

# JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

## **Report on the Verification of the Performance of 1507, MON 810, MIR 162 and NK603 event- specific PCR-based Methods applied to DNA extracted from GM Stack 1507 x MON 810 x MIR 162 x NK603 maize**

European Union Reference Laboratory for  
Genetically Modified Food and Feed

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**Contact information**

Name: EURL GMFF

Email: JRC-EURL-GMFF@ec.europa.eu

**JRC Science Hub**

<https://ec.europa.eu/jrc>

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# **Report on the Verification of the Performance of 1507, MON 810, MIR 162 and NK603 event-specific PCR-based Methods applied to DNA extracted from GM Stack 1507 x MON 810 x MIR 162 x NK603 maize**

**18 October 2018**

**European Union Reference Laboratory for GM Food and Feed**

## **Executive Summary**

An application was submitted by Pioneer Hi-Bred International, Inc., as represented by Pioneer Overseas Corporation to request the authorisation of genetically modified stack (GM stack) 1507 x MON 810 x MIR 162 x NK603 maize (herbicide tolerance to glyphosate and glufosinate-ammonium herbicides, and protection against lepidopteran target pests) and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, 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 GM stack 1507 x MON 810 x MIR 162 x NK603 maize is DAS-Ø15Ø7-1xMON-ØØ81Ø-6xSYN-IR162-4xMON-ØØ6Ø3-6.

The GM stack 1507 x MON 810 x MIR 162 x NK603 maize has been obtained by conventional crossing between the genetically modified maize events: 1507, MON 810, MIR 162 and NK603, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events 1507, MON 810, MIR 162 and NK603 (see <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). In line with the approach defined by the ENGL ([http://gmo-crl.jrc.ec.europa.eu/doc/Min\\_Perf\\_Requirements\\_Analytical\\_methods.pdf](http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf)) the EURL GMFF has carried out only an *in-house* verification of the performance of each validated method when applied to genomic DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

The results of the *in-house* verification led to the conclusion that the individual methods meet the ENGL performance criteria also when applied to genomic DNA extracted from the GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

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

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## Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: Belac 268 TEST (Flexible Scope for DNA extraction, DNA identification and real Time PCR) and ISO 17043:2010 accredited (certificate number: Belac 268 PT, proficiency test provider).

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

### Address of contact laboratory:

European Commission  
Directorate General Joint Research Centre  
Directorate F – Health, Consumers and Reference Materials  
European Union Reference Laboratory for GM Food and Feed  
Food & Feed Compliance (F.5)  
Via E. Fermi, 2749. TP201  
I-21027 Ispra (VA), Italy

Functional mailbox: [JRC-EURL-GMFF@ec.europa.eu](mailto:JRC-EURL-GMFF@ec.europa.eu)

## 1. Introduction

The EU legislative system <sup>(1, 2)</sup> for genetically modified food and feed foresees that any GMO for food and feed use shall undergo the authorisation process before it can be placed on the 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 GM stack 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 EURL 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 EURL GMFF report in the overall opinion concerning the risk assessment and potential authorisation of the assessed stack. In line with the approach defined by the ENGL ([http://gmo-crl.jrc.ec.europa.eu/doc/Min\\_Perf\\_Requirements\\_Analytical\\_methods.pdf](http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf)) the EURL GMFF carries out an *in-house* verification of the performance of each event-specific methods if this method has previously been validated by the EURL GMFF for the parental single-line event and these events have been stacked by conventional crossing. These criteria are met for the GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

Upon reception of methods, samples and related data (step 1), the EURL 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 (EU) No 503/2013 (Annex III).

The results of the *in-house* verification study were evaluated with reference to ENGL method performance requirements <sup>(3)</sup> 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 when applied to genomic DNA extracted from the stack, and the corresponding control samples of DNA extracted from the GM stack maize 1507 x MON 810 x MIR 162 x NK603 and from non GM maize.

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

### 3. Step 2 (dossier scientific assessment)

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL <sup>(3)</sup> and with regard to their documentation and reliability.

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSDr %) calculated by the applicant for the four methods applied to 1507 x MON 810 x MIR 162 x NK603 maize genomic DNA. Means are the average of fifteen replicates for 1507 method as validated, MIR162 and NK603 methods, and twenty-four replicates for MON 810 method, obtained through three runs for 1507 method as validated, MIR162 and NK603 methods, and four runs for the MON 810 method. Percentages are expressed as GM DNA / total DNA x 100, in copy number ratio.

*Note: Numerical values presented in the following tables were rounded keeping two digits for values  $\leq 1$ , one digit for values between 1 and 10 and no digit for values  $\geq 10$ , unless otherwise stated. The calculations in the MS Excel files however were done over not rounded data. This approach might create small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.*

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) provided by the applicant for the 1507, MON 810, MIR 162 and NK603 methods applied to GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

1507 with GeneAmp 10X PCR Buffer II*					
Sample GM %	Expected value (GMO %)				
	0.10	0.50	0.90	2.00	5.00
Mean	0.08	0.40	0.74	1.86	4.59
RSD <sub>r</sub> (%)	12.5	7.5	4.1	5.4	5.2
Bias (%)	-20.0	-20.0	-17.8	-7.0	-8.2
MON 810*					
Sample GM %	Expected value (GMO %)				
	0.10	0.50	1.00	2.00	5.00
Mean	0.096	0.491	1.147	2.255	5.874
RSD <sub>r</sub> (%)	24.00	12.934	8.267	6.456	5.413
Bias (%)	-4.44	-1.80	14.70	12.75	17.48
MIR 162*					
Sample GM %	Expected value (GMO %)				
	0.10	0.50	0.90	2.00	5.00
Mean	0.10	0.52	0.9	1.99	4.68
RSD <sub>r</sub> (%)	10.0	9.6	12.2	8.5	9.0
Bias (%)	0.0	4.0	0.0	-0.5	-6.4

<b>NK603*</b>					
<b>Sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>0.10</b>	<b>0.50</b>	<b>0.90</b>	<b>2.00</b>	<b>5.00</b>
<b>Mean</b>	0.08	0.47	0.88	2.05	5.09
<b>RSD<sub>r</sub> (%)</b>	12.5	6.4	8.0	5.9	4.1
<b>Bias (%)</b>	-20.0	-6.0	-2.2	2.5	1.8

\* Numbers are not rounded but are presented as reported by the applicant

The EURL GMFF verified the data and concluded that they were reliable and seemed to confirm that the methods meet the ENGL performance criteria <sup>(3)</sup>.

Five requests of complementary information regarding the method, the control samples and DNA sequences were addressed to the applicant.

In accordance with the requirements set in Annex 2 of the ENGL document for method verification criteria, the applicant can refer to existing data for applicability, practicability, specificity, LOD and robustness. However, for method 1507 the applicant substituted the 10 x PCR buffer with the Bio-Rad Sso Advanced™ Universal Probes Supermix. Hence additional data had to be produced in support of the 1507 method. Therefore, the applicant also submitted information and bridging data on specificity, LOD, robustness and quantification.

The specificity of the modified event-specific assay was assessed by the applicant with genomic DNA extracted from 1507 as positive control sample and from 16 GM maize events (MON810, GA21, Bt176, Bt11, NK603, MON 863, 5307, MIR 604, MON88017, 59122, 3272, T25, MON89034, 98140, MON 87460, MON 87427), 15 GM soybean events (44406, A2704-12, A5547-127, 305423, 356043, GTS 40-3-2, MON 89788, MON 87701, FG72, DAS 68416-4, CV127, DAS-81419-2, MON 87705, MON 87769, MON 87708), 10 GM oilseed rape events (Ms8, Rf3, T45, GT73/RT73, MS1, Rf1, RF2, MON 88302, Topas 19/2, 073496), H71 sugar beet, 9 GM cotton events (MON 531, MON 15985, MON 1445, GHB614, GHB119, T304-40, LLCotton25, 281-24-236, MON 88913), EH92-527-1 potato, LLRice62 rice and conventional soybean, oilseed rape, maize, cotton, wheat, rice, potato, sugar beet.

According to the method developer the modified 1507 method did not react with any sample except the positive control. The LOD of the modified 1507 method was assessed by the applicant to be at least 0.02 % in mass fraction of GM material, with 200 ng of genomic DNA per reaction. The robustness was assessed on Applied Biosystems 7500 Fast, ViiA7 and 7900 HT real-time PCR instruments, on and Roche Light Cycler 480, in combination with the following variations: +/-10 % primer, +/-10 % probe, +/-5 % master mix, +/-2 °C in annealing temperature, +/-10 % reaction volume of master mix. The RSD<sub>r</sub> and trueness calculated for a combination of changes did not exceed 30 %, thus meeting the ENGL requirement for robustness. The performance data submitted by the applicant for the modified 1507 method are reported in Table 2 below. Means are the average of thirty replicates obtained through five runs. Percentages are expressed as GM DNA / total DNA x 100, in mass fraction of GM material.



Table 2. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSD<sub>r</sub> %) provided by the applicant for the 1507 modified method applied to GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

<b>1507 with Bio-Rad SsoAdvanced™ Universal Probes Supermix*</b>				
<b>Sample GM %</b>	<b>Expected value (GMO %)</b>			
	<b>0.08</b>	<b>0.9</b>	<b>2.0</b>	<b>5.0</b>
<b>Mean</b>	0.0996	1.007	2.163	5.363
<b>RSD<sub>r</sub> (%)</b>	18.07	6.16	5.36	8.20
<b>Bias (%)</b>	25.54	11.89	8.15	7.26

\* Numbers are not rounded but are presented as reported by the applicant

The EURL GMFF verified the data and the complementary information received and accepted the received clarifications as satisfactory.

The dossier was therefore moved to step 3.

## 4. Step 3 (EURL GMFF experimental testing)

In step 3 the EURL GMFF implemented the four methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

### 4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from ground seeds of GM stack 1507 x MON 810 x MIR 162 x NK603 maize, hemizygous for the loci 1507, MON 810 (male contribution), MIR 162 and NK603 (female contribution), as positive control sample.
- genomic DNA extracted from ground seeds of conventional (non-GM) maize whose genetic background is similar to the positive control sample, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize with the non-GM maize genomic DNA, in a constant amount of total maize genomic DNA. The same GM concentrations as in the validation of the methods for the single lines were achieved. Table 3 shows the five GM concentrations used in the verification of the 1507, MON 810, MIR 162 and NK603 methods when applying them to genomic DNA extracted from the GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

Table 3. Percentage (GM %) of 1507, MON 810, MIR 162 and NK603 in 1507 x MON 810 x MIR 162 x NK603 maize stack genomic DNA contained in the verification samples.

<b>1507 GM %*</b> [(GM DNA / total maize DNA x 100)]	<b>MON 810 GM %*</b> [(GM DNA / total maize DNA x 100)]	<b>MIR 162 GM %*</b> [(GM DNA / total maize DNA x 100)]	<b>NK603 GM %*</b> [(GM DNA / total maize DNA x 100)]
0.10	0.10	0.10	0.10
0.50	0.50	0.40	0.50
0.90	1.00	0.90	1.00
2.0	2.0	2.0	2.0
5.0	5.0	5.0	5.0

\* percentage expressed in copy number ratio

The applicant introduced deviations to the protocols, which are described in §4.4.1. The EURL GMFF laboratory implemented the protocols already published for the individual 1507, MON 810, MIR 162 and NK603 GM events (available at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>), with the deviations described in in §4.4.1.

## 4.2 DNA extraction

A method for DNA extraction from maize was previously evaluated by the EURL GMFF with regard to its performance characteristics and was considered valid, i.e. fit the purpose of providing maize DNA of appropriate quality and amount for being used in subsequent PCR experiments.

Annex III to Reg. (EU) No 503/2013 <sup>(2)</sup> requires the applicant to discuss the validity and limitations of the detection methods in the various types of foods and feeds (matrices) that are expected to be placed on the market. To this regard the applicant stated that the applicability of the quantitative real-time PCR methods developed for 1507, MON 810, MIR 162 and NK603 depends on the isolation of sufficient quantity and quality of purified DNA. The provided DNA extraction method is intended for extraction of genomic DNA from seeds, which results in primarily high molecular weight DNA. The applicant also informed the EURL GMFF that during the processing of maize seeds into food and feed ingredients a number of steps are typically followed, which can influence the quality and intactness of the DNA contained in the final processed soybean products <sup>(4,5,6)</sup>. DNA extraction from certain of these processed matrices may require additional rounds of purification in order to achieve the quality standards needed for quantitative real-time PCR <sup>(7,8)</sup>.

On a general note, the EURL GMFF recommends that laboratories using this validated method for testing complex or difficult matrices always verify that the extracted genomic DNA is of sufficient quality.

The protocol for the DNA extraction method is available at <http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-02-14-XP.pdf>.

Consequently, the EURL GMFF did not verify the DNA extraction method proposed by the applicant.

### 4.3 *Experimental design*

Eight PCR runs were carried out for each method. In each run, samples were analysed in parallel with both the GM-specific system and the reference system high mobility group (*hmg*) for 1507, MON 810 and NK603 events or the reference system alcohol dehydrogenase 1 (*Adh1*) for MIR 162 event. Five GM levels were examined per run, each GM level in duplicate. PCR analysis was performed in triplicate for all samples. In total, for each method 1507, MON 810, MIR 162 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). The 1507 method was assessed in parallel as validated, with the original 10 x PCR buffer and with the method modified by the applicant, with Bio-Rad SsoAdvanced™ Universal Probes Supermix (see <http://gmo-crl.jrc.ec.europa.eu/summaries/TC1507-WEB-Protocol-Validation-VERSION-B.pdf> for the modified method). An Excel spreadsheet was used for determination of the GM %.

### 4.4 *PCR methods*

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize using the single detection methods previously validated for the respective single GM events 1507, MON 810, MIR 162 and NK603.

For detection of GM maize events 1507, MON 810, MIR 162 and NK603, DNA fragments of 58-bp, 92-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 (6-carboxyfluorescein) as reporter dye at their 5'-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at their 3'-end for all four events.

For quantification of GM maize events 1507, MON 810 and NK603, a taxon-specific reference system amplifies a 79-bp fragment of high mobility group *hmg* a maize endogenous gene (GenBank AJ131373.1), using two *hmg* gene-specific primers and a gene-specific probe labelled with FAM as reporter dye at its 5' end and TAMRA as quencher dye at its 3' end.

For quantification of GM maize event MIR 162, a taxon-specific reference system amplifies a 135-bp fragment of alcohol dehydrogenase 1 *Adh1* a maize endogenous gene (GenBank X04050), using two *Adh1* gene-specific primers and a gene-specific probe labelled with VIC as reporter dye at its 5' end and TAMRA as quencher dye at its 3' end.

For the relative quantification of GM maize events 1507, MON 810 and NK603 standard curves are generated both for the 1507, MON 810 and NK603 and for the *hmg* specific system by plotting Cq values of the calibration standards against the logarithm of the DNA amount and by fitting a linear regression into these data. Thereafter, the Cq values of the unknown samples are measured and, by means of the regression formula, the relative amount of 1507, MON 810 and NK603 DNA is estimated.

For relative quantification of GM maize event MIR 162 DNA in a test sample, the  $\Delta C_q$  values of calibration samples are used to calculate, by linear regression, a standard curve (plotting  $\Delta C_q$  values against the logarithm of the relative amount of MIR 162 event DNA). The  $\Delta C_q$  values of the unknown samples are measured and, by means of the regression formula, the relative amount of MIR 162 event is estimated.

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/StatusOfDossiers.aspx>. For the protocol of the modified 1507 method please refer to Annex I to this report.

#### **4.4.1 Deviations from the validated methods**

The applicant introduced the following deviations to the validated methods:

- reduction of the reaction volume from 25  $\mu$ l to 20  $\mu$ l for the 1507 method;
- reduction of the reaction volume from 50  $\mu$ l to 20  $\mu$ l for the NK603 method;
- replacement of the 10 x PCR buffer described in 1507 validated protocol (<http://gmo-crl.jrc.ec.europa.eu/summaries/TC1507-WEB-Protocol-Validation.pdf>) with GeneAmp 10X PCR Buffer II;
- quantification of the NK603 event relative to the *hmg* taxon-specific module, with the reaction mix described in 98140 maize validation report: [http://gmo-crl.jrc.ec.europa.eu/summaries/DP-098140-6\\_validated\\_Method.pdf](http://gmo-crl.jrc.ec.europa.eu/summaries/DP-098140-6_validated_Method.pdf).

The verification of the NK603 method was done by the EURL GMFF using *hmg* as reference system in a final reaction volume of 25  $\mu$ l for both the GM and the reference system in line with the NK603 protocol already modified and verified in the context of the maize stacked event verification (bridging study) EURL-VL-01/11VR, (page 19 of <http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-1507-59122-MON810-NK603%20.pdf>). The NK603 quantification was performed relative to the validated maize reference system *hmg* in substitution of the maize reference system *adh1* that was originally validated but later demonstrated to be suboptimal for quantitative purposes (pages 10-11 of <http://gmocrl.jrc.ec.europa.eu/summaries/EURL-VL-03-10-VR.pdf>).

## **4.5 Results**

Tables 4-7 report the values of the slopes of the different standard curves generated by the EURL GMFF when using DNA extracted from the GM stack, from which the PCR efficiency is calculated using the formula  $[10^{(-1/\text{slope})} - 1] \times 100$ , and of the coefficient of determination ( $R^2$ ) reported for all PCR systems in the eight runs, for GM maize events 1507, MON 810, MIR 162 and NK603. Slope values were rounded to two digits.

Table 4a. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the 1507 method as validated on GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

Run	1507			hmg		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.30	101	1.00	-3.48	94	1.00
2	-3.21	105	1.00	-3.34	99	1.00
3	-3.32	100	1.00	-3.50	93	1.00
4	-3.23	104	1.00	-3.35	99	1.00
5	-3.40	97	1.00	-3.31	101	1.00
6	-3.32	100	1.00	-3.37	98	1.00
7	-3.17	107	1.00	-3.31	100	1.00
8	-3.28	102	1.00	-3.33	100	1.00
Mean	-3.28	102	1.00	-3.38	98	1.00

Table 4b. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the 1507 method with Bio-Rad SsoAdvanced™ Universal Probes Supermix on GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

Run	1507			hmg		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.37	98	1.00	-3.38	98	1.00
2	-3.56	91	1.00	-3.37	98	1.00
3	-3.36	98	1.00	-3.32	100	1.00
4	-3.58	90	1.00	-3.38	98	1.00
5	-3.37	98	1.00	-3.35	99	1.00
6	-3.42	96	1.00	-3.32	100	1.00
7	-3.48	94	1.00	-3.32	100	1.00
8	-3.50	93	1.00	-3.33	99	1.00
Mean	-3.46	95	1.00	-3.35	99	1.00

Table 5. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the MON 810 method on GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

Run	MON 810			<i>hmg</i>		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.24	104	0.99	-3.27	102	1.00
2	-3.21	105	0.99	-3.36	98	1.00
3	-3.36	99	0.99	-3.34	99	1.00
4	-3.20	105	1.00	-3.42	96	1.00
5	-3.20	105	0.99	-3.23	104	1.00
6	-3.49	93	0.98	-3.35	99	1.00
7	-3.27	102	0.97	-3.33	100	1.00
8	-3.22	105	0.98	-3.36	98	1.00
Mean	-3.27	102	0.99	-3.33	100	1.00

Table 6. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the MIR 162 method on GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

Run	MIR 162		
	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.46	94	0.99
2	-3.47	94	1.00
3	-3.40	97	1.00
4	-3.60	90	1.00
5	-3.46	95	1.00
6	-3.44	95	1.00
7	-3.43	96	0.99
8	-3.20	105	1.00
Mean	-3.43	96	1.00

Table 7. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the NK603 method on GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

Run	NK603			<i>hmg</i>		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.69	87	0.99	-3.40	97	1.00
2	-3.83	83	0.99	-3.35	99	1.00
3	-3.71	86	0.99	-3.35	99	1.00
4	-3.67	87	0.99	-3.38	98	1.00
5	-3.79	84	0.99	-3.40	97	1.00
6	-3.54	92	0.99	-3.35	99	1.00
7	-3.73	85	0.99	-3.32	100	1.00
8	-3.84	82	0.99	-3.39	97	1.00
Mean	-3.72	86	0.99	-3.37	98	1.00

The mean PCR efficiencies of the GM and taxon-specific systems were above 90 % (102 % for 1507 system as validated and for MON 810 system, 95 % for 1507 system with Bio-Rad SsoAdvanced™ Universal Probes Supermix, and 96 % for MIR 162 system; the efficiency of the maize reference system (*hmg*) ranged between 98 % and 100 %), except for the value of NK603, which falls slightly below 90 % (86 %) and was deemed acceptable as it was in line with previous verifications performed by the EURL GMFF (e.g. <http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-TC1507-59122-MON810-NK603%20.pdf>).

The mean  $R^2$  coefficient of the methods was 1.00 for all systems except MON 810 and NK603, for which it was 0.99. The data presented in Tables 4-7 confirm the appropriate performance characteristics of the four methods and the modified 1507 method, when tested on GM stack 1507 x MON 810 x MIR 162 x NK603 maize in terms of PCR efficiency and  $R^2$  coefficient.

The EURL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation,  $RSD_r$  %) of the four methods applied to samples of DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize see tables 8-11.

Table 8a. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ( $RSD_r$  %) of the 1507 method as validated applied to genomic DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

<b>1507</b>					
<b>Unknown sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>5.0</b>	<b>2.0</b>	<b>0.90</b>	<b>0.50</b>	<b>0.10</b>
<b>Mean</b>	4.6	1.9	0.81	0.44	0.08
SD	0.28	0.09	0.06	0.04	0.01
$RSD_r$ (%)	6.1	5.1	7.2	8.1	12
Bias (%)	-7.4	-7.4	-10	-13	-17

Table 8b. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ( $RSD_r$  %) of the 1507 method with Bio-Rad SsoAdvanced™ Universal Probes Supermix applied to genomic DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

<b>1507</b>					
<b>Unknown sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>5.0</b>	<b>2.0</b>	<b>0.90</b>	<b>0.50</b>	<b>0.10</b>
<b>Mean</b>	4.9	2.0	0.87	0.51	0.09
SD	0.31	0.11	0.11	0.08	0.02
$RSD_r$ (%)	6.4	5.3	13	16	20
Bias (%)	-2.0	1.7	-3.5	2.0	-6.2

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ( $RSD_r$  %) of the MON 810 method applied to genomic DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

<b>MON 810</b>					
<b>Unknown sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>5.0</b>	<b>2.0</b>	<b>1.00</b>	<b>0.50</b>	<b>0.10</b>
<b>Mean</b>	4.9	1.8	0.95	0.42	0.08
SD	0.45	0.26	0.16	0.06	0.02
$RSD_r$ (%)	9.2	15	17	16	23
Bias (%)	-2.1	-11	-4.7	-17	-18



Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ( $RSD_r$  %) of the MIR 162 method applied to genomic DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

<b>MIR 162</b>					
<b>Unknown sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>5.0</b>	<b>2.0</b>	<b>0.90</b>	<b>0.40</b>	<b>0.10</b>
<b>Mean</b>	4.9	2.0	0.96	0.41	0.11
SD	0.57	0.28	0.14	0.04	0.02
$RSD_r$ (%)	12	14	14	9.7	14
Bias (%)	-2.8	2.4	6.8	3.2	7.8

Table 11. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ( $RSD_r$  %) of the NK603 method applied to genomic DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

<b>NK603</b>					
<b>Unknown sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>5.0</b>	<b>2.0</b>	<b>1.00</b>	<b>0.50</b>	<b>0.10</b>
<b>Mean</b>	4.6	1.7	0.93	0.44	0.11
SD	0.34	0.24	0.06	0.04	0.01
$RSD_r$ (%)	7.4	14	6.2	8.1	12
Bias (%)	-7.8	-13	-6.7	-11	7.4

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 should be less or equal to  $\pm 25$  % across the entire dynamic range. As shown in Tables 8-11, the values range from -17 % to -7.4 % for 1507 method as validated, from -6.2 % to 2.0 % for 1507 method with Bio-Rad SsoAdvanced™ Universal Probes Supermix, from -18 % to -2.1 % for MON 810, from -2.8 % to 7.8 % for MIR 162, and from -13 % to 7.4 % for NK603. Therefore, the four methods and the modified 1507 method satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

Tables 8-11 also show the relative repeatability standard deviation ( $RSD_r$ ) estimated for each GM level. According to the ENGL acceptance criteria and method performance requirements, the  $RSD_r$  values should be equal to or below 25 %. As the values range between 5.1 % and 12 % for 1507 method as validated, between 5.3 % and 20 % for 1507 method with Bio-Rad SsoAdvanced™ Universal Probes Supermix, between 9.2 % and 23 % for MON 810, between 9.7 % and 14 % for MIR 162 and between 6.2 % and 14 % for NK603, the four methods and the modified 1507 method satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize.

## 5. Conclusions

The performance of the four event-specific methods for the detection and quantification of maize single line events 1507, MON 810, MIR 162 and NK603, when applied to genomic DNA extracted from GM stack 1507 x MON 810 x MIR 162 x NK603 maize, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant.

Event 1507 can be quantified with both the original validated method, and the modified method proposed by the applicant.

Therefore these methods, developed and validated to detect and quantify the single maize events 1507, MON 810, MIR 162 and NK603, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack 1507 x MON 810 x MIR 162 x NK603 maize or any of its sub-combinations, supposed that sufficient genomic DNA of appropriate quality is available.

## 6. References

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