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Report on the Verification of the Performance of 1507, 59122, MON 810 and NK603 Event-specific PCR-based Methods Applied to DNA Extracted from Stack Maize 1507 x 59122 x MON 810 x NK603

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Report on the Verification of the Performance of 1507, 59122, MON 810 and NK603 Event-specific PCR-based Methods Applied to DNA Extracted from Stack Maize 1507 x 59122 x MON 810 x NK603

13 November 2013

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Pioneer Overseas Corporation to request the authorization of the genetically modified maize stack 1507 x 59122 x MON 810 x NK603, resistant against certain lepidopteran pests, protected against corn rootworm larvae, and glufosinate-ammonium and glyphosate tolerant, and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, and import and processing, in accordance with articles 5 and 17 of Regulation (EC) N° 1829/2003 GM Food and GM Feed. The unique identifier assigned to 1507 x 59122 x MON 810 x NK603 maize is DAS-Ø15Ø7-1xDAS-59122-7xMON-ØØ81Ø-6xMON-ØØ6Ø3-6.

The genetically modified maize line 1507 x 59122 x MON 810 x NK603 has been obtained by conventional crossing of four genetically modified single maize events: 1507, 59122, MON 810 and NK603 without any new genetic modification.

The EU-RL GMFF has previously validated, and declared fit for purpose, the detection methods for the single events 1507, 59122, MON 810 and NK603 (see: http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EU-RL GMFF therefore has carried out only an in-house verification of the performance of each validated method when applied to DNA extracted from 1507 x 59122 x MON 810 x NK603.

The herewith reported in-house verification study lead to the conclusion that the individual methods meet the ENGL performance criteria also when applied to DNA extracted from the GM maize stack 1507 x 59122 x MON 810 x NK603.

This report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

Quality assurance

The EU-RL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172 (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EU-RL GMFF quality system.

The EU-RL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EU-RL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection (IHCP) provided by CERMET.

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Content

1. Introduction

The EU legislative system [1, 2] for genetically modified food and feed provides that any GMO for food and feed use shall undergo an authorisation process before it can be placed on the EU market. This holds true also for a GMO containing more than one single GM event obtained by conventional crossing, co-transformation or re-transformation (genetically modified stack).

Consequently, the application for authorisation of a GMO shall be accompanied, among others, by an event-specific method for detection, identification and quantification for each GM event composing the stack, and by samples of the stack and food and feed derived from it. The EU-RL GMFF shall validate the event specific methods of detection proposed by the applicant with regard to their performance when applied to DNA extracted from the stack, and shall report to the European Food Safety Authority, who will include the EU-RL GMFF report in the overall Opinion concerning the risk assessment and potential authorization of the assessed stack.

Upon reception of methods, samples and related data (step 1), the EU-RL GMFF carried out the assessment of the documentation (step 2) and the in-house verification of the methods (step 3) according to the requirements of Regulation (EC) No 641/2004 (Annex I)

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements and to the validation results on the individual events.

2. Step 1 (dossier reception and acceptance)

Pioneer Overseas Corporation submitted the detection methods, data demonstrating their adequate performance, and as corresponding control samples DNA extracted from the GM maize stack 1507 x 59122 x MON 810 x NK603, and from conventional non GM maize.

The dossier was found to be complete and thus was moved to step 2.

3. Step 2 (scientific dossier assessment)

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL [3] and with regard to their documentation and reliability.

Four requests for complementary information regarding methods, control samples, DNA sequences and experimental design were addressed to and adequately responded to by the applicant.

In the dossier, the applicant reported that for 3 of the stacked events the previously validated methods were used without modifications. Only the proposed NK603 method is using a different reference system (see Section 5.5 for details) in comparison to the previously validated method. For all four methods the applicant submitted data showing that the ENGL method acceptance criteria were met also when applied to DNA extracted from the stack.

The EU-RL GMFF verified the data and the complementary information received and concluded that the data were reliable and seemed to confirm that the methods meet the ENGL method performance criteria [3], i.e. PCR efficiency, linearity, repeatability and trueness. Based on the considerations above, the dossier was moved to step 3.

4. Step 3 (EU-RL GMFF experimental testing)

In step 3 the EU-RL GMFF implemented all 4 methods in its own laboratory and performed a verification of their key performance parameter when applied to DNA extracted from GM-maize stack 1507 x 59122 x MON 810 x NK603.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from seeds of the GM-maize stack 1507 x 59122 x MON 810 x NK603
- genomic DNA extracted from ground non GM (conventional) maize seeds.

Test samples containing mixtures of GM maize stack 1507 x 59122 x MON 810 x NK603 and non GM maize genomic DNA at different target GMO concentrations were prepared by the EU-RL GMFF.

The protocols (reagents, concentrations, primer/probe sequences) described by the applicant were implemented precisely in the EU-RL GMFF laboratory. For GM event 1507, 59122 and MON 810 followed exactly those already published as validated methods for the individual 1507, 59122, MON 810 single-line events (available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). For NK603, the previously published method as well as the modified version were implemented in order to verify the impact of the proposed change of the reference system.

Table 1 shows the five GM levels used in the verification of the 1507, 59122, MON 810 and NK603 methods. It should be noted that slight deviations of the concentrations were introduced to avoid unintentional biases.

Table 1. Percentage of 1507, 59122, MON 810 and NK603 in 1507 x 59122 x MON 810 x NK603 in the verification samples

1507 GM% (GM DNA / total maize DNA x 100)	**59122 GM%** (GM DNA / total maize DNA x 100)	**MON 810 GM%** (GM DNA / total maize DNA x 100)	**NK603 GM%** (GM DNA / total maize DNA x 100)
0.1	0.1	0.1	0.1
0.5	0.4	0.5	0.5
0.9	0.9	1.0	1.0
2.0	2.0	2.0	2.0
5.0	4.5	5.0	5.0

4.2 DNA extraction

A method for DNA extraction from maize seeds was previously evaluated by the EU-RL GMFF with regard to its performance characteristics and was considered valid, i.e. fit for the purpose of providing maize-DNA of appropriate quality and amount for being used in subsequent PCR experiments. The protocol for the DNA extraction method is available at http://gmo-crl.jrc.ec.europa.eu/summaries/59122_DNAExtr_sampl.pdf.

Consequently, the EU-RL GMFF did not verify the DNA extraction method proposed by the applicant.

4.3 Experimental design

Eight PCR runs for each of the four methods were carried out. In each run, samples were analysed in parallel with both the GM specific system and the reference system *hmg* (*high mobility group*). Five GM levels were examined per run, for each GM level in duplicate. In total, for each method (1507, 59122, MON 810 and NK603), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level (8 runs x 2 replicated levels per run). An Excel spreadsheet was used for determination of GM%.

4.4 PCR methods

During the verification study, the EU-RL GMFF carried out parallel tests on DNA extracted from GM-maize stack 1507 x 59122 x MON 810 x NK603 using the methods previously validated for the respective single line GM-events 1507, 59122, MON 810 and NK603.

For the detection of GM-maize events 1507, 59122, MON 810 and NK603, DNA fragments of 58-bp, 86-bp, 92-bp and 108-bp respectively are amplified using specific primers. PCR products are measured during each cycle (real-time) by means of target-specific oligonucleotide probes 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 quantification of GM Maize events 1507, 59122, MON 810 and NK603, a taxon-specific reference system amplifies a 79-bp fragment of *hmg* (*high mobility group*) maize endogenous gene (AJ131373.1) using two *hmg* gene-specific primers and one *hmg* gene-specific probe labelled with FAM and TAMRA.

Standard curves are generated both for the respective GM event (1507, 59122, MON 810 and NK603) and the reference (*hmg*) specific system by plotting Ct values of the calibration standards against the logarithm of the DNA copy numbers of 1507, 59122, MON 810 and NK603 events, respectively, and fitting a linear regression into these data. Thereafter, the normalised Ct values of the unknown samples are measured and the amount of DNA of 1507, 59122, MON 810 and NK603 events, respectively, is estimated using the regression formula.

For detailed information on the preparation of the respective standard curve calibration samples please refer to the protocols of the validated methods at http://gmo-crl.jrc.ec.europa.eu/.

4.5 Deviations from the validated single-line methods

The *adh1* reference system originally validated for the quantification of NK603 single event was shown to target a DNA region harbouring a SNP (single nucleotide polymorphism) in the annealing site of the *adh1* primer F. The presence of this SNP was shown to be related to a quantification bias, depending on the maize variety and on the calibrant used [(6)].

Therefore the applicant proposed to use the *hmg* reference system. Accordingly the EU-RL GMFF also implemented this variation and verified the performance of the method for relative quantification of GM maize NK603, which was previously validated by the EU-RL GMFF for GM maize MON89034, published at http://gmo-crl.jrc.ec.europa.eu/summaries/MON89034_validated_Method.pdf.

The performance of the NK603 method was also verified in combination with the validated *hmg* reference system described in the validation of GM maize MON87460 (http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27_MON87460_validated_Method.pdf) because, although the target sequence and the primers used are the same as those validated by the EU-RL GMFF for maize MON 89034, some modifications in the PCR conditions were introduced.

These modifications concern the use of a 25 µL final volume of reaction and the use of a different master mix reagent; consequently, the reaction volume of the NK603 event-specific system was also adjusted to 25 µL to have the same reaction volume for both the reference and the event-specific systems, while maintaining the validated concentrations of NK603 primers and probe in reaction. The amplification mixtures tested for the verification of NK603 method are described in Annex I to this report.

4.6 Results

The values of the slopes of the standard curves established by the EU-RL GMFF, from which the PCR efficiency is calculated using the formula $[10^{(-1/slope)} - 1] \times 100$, and of the R^2 (expressing the linearity of the regression) reported for all PCR systems in the eight runs, are presented in Tables 2, 3, 4, 5, 6 and 7 for GM-maize events 1507, 59122, MON 810 and NK603, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 1507 method on 1507 x 59122 x MON 810 x NK603

Run	1507			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.49	93	0.98	-3.41	96	1.00
2	-3.35	99	1.00	-3.43	96	1.00
3	-3.52	92	0.97	-3.31	100	1.00
4	-3.19	106	0.98	-3.44	95	1.00
5	-3.23	104	0.98	-3.34	99	1.00
6	-3.14	108	0.97	-3.33	100	1.00
7	-3.31	101	1.00	-3.44	95	1.00
8	-3.12	109	0.97	-3.40	97	1.00
Mean	-3.29	101	0.98	-3.39	97	1.00

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 59122 method on 1507 x 59122 x MON 810 x NK603

Run	59122			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.15	108	0.99	-3.30	101	1.00
2	-3.19	106	0.97	-3.20	105	1.00
3	-2.88	123	0.99	-3.13	109	0.99
4	-3.01	115	0.98	-3.04	113	0.99
5	-3.14	108	0.99	-3.29	101	1.00
6	-2.90	121	0.98	-3.22	105	1.00
7	-3.19	106	0.99	-3.27	102	1.00
8	-3.34	99	0.98	-3.15	108	0.99
Mean	-3.10	111	0.98	-3.20	105	0.99

The first tests on the 0.1% GM sample showed a sub-optimal bias % for the 59122 method; the samples was therefore re-prepared and analysed in sixteen replicates in two runs. The values of the slopes of these two additional standard curves, together with the PCR efficiency and the R^2 are presented in Table 4.

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 59122 method on 1507 x 59122 x MON 810 x NK603 for the quantification of the re-prepared sample at 0.1% GM

Run	59122			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.21	105	0.99	-3.29	101	1.00
2	-3.17	107	0.98	-3.33	100	1.00
Mean	-3.19	106	0.98	-3.31	101	1.00

Table 5. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 810 method on 1507 x 59122 x MON 810 x NK603

Run	MON 810			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.22	105	0.97	-3.21	105	0.99
2	-3.11	110	0.97	-3.29	101	1.00
3	-3.35	99	0.99	-3.42	96	1.00
4	-3.26	103	0.99	-3.38	98	1.00
5	-3.35	99	0.99	-3.40	97	0.96
6	-3.30	101	0.99	-3.34	99	1.00
7	-3.19	106	0.99	-3.33	100	1.00
8	-3.32	100	0.99	-3.15	108	1.00
Mean	-3.26	103	0.99	-3.32	100	0.99

Table 6. Values of standard curve slope, PCR efficiency and linearity (R^2) for the NK603 method with the *hmg* reference system described in the validation of MON89034 maize on 1507 x 59122 x MON 810 x NK603

Run	NK603			*hmg**		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.75	85	1.00	-3.42	96	1.00
2	-3.43	96	0.99	-3.20	105	1.00
3	-3.74	85	1.00	-3.32	100	1.00
4	-3.69	87	0.99	-3.32	100	1.00
5	-3.64	88	0.99	-3.33	100	1.00
6	-3.70	86	0.99	-3.30	101	1.00
7	-3.57	91	0.99	-3.28	102	1.00
8	-3.60	89	1.00	-3.19	106	1.00
Mean	-3.64	88	0.99	-3.29	101	1.00

* *hmg* validated in the context of method for detection of event MON89034

Table 7. Values of standard curve slope, PCR efficiency and linearity (R^2) for the NK603 method with the *hmg* reference system described in the validation of MON87460 maize on 1507 x 59122 x MON 810 x NK603

Run	NK603			*hmg**		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.60	90	1.00	-3.41	96	1.00
2	-3.61	89	0.99	-3.43	96	1.00
3	-3.72	86	1.00	-3.41	97	1.00
4	-3.62	89	0.99	-3.43	96	1.00
5	-3.73	85	0.99	-3.45	95	1.00
6	-3.88	81	1.00	-3.40	97	1.00
7	-3.54	92	0.99	-3.41	97	1.00
8	-3.49	94	0.99	-3.43	96	0.99
Mean	-3.65	88	0.99	-3.42	96	1.00

* *hmg* validated in the context of method for detection of event MON87460

The mean PCR efficiencies of the calibration curves for each of the four event-specific methods were above 90% (101% for 1507, 111% and 106% for 59122, and 103% for MON 810, respectively) except for NK603 (88%). The linearity of the methods (R^2) was between 0.98 (1507 and 59122) and 0.99 (MON 810 and NK603). The data presented in Tables 2, 3, 4, 5, 6 and 7 confirm the appropriate performance characteristics of the four methods when tested on 1507 x 59122 x MON 810 x NK603 in terms of PCR efficiency and linearity.

The EU-RL GMFF also assessed the values of trueness and precision (expressed as RSDr %, relative repeatability standard deviation) of the four methods applied to samples of DNA extracted from GM maize stack 1507 x 59122 x MON 810 x NK603.

Tables 8 to 12 report the trueness and precision for each GM level for each of the four methods.

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 1507 method applied to 1507 x 59122 x MON 810 x NK603 maize DNA

1507					
Unknown sample GM%	Expected value (GMO%)				
	0.1	0.5	0.9	2.0	5.0
Mean	0.11	0.50	0.96	1.93	5.9
SD	0.02	0.10	0.09	0.23	0.57
RSD_r (%)	20	20	9.1	12	9.7
Bias (%)	11	-0.48	6.7	-3.4	17

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 59122 method applied to 1507 x 59122 x MON 810 x NK603 maize DNA

59122					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.4**	**0.9**	**2.0**	**4.5**
Mean	0.12	0.34	0.74	1.9	4.2
SD	0.01	0.06	0.09	0.20	0.50
RSD_r (%)	11	16	12	11	12
Bias (%)	24	-15	-18	-6.9	-5.8

Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 810 method applied to 1507 x 59122 x MON 810 x NK603 maize DNA

MON 810					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**1.0**	**2.0**	**5.0**
Mean	0.09	0.43	0.93	1.8	5.1
SD	0.02	0.06	0.09	0.23	0.59
RSD_r (%)	18	14	9.4	12	12
Bias (%)	-14	-13	-7.3	-8.4	1.4

Table 11. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the NK603 method with the *hmg* reference system described in the validation of MON89034 maize applied to 1507 x 59122 x MON 810 x NK603 maize DNA

NK603					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**1.0**	**2.0**	**5.0**
Mean	0.11	0.47	0.94	1.8	4.6
SD	0.01	0.06	0.09	0.15	0.34
RSD_r (%)	12	12	9.8	8.3	7.4
Bias (%)	7.4	-5.0	-5.8	-12	-8.6

Table 12. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the NK603 method with the *hmg* reference system described in the validation of MON87460 maize applied to 1507 x 59122 x MON 810 x NK603 maize DNA

NK603					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**1.0**	**2.0**	**5.0**
Mean	0.12	0.51	1.00	1.8	5.0
SD	0.01	0.07	0.07	0.15	0.20
RSD_r (%)	11	15	7.3	8.1	4.0
Bias (%)	18	2.3	0.28	-8.4	-0.55

The trueness of the method is estimated using the measurements 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 ± 25% across the entire dynamic range. As shown in Tables 8, 9, 10, 11 and 12, the values range from -3.4% to 17% for 1507, from -18% to 24% for 59122, from -14% to 1.4% for MON 810. For NK603, the bias is from -12 to 7.4 when the *hmg* reference system described in the validation of MON89034 maize is used and from -8.4 to 18 when the *hmg* reference system described in the validation of MON87460 maize is used. Therefore, the four methods satisfy the above mentioned requirement throughout their respective dynamic ranges.

Tables 8, 9, 10, 11 and 12 also document the relative repeatability standard deviation (RSD_r) for each GM level. As indicated by the ENGL, the EU-RL GMFF requires RSD_r values to be below 25%. As it can be observed from Tables 8 to 12, the values range between 9.1% and 20% for 1507, between 11% and 16% for 59122, between 9.4% and 18% for MON 810. For NK603, the bias is between 7.4% and 12% when the *hmg* reference system described in the validation of MON89034 maize is used, and between 4% and 15% when the *hmg* reference system described in the validation of MON87460 maize is used. Therefore, the four methods satisfy this requirement throughout their respective dynamic ranges when applied to stack-derived DNA.

5. Comparison of NK603 method performance using the *hmg* reference system from MON89034 and MON87460 on 1507 x 59122 x MON 810 x NK603

An indicative comparison of the performance of the NK603 method with the *hmg* reference system described in the validation of MON89034 maize and with the *hmg* reference system described in the validation of MON87460 maize applied to maize 1507 x 59122 x MON 810 x NK603 is shown in Table 13.

Table 13. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the NK603 detection method applied to 1507 x 59122 x MON 810 x NK603 with the two *hmg* reference systems tested

Trueness and repeatability of NK603 quantification with *hmg* (validated with MON89034) on 1507 x 59122 x MON 810 x NK603			**Trueness and repeatability of NK603 quantification with *hmg* (validated with MON87460) on 1507 x 59122 x MON 810 x NK603**		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	7.4	12	**0.1**	18	11
0.5	-5.0	12	**0.5**	2.3	15
1.0	-5.8	9.8	**1.0**	0.28	7.3
2.0	-12	8.3	**2.0**	-8.4	8.1
5.0	-8.6	7.4	**5.0**	-0.55	4.0

The trueness of the NK603 method is within the acceptance range set by ENGL (± 25%) for the whole dynamic range with both *hmg* reference systems, when applied to maize 1507 x 59122 x MON 810 x NK603.

The relative repeatability standard deviation (RSD_r %) of the NK603 method is within the ENGL acceptance level established at maximum 25% with both *hmg* reference systems, when applied to maize 1507 x 59122 x MON 810 x NK603.

The data presented in Table 13 show that the method performs according to the ENGL performance criteria when applied to DNA extracted from maize 1507 x 59122 x MON 810 x NK603 with both the *hmg* reference system described in the validation of MON89034 maize and in the validation of MON87460 maize.

6. Comparison of method performance on 1507 x 59122 x MON 810 x NK603 and on the single events

An indicative comparison of the performance of the four methods applied to maize 1507 x 59122 x MON 810 x NK603 and on the single events is shown in Tables 14, 15, 16, 17 and 18. The performance of the methods on the single lines was previously validated through international collaborative trials (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

The comparison of data generated in different testing conditions and different times is intended to be only of qualitative nature; differences in the figures reported are not necessarily statistically significant.

Table 14. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the 1507 detection method applied to 1507 x 59122 x MON 810 x NK603 and to the single event 1507

Trueness and repeatability of 1507 quantification on 1507 x 59122 x MON 810 x NK603			**Trueness and repeatability of 1507 quantification on single event 1507***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	11	20	**0.1**	6.0	18
0.5	-0.48	20	**0.5**	-4.0	12
0.9	6.7	9.1	**0.9**	3.7	7.7
2.0	-3.4	12	**2.0**	-1.7	8.5
5.0	17	9.7	**5.0**	8.4	14

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 15. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the 59122 detection method applied to 1507 x 59122 x MON 810 x NK603 and to the single event 59122

Trueness and repeatability of 59122 quantification on 1507 x 59122 x MON 810 x NK603			**Trueness and repeatability of 59122 quantification on single event 59122***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	24	11	**0.1**	29	18
0.4	-15	16	**0.4**	15	14
0.9	-18	12	**0.9**	9	16
2.0	-6.9	11	**2.0**	7	14
4.5	-5.8	12	**4.5**	-1	8.5

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 16. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 810 detection method applied to 1507 x 59122 x MON 810 x NK603 and to the single event MON 810

Trueness and repeatability of MON 810 quantification on 1507 x 59122 x MON 810 x NK603			**Trueness and repeatability of MON 810 quantification on single event MON 810***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	-14	18	**0.1**	-14	19
0.5	-13	14	**0.5**	-9.0	22
1.0	-7.3	9.4	**1.0**	-5.0	7.5
2.0	-8.4	12	**2.0**	-7.0	11
5.0	1.4	12	**5.0**	-8.0	11

* method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 17. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the NK603 detection method applied to 1507 x 59122 x MON 810 x NK603 and of the NK603 detection method applied to the single line event NK603. Event NK603 is quantified relatively to the *hmg* reference system validated in the context of the method of detection of event MON89034 maize in 1507 x 59122 x MON 810 x NK603 maize

Trueness and repeatability of NK603 quantification with *hmg* validated with MON89034 on 1507 x 59122 x MON810 x NK603			**Trueness and repeatability of NK603 quantification on single event NK603***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	7.4	12	**0.10**	83	24
0.5	-5.0	12	**0.49**	73	15
1.0	-5.8	9.8	**0.98**	47	17
2.0	-12	8.3	**1.96**	14	7.7
5.0	-8.6	7.4	**4.91**	22	22

* method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 18. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the NK603 detection method applied to 1507 x 59122 x MON 810 x NK603 and of the NK603 detection method applied to single line event NK603. Event NK603 is quantified relatively to the *hmg* reference system validated in the context of the method of detection of event MON87460 maize in 1507 x 59122 x MON 810 x NK603 maize.

Trueness and repeatability of NK603 quantification with *hmg* validated in MON87460 application on 1507 x 59122 x MON 810 x NK603			**Trueness and repeatability of NK603 quantification on single event NK603***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	18	11	**0.10**	83	24
0.5	2.3	15	**0.49**	73	15
1.0	0.28	7.3	**0.98**	47	17
2.0	-8.4	8.1	**1.96**	14	7.7
5.0	-0.55	4.0	**4.91**	22	22

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

The trueness of the four event-specific methods when applied to maize 1507 x 59122 x MON 810 x NK603 is within the acceptance range set by ENGL (± 25%) for the whole dynamic ranges studied.

The relative repeatability standard deviation (RSD_r %) of the four event-specific methods when applied to maize 1507 x 59122 x MON 810 x NK603 are below the ENGL acceptance level established at maximum 25%.

The data presented in tables 14, 15, 16, 17 and 18 show that the methods perform according to the ENGL performance criteria when applied to DNA extracted from the maize single events 1507, 59122, MON 810 and NK603 and to the DNA extracted from maize 1507 x 59122 x MON 810 x NK603.

7. Conclusions

The performance of the four event-specific methods for the detection and quantification of events 1507, 59122, MON 810 and NK603, when applied to DNA extracted from maize 1507 x 59122 x MON 810 x NK603, meets the ENGL performance criteria (ENGL), as assessed on the control samples provided by the applicant.

The method verification has demonstrated that the PCR efficiency, linearity, trueness and repeatability of the methods were within the limits established by the ENGL.

In conclusion, the verification study confirmed that the four methods are capable to detect, identify and quantify each of the GM events when applied to genomic DNA of suitable quality, extracted from maize 1507 x 59122 x MON 810 x NK603.

Event NK603 can be quantified with both the *hmg* reference systems validated in the context of the method of detection of event MON890340 and of event MON87460.

Therefore these methods, developed and validated to detect and quantify the single events, can be equally applied for the quantification of the respective events combined in maize 1507 x 59122 x MON 810 x NK603.

8. References

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Annex 1: Real-time PCR amplification reaction conditions tested for the verification of NK603 quantitative real-time PCR method on DNA from 1507 x 59122 x MON 810 x NK603 maize

The verification study for NK603 method, applied to 1507 x 59122 x MON 810 x NK603 maize, has been performed by the EU-RL GMFF using the originally validated method (available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) with two different *hmg* reference systems: the one described in the validation of MON89034 maize, and the one described in the validation of MON87460 maize. The amplification reaction mixtures for the NK603 event-specific and the *hmg* reference systems used in the verification of NK603 method are reported below in Tables 1, 2, 3 and 4.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the NK603 assay used in combination with the *hmg* reference system described in the validation of MON89034 maize.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
NK603 primer F (10 µM)	150 nM	0.75
NK603 primer R (10 µM)	150 nM	0.75
NK603 probe (10 µM)	50 nM	0.25
Nuclease free water	-	18.25
DNA (max 200 ng)	-	5
Total reaction volume:		50 µL

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* assay described in the validation of MON89034 maize.

Component	Final concentration	µL/reaction
TaqMan® Buffer A (10x)	1x	2.5
$MgCl_2$ (25 mM)	6.5 mM	6.5
dNTP mix (10 mM each)	200 µM each	0.5
AmpliTaq® Gold Polymerase (5U/µl)	1.25 U	0.25
hmg primer 1 (10 µM)	300 nM	0.75
hmg primer 2 (10 µM)	300 nM	0.75
hmg probe (10 µM)	160 nM	0.40
Nuclease free water	-	6.85
DNA (max 200 ng)	-	5
Total reaction volume:		25 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the NK603 assay used in combination with the *hmg* reference system described in the validation of MON87460 maize.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
NK603 primer F (10 µM)	150 nM	0.38
NK603 primer R (10 µM)	150 nM	0.38
NK603 probe (10 µM)	50 nM	0.13
Nuclease free water	-	6.63
DNA (max 200 ng)	-	5
Total reaction volume:		25 µL

Table 4. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* assay described in the validation of MON87460 maize.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
hmg primer 1 (10 µM)	300 nM	0.75
hmg primer 2 (10 µM)	300 nM	0.75
hmg probe (10 µM)	160 nM	0.40
Nuclease free water	-	5.6
DNA (max 200 ng)	-	5
Total reaction volume:		25 µL

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Abstract

An application was submitted by Pioneer Overseas Corporation to request the authorization of the genetically modified maize stack 1507 x 59122 x MON 810 x NK603, resistant against certain lepidopteran pests, protected against corn rootworm larvae, and glufosinate-ammonium and glyphosate tolerant, and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, and import and processing, in accordance with articles 5 and 17 of Regulation (EC) N° 1829/2003 GM Food and GM Feed. The unique identifier assigned to 1507 x 59122 x MON 810 x NK603 maize is DAS-Ø1507-1xDAS-59122-7xMON-ØØ81Ø-6xMON-ØØ6Ø3-6.

The genetically modified maize line 1507 x 59122 x MON 810 x NK603 has been obtained by conventional crossing of four genetically modified single maize events: 1507, 59122, MON 810 and NK603 without any new genetic modification.

The EU-RL GMFF has previously validated, and declared fit for purpose, the detection methods for the single events 1507, 59122, MON 810 and NK603 (see: http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EU-RL GMFF therefore has carried out only an in-house verification of the performance of each validated method when applied to DNA extracted from 1507 x 59122 x MON 810 x NK603.

The herewith reported in-house verification study lead to the conclusion that the individual methods meet the ENGL performance criteria also when applied to DNA extracted from the GM maize stack 1507 x 59122 x MON 810 x NK603.

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