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

Event-specific method for the quantitation of maize 1507xNK603 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 two quantitative, event-specific methods, previously validated on the parental lines, to detect and quantify the TC1507 and the NK603 maize transformation events on flour from the "stacked" maize line combining the two thereof traits (unique identifiers DAS-01507-1 and MON-00603-6). The study was conducted according to internationally accepted guidelines.

Pioneer Hi-Bred International provided the method-specific samples (semi-ground seeds 1507xNK603 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 1507 and NK603 in the stacked maize line combining the two traits derived through traditional breeding techniques between progeny of 1507 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 in February-March 2005.

Genomic DNA from wild type maize and from the maize line 1507xNK603 was extracted following the methods enclosed in the validated protocols for events 1507 and NK603 respectively (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 1507 and NK603 DNA to total maize DNA from the stacked line. The 1507 event was quantified in reference to a maize endogenous system obtained from a *hmg* gene (high mobility group). The NK603 event was 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 1507xNK603 genomic DNA was extracted from maize seeds, lot PIV1PNE11040-00, while the control DNA was extracted from a non-GM maize line (Lot Number PIP20LBN).
Samples containing mixtures of 0% and 100% 1507xNK603 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 1507 and for NK603 events.

Table 1 shows the five levels of unknown samples used in the verification of the 1507 and NK603 methods on genomic DNA of 1507xNK603

Table 1. GM contents in the unknown samples

1507 GM % (GM copy number/maize genome copy number *100)	NK603 GM % (GM copy number/maize genome copy number *100)
0.10	0.10
0.50	0.50
0.90	1.00
2.00	2.00
5.00	5.00

3. Experimental design

Five experiments 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 in two replicate samples. Each sample was analyzed in triplicate. On the whole, for each method (1507 and NK 603), quantification of the five GM levels was performed as an average of ten replicate samples/GM level, each resulting from an average of three repetitions. The 5.0% sample analysed with the NK603 method was quantified according to eleven measurements.
An internally validated Excel spreadsheet was used for the calculations of the GM% of all samples.

4. Method

4.1 Description of the operational steps

For specific detection of event 1507 genomic DNA, a 58-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 1507 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 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 NK603 DNA, a maize-specific reference system amplifies a 70-bp fragment of *Adh1* (alcohol dehydrogenase 1), 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 1507 system as well as for the *Adh1* 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 1507 (or NK603) DNA in the unknown sample, the 1507 (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 http://gmo-crl.jrc.it/summaries/NK603-WEB-Protocol%20Validation.pdf and http://gmo-crl.jrc.it/summaries/TC1507-WEB-Protocol-Validation.pdf.

5. Deviations reported

No deviation from the protocols 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 1 and 2.

Table 1. Values of standard curve slope, PCR efficiency and linearity (R^2) for the TC1507 method on 1507xNK603

Run	1507			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3,14	91,79	0,99	-3,22	95,64	1,00
2	-3,10	89,96	0,99	-3,18	93,95	1,00
3	-3,16	92,66	1,00	-3,18	93,56	1,00
4	-3,19	94,02	0,99	-3,19	93,95	1,00
5	-3,09	89,55	0,99	-3,18	93,83	1,00
Mean	-3,14	91,60	0,99	-3,19	94,18	1,00

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the NK603 method on 1507xNK603

Run	NK603			*Adh1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-4,03	76,95	0,99	-3,39	97,38	1,00
2	-3,82	82,84	1,00	-3,40	96,86	1,00
3	-3,73	85,40	1,00	-3,35	98,98	1,00
4	-3,85	81,88	0,99	-3,28	101,88	1,00
5	-3,84	82,04	0,99	-3,27	102,22	1,00
Mean	-3,85	81,82	0,99	-3,34	99,46	1,00

Data reported in Table 1 and 2 confirm the good performance characteristics of the method tested.

In fact, the R^2 value of the regression line for the 1507 method is above 0.99; similarly the R^2 value of the regression line for the NK603 system is close to 1.00.

PCR efficiencies are above 90%, with the exception of the NK603 specific system (81.8%).

6.2. Method performance requirements

The results of the in-house verification for the 1507 and NK603 methods are reported in Table 3. These are evaluated with respect to the method acceptance criteria and performance requirements, as established by ENGL and adopted by CRL.

In table 3 estimates of both accuracy and precision for each GM level and for both methods are reported.

Table 3. Estimates of accuracy and precision for the 1507 and NK603 systems on maize 1507xNK603

1507					
Unknown sample GM%	**Expected value (GMO %)**				
	0,10	**0,50**	**0,90**	**2,00**	**5,00**
Mean	**0,074**	**0,41**	**0,88**	**2,08**	**5,51**
SD	0,01	0,04	0,11	0,19	0,27
RSDr (%)	13,14	10,75	12,37	9,33	4,94
Bias%	-26,39	-17,73	-1,73	3,80	10,29
NK603					
Unknown sample GM%	**Expected value (GMO %)**				
	0,10	**0,50**	**1,00**	**2,00**	**5,00**
Mean	**0,09**	**0,46**	**0,89**	**1,57**	**5,37**
SD	0,01	0,10	0,14	0,24	0,87
RSDr%	16,24	21,69	15,67	15,24	16,12
Bias%	-9,30	-8,60	-11,11	-21,73	7.37

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.

As shown in Table 3, it can be observed that the accuracy of the DAS1507 system is acceptable over the whole dynamic range, with a minor deviation at the lowest GM level, namely 0.10%. The relative repeatability standard deviation (RSDr) is well within the limits set by the acceptance criteria. Similarly, the RSDr of the NK603 system is definitely acceptable at all tested GM concentrations and its bias maintains below the 25% at each GM

concentration showing a maximum (-21.73%) at the 2.00% level. Thus, the two methods 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 stacked and parental line

A synoptic comparison of the two method performances in the stacked maize and parental lines respectively, is shown in Table 4 and 5.

The 1507 method has similar performance characteristics in the stacked product as in the parental line with a minor exception at the 0.10% level, as evaluated by checking both accuracy and precision of the method in respect of the ENGL minimum acceptance criteria.

Table 4. Comparison of accuracy and precision of DAS1507 method in the stacked and parental line

Accuracy and precision of 1507 quantitation in 1507xNK603			Accuracy and precision of 1507 quantitation in parental line 1507*		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
-	-	-	**0.00**	0.00	0.00
0,10	-26,39	13,14	**0.10**	6.00	18.11
0,50	-17,73	10,75	**0.50**	-4.00	11.70
0.90	-1,73	12,37	**0.90**	3.70	7.68
2,00	3,80	9,33	**2.00**	-1.70	8.48
5,00	10,29	4,94	**5.00**	8.40	14.41

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

Table 5. Comparison of accuracy and precision of NK603 method in the stacked and parental line

Accuracy and precision of NK603 quantitation in 1507xNK603			Accuracy and precision of NK603 quantitation in parental line NK603*		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0,10	-9,30	16,24	**0.10**	83.00	24.25
0,50	-8,60	21,69	**0.49**	72.86	15.24
1,00	-11,11	15,67	**0.98**	46.50	17.16
2,00	-21,73	15,24	**1.96**	14.03	7.69
5,00	6,28	16,07	**4.91**	22.08	21.63

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

The NK603 method shows better performances in the stacked product as compared to the performances displayed on the parental line in terms of accuracy of quantitation. Therefore, the in-house method verification has demonstrated that the 1507 and the NK603 methods can be equally applied in quantitation of the respective events in the stacked 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

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