.60
0.50	-15.06	18.28	**0.50**	-7.74	20.82
1.00	-17.13	11.60	**1.00**	-16.73	16.51
2.00	-18.32	9.40	**2.00**	-10.93	15.93
5.00	-14.30	3.73	**5.00**	-9.69	28.65

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

Therefore, the in-house method verification has demonstrated that the MON863 and the MON810 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 by 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.