



# JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of  
DP4114, MON810, MIR604 and NK603 event-  
specific PCR-based Methods applied to DNA  
extracted from GM Stack DP4114 x MON810 x  
MIR604 x NK603 maize

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# **Report on the Verification of the Performance of DP4114, MON810, MIR604 and NK603 event-specific PCR-based Methods applied to DNA extracted from GM Stack DP4114 x MON810 x MIR604 x NK603 maize**

**9 October 2020**

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

## **Executive Summary**

An application was submitted by Pioneer Overseas Corporation to request the authorisation of genetically modified stack (GM stack) DP4114 x MON810 x MIR604 x NK603 maize (herbicide tolerance to glyphosate and glufosinate-ammonium herbicides, protection against lepidopteran target pests and protection against coleopteran 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) No 1829/2003 GM Food and GM Feed. The unique identifier assigned to GM stack DP4114 x MON810 x MIR604 x NK603 maize is DP-ØØ4114-3xMON-ØØ81Ø-6xSYN-IR6Ø4-5xMON-ØØ6Ø3-6.

The GM stack DP4114 x MON810 x MIR604 x NK603 maize has been obtained by conventional crossing between the genetically modified maize events: DP4114, MON810, MIR604 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 DP4114, MON810, MIR604 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 DP4114 x MON810 x MIR604 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 DP4114 x MON810 x MIR604 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 17025:2017 accredited [certificate number: Belac 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) in food and feed by DNA extraction, DNA identification and Real-time PCR and for determination of Genetically Modified content in % (cp/cp) in food and feed by DNA extraction and digital 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.

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## 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 DP4114 x MON810 x MIR604 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. Dossier reception and acceptance (step 1)

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 DP4114 x MON810 x MIR604 x NK603 and from non GM maize.

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

### 3. Dossier scientific assessment (step 2)

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.

#### 3.1 DNA extraction

A method for DNA extraction from maize (EURL-VL-02/14XP) 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 DP4114, MON810, MIR604 and NK603 *'in seed, food and feed matrices is primarily based on the quality of genomic DNA template that is utilized. Exceptionally pure DNA, applicable for molecular biology procedures, such as Polymerase Chain Reaction (PCR) amplification, is imperative to provide adequate source template suitable for use for a wide variety of agricultural products, including maize grain and derived matrices supporting food and feed products. The processing of maize grain involves varying degrees of mechanical, enzymatic, solvent, heat, acid, pressure treatment, or combinations of these steps<sup>(4-8)</sup>. These steps influence the quality and intactness of DNA contained in the final processed maize products<sup>(9-12)</sup> which may result in significant degradation of high molecular weight DNA and failure to PCR amplify products greater than a few 100 base pairs<sup>(9, 10)</sup>. Random DNA fragmentation is known to lead to variability in quantifying DNA by qPCR<sup>(13)</sup>, thus affecting the ability to accurately quantify the presence of a GM event and taxon-specific target in processed fractions. Moreover, the DNA extraction procedure necessary for some of these processed matrices may need additional rounds of processing to clean-up the DNA, to eliminate PCR inhibitors in order to achieve quality genomic DNA suitable for PCR testing<sup>(14, 15)</sup>.*

*Pure DNA extractions such as the cetyltrimethyl ammonium bromide (CTAB) method or the Wizard (Promega) method are suitable for the isolation of pure genomic DNA from a wide variety of cereal based matrices<sup>(11, 16, 17)</sup>. These extraction methods are widely used for plant based materials, are economical and can be easily scaled<sup>(18)</sup>.*

*Internal testing of DNA extracted from food matrices derived from maize grain prove that these matrices can yield DNA template suitable for PCR and thus for the detection of adventitious mixing of genetically modified ingredients. Two maize food matrices: corn tortilla chips and corn meal were selected to test their DNA extraction capability. Processing steps for these matrices include a nixtamalization process involving lime, ash and multiple heating steps to remove the pericarp and germ followed by grinding<sup>(19, 20)</sup>. Corn tortilla chips undergo high temperature baking followed by frying to create the chips<sup>(19)</sup>. Subsequent real-time PCR*

*analysis indicates reproducible and expected detection of both high mobility group protein A (hmg-A) and alcohol dehydrogenase 1 (adh1) reference genes.'*

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 <https://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.

### 3.2 PCR methods

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSD<sub>r</sub> %) calculated by the applicant for the four methods applied to DP4114 x MON810 x MIR604 x NK603 maize genomic DNA. Means are the average of thirty replicates obtained through five runs performed with ABI 7500 Fast real-time PCR equipment. Percentages are expressed as GM DNA / total DNA x 100.

*Note: Numerical values presented in the following tables were rounded keeping two digits for values ≤ 1, one digit for values between 1 and 10 and no digit for values ≥ 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, RSD<sub>r</sub> %) provided by the applicant for the DP4114, MON810, MIR604 and NK603 methods applied to GM stack DP4114 x MON810 x MIR604 x NK603 maize.

DP4114 *				
Sample GM %	Expected value (GM0 %)			
	8.0	4.5	2.0	0.09
Mean	9.629	5.226	2.499	0.110
RSD <sub>r</sub> (%)	8.038	13.222	5.802	18.182
Bias (%)	20.36	16.13	24.95	22.22
MON810 *				
Sample GM %	Expected value (GM0 %)			
	8.0	4.5	2.0	0.09
Mean	9.092	4.833	2.012	0.100
RSD <sub>r</sub> (%)	10.504	10.697	12.575	19.000
Bias (%)	13.65	7.40	0.60	0.00



<b>MIR604 *</b>				
<b>Sample GM %</b>	<b>Expected value (GM0 %)</b>			
	<b>8.0</b>	<b>4.5</b>	<b>2.0</b>	<b>0.09</b>
<b>Mean</b>	<b>8.005</b>	<b>4.415</b>	<b>2.165</b>	<b>0.098</b>
RSD <sub>r</sub> (%)	6.796	8.901	6.467	20.408
Bias (%)	0.06	-1.89	8.25	8.89
<b>NK603 *</b>				
<b>Sample GM %</b>	<b>Expected value (GM0 %)</b>			
	<b>8.0</b>	<b>4.5</b>	<b>2.0</b>	<b>0.09</b>
<b>Mean</b>	<b>7.097</b>	<b>4.422</b>	<b>2.026</b>	<b>0.092</b>
RSD <sub>r</sub> (%)	10.244	7.734	15.499	21.739
Bias (%)	-11.29	-1.73	1.30	-8.00

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

### 3.2.1 Deviations from the validated methods introduced by the applicant

The applicant introduced the following modifications to the validated methods:

- MIR604: the quantification was performed by using a calibration standard curve made by serial dilution of a 10 % reference material instead of the  $\Delta Cq$  standard curve described in the validated method;
- NK603: the method CRLVL27/04VP was used with a reaction volume of 25  $\mu$ L and with *hmg* as taxon-specific method. The modifications are described and assessed in EURL-VL-01/11VR.

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>.

The dossier was therefore moved to step 3.

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

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 DP4114 x MON810 x MIR604 x NK603 maize.

## 4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from ground seeds of GM stack DP4114 x MON810 x MIR604 x NK603 maize, hemizygous for the loci, as positive control sample.
- genomic DNA extracted from ground seeds of conventional (non-GM) maize whose genetic background is near isogenic line (NIL), as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack DP4114 x MON810 x MIR604 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 2 shows the five GM concentrations used in the verification of the DP4114, MON810, MIR604 and NK603 methods when applying them to genomic DNA extracted from the GM stack DP4114 x MON810 x MIR604 x NK603 maize.

Table 2. Percentage (GM %) of DP4114, MON810, MIR604 and NK603 in DP4114 x MON810 x MIR604 x NK603 stack genomic DNA contained in the verification samples.

<b>DP4114 GM %*</b> [(GM DNA / total maize DNA x 100)]	<b>MON810 GM %*</b> [(GM DNA / total maize DNA x 100)]
0.06	0.10
0.50	0.50
0.90	1.0
2.6	2.0
5.0	5.0
<b>MIR604 GM %*</b> [(GM DNA / total maize DNA x 100)]	<b>NK603 GM %*</b> [(GM DNA / total maize DNA x 100)]
0.10	0.10
0.40	0.50
0.90	1.0
2.5	2.0
6.0	5.0

\* percentage expressed in copy number ratio.

The protocols were implemented precisely in the EURL GMFF laboratory and were in accordance with the protocols already published for the individual DP4114, MON810 and MIR604 GM events (available at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>).

The protocol for NK603 event described in Annex 1 to EURL-VL-01/11VR was used (<https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-1507-59122-MON810-NK603%20.pdf>).

## 4.2 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 *hmg*, *high mobility group* gene, or *Adh1*, *alcohol dehydrogenase 1* gene. 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 DP4114, MON810, MIR604 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 the GM %.

## 4.3 PCR methods

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack DP4114 x MON810 x MIR604 x NK603 maize using the single detection methods previously validated for the respective single GM events DP4114, MON810, MIR604 and NK603.

For detection of GM maize events DP4114, MON810, MIR604 and NK603. DNA fragments of 80-bp, 92-bp, 76-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 for all four events. As a quencher dye at their 3'-end BHQ (Black Hole quencher) was used for DP4114 and TAMRA (6-carboxytetramethylrhodamine) for the other three events.

For quantification of GM maize events DP4114, MON810 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 and Minor Groove Binding (MGB) or TAMRA. For quantification of GM maize event MIR604, a taxon-specific reference system amplifies a 135-bp fragment of *alcohol dehydrogenase 1* gene (*Adh1*), a maize endogenous gene (GenBank AY691949), using two *Adh1* gene-specific primers and a gene-specific probe labelled with VIC® and TAMRA.

For the relative quantification of GM maize events DP4114, MON810 and NK603 standard curves are generated both for the DP4114, MON810 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 DP4114, MON810 and NK603 DNA is estimated.

For relative quantification of GM maize event MIR604 DNA in a test sample, the  $\Delta Cq$  values of calibration samples are used to calculate, by linear regression, a standard curve (plotting  $\Delta Cq$  values against the logarithm of the relative amount of MIR604 event DNA). The  $\Delta Cq$  values of

the unknown samples are measured and, by means of the regression formula, the relative amount of MIR604 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>.

#### 4.3.1 Deviations from the validated methods introduced by the EURL GMFF

No deviations from the original validated methods were introduced.

### 4.4 Results

Tables 3-6 present 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 DP4114, MON810, MIR604 and NK603. Slope values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the DP4114 method on GM stack DP4114 x MON810 x MIR604 x NK603 maize.

Run	DP4114			hmg		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.32	100	1.00	-3.36	99	1.00
2	-3.37	98	1.00	-3.36	98	1.00
3	-3.33	99	1.00	-3.36	98	1.00
4	-3.56	91	1.00	-3.22	105	1.00
5	-3.53	92	1.00	-3.38	98	1.00
6	-3.37	98	1.00	-3.41	96	1.00
7	-3.36	98	1.00	-3.38	98	1.00
8	-3.32	100	1.00	-3.39	97	1.00
Mean	-3.40	97	1.00	-3.36	99	1.00

Table 4. Values of standard curve slope. PCR efficiency and  $R^2$  coefficient for the MON810 method on GM stack DP4114 x MON810 x MIR604 x NK603 maize.

Run	MON810			<i>hmg</i>		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
<b>1</b>	-3.37	98	1.00	-3.42	96	1.00
<b>2</b>	-3.18	106	1.00	-3.40	97	1.00
<b>3</b>	-3.30	101	0.99	-3.35	99	1.00
<b>4</b>	-3.30	101	0.99	-3.39	97	1.00
<b>5</b>	-3.27	102	0.99	-3.33	100	1.00
<b>6</b>	-3.32	100	1.00	-3.35	99	1.00
<b>7</b>	-3.24	104	0.99	-3.29	101	1.00
<b>8</b>	-3.19	106	1.00	-3.33	100	1.00
<b>Mean</b>	<b>-3.27</b>	<b>102</b>	<b>1.00</b>	<b>-3.36</b>	<b>99</b>	<b>1.00</b>

Table 5. Values of standard curve slope. PCR efficiency and  $R^2$  coefficient for the MIR604 method on GM stack DP4114 x MON810 x MIR604 x NK603 maize.

Run	MIR604/ <i>Adh1</i>		
	Slope	PCR Efficiency (%)	$R^2$ coefficient
<b>1</b>	-3.35	99	1.00
<b>2</b>	-3.39	97	1.00
<b>3</b>	-3.24	103	1.00
<b>4</b>	-3.28	102	1.00
<b>5</b>	-3.33	100	1.00
<b>6</b>	-3.32	100	1.00
<b>7</b>	-3.30	101	1.00
<b>8</b>	-3.35	99	1.00
<b>Mean</b>	<b>-3.32</b>	<b>100</b>	<b>1.00</b>

Table 6. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the NK603 method on GM stack DP4114 x MON810 x MIR604 x NK603 maize.

Run	NK603			<i>hmg</i>		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.43	96	1.00	-3.38	98	1.00
2	-3.44	95	1.00	-3.38	98	1.00
3	-3.36	98	1.00	-3.34	99	1.00
4	-3.49	94	1.00	-3.35	99	1.00
5	-3.54	92	1.00	-3.36	98	1.00
6	-3.49	93	1.00	-3.38	98	1.00
7	-3.44	95	1.00	-3.37	98	1.00
8	-3.42	96	1.00	-3.36	98	1.00
Mean	-3.45	95	1.00	-3.36	98	1.00

The mean PCR efficiencies of the GM and species-specific systems were above 90 % (97 % for DP4114 and 99 % for *hmg*, 102 % for MON810 and 99 % for *hmg*, 100 % for MIR604/*Adh1* systems, 95 % for NK603 and 98 % for *hmg* systems, respectively). The mean  $R^2$  coefficient of the methods was 1.00 for all systems in all cases. The data presented in Tables 3-6 confirm the appropriate performance characteristics of the four methods when tested on GM stack DP4114 x MON810 x MIR604 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 DP4114 x MON810 x MIR604 x NK603 maize see Tables 7-10.

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ( $RSD_r$  %) of the DP4114 method applied to genomic DNA extracted from GM stack DP4114 x MON810 x MIR604 x NK603 maize.

DP4114					
Unknown sample GM %	Expected value (GMO %)				
	5.0	2.6	0.90	0.52	0.06
Mean	5.1	2.9	0.97	0.59	0.07
SD	0.29	0.21	0.06	0.05	0.01
$RSD_r$ (%)	5.7	7.5	6.6	9.0	19
Bias (%)	2.03	9.3	7.8	13	14

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD<sub>r</sub> %) of the MON810 method applied to genomic DNA extracted from GM stack DP4114 x MON810 x MIR604 x NK603 maize.

<b>MON810</b>					
<b>Unknown sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>5.0</b>	<b>2.0</b>	<b>1.0</b>	<b>0.50</b>	<b>0.10</b>
<b>Mean</b>	5.0	2.2	1.1	0.55	0.10
SD	0.16	0.17	0.04	0.04	0.01
RSD <sub>r</sub> (%)	3.1	7.8	3.6	8.1	7.9
Bias (%)	0.17	8.7	7.1	9.7	0.0

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD<sub>r</sub> %) of the MIR604 method applied to genomic DNA extracted from GM stack DP4114 x MON810 x MIR604 x NK603 maize.

<b>MIR604</b>					
<b>Unknown sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>6.0</b>	<b>2.5</b>	<b>0.90</b>	<b>0.40</b>	<b>0.10</b>
<b>Mean</b>	5.9	2.5	0.90	0.39	0.10
SD	0.25	0.12	0.05	0.02	0.01
RSD <sub>r</sub> (%)	4.3	4.9	5.9	4.6	6.1
Bias (%)	-1.5	0.66	-0.45	-2.9	-0.93

Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD<sub>r</sub> %) of the MON810 method applied to genomic DNA extracted from GM stack DP4114 x MON810 x MIR604 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>1.0</b>	<b>0.50</b>	<b>0.10</b>
<b>Mean</b>	4.9	2.1	1.1	0.55	0.11
SD	0.16	0.08	0.04	0.03	0.01
RSD <sub>r</sub> (%)	3.3	3.6	3.4	5.5	8.5
Bias (%)	-2.3	6.2	5.5	10	11

The trueness of the method is estimated using the measurements of the method's 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 7-10, the values range from 2.0 % to 14 % for DP4114, 0.0 % to 9.7 % for MON810, -2.9 % to 0.66 % for MIR604 and from -2.3 % to 11 % for NK603. Therefore, the four methods satisfy the above mentioned requirement throughout

their respective dynamic ranges, also when applied to DNA extracted from GM stack DP4114 x MON810 x MIR604 x NK603 maize.

Tables 7-10 also show the relative repeatability standard deviation (RSD<sub>r</sub>) estimated for each GM level. According to the ENGL acceptance criteria and method performance requirements, the RSD<sub>r</sub> values should be equal to or below 25 %. As the values range between 5.7 % and 19 % for DP4114, between 3.1 % and 8.1 % for MON810, between 4.3 % and 6.1 % for MIR604 and between 3.3 % and 8.5 % for NK603, the four methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack DP4114 x MON810 x MIR604 x NK603 maize.

## 5. Conclusions

The performance of the four event-specific methods for the detection and quantification of maize single line events DP4114, MON810, MIR604 and NK603, when applied to genomic DNA extracted from GM stack DP4114 x MON810 x MIR604 x NK603 maize, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant.

Therefore these methods, developed and validated to detect and quantify the single maize events DP4114, MON810, MIR604 and NK603, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack DP4114 x MON810 x MIR604 x NK603 maize or any of its sub-combinations supposed that sufficient genomic DNA of appropriate quality is available.

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