



# JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of  
MON89034, 1507, NK603, MIR162 and DAS-  
40278-9 event-specific PCR-based Methods  
applied to DNA extracted from GM Stack  
MON89034 x 1507 x NK603 x MIR162 x DAS-  
40278-9 maize

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# **Report on the Verification of the Performance of MON89034, 1507, NK603, MIR162 and DAS-40278-9 event-specific PCR-based Methods applied to DNA extracted from GM Stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize**

**15 October 2020**

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

## **Executive Summary**

An application was submitted by DowAgroSciences Ltd to request the authorisation of genetically modified stack (GM stack) MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize (resistant against certain insect pests and tolerant to application of 2,4-D, glufosinate-ammonium and glyphosate herbicides) 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 MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize is MON-89034-3 x DAS-01507-1 x MON-00603-6 x SYN-IR162-4 x DAS-40278-9.

The GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize has been obtained by conventional crossing between the genetically modified maize events: MON89034, 1507, NK603, MIR162 and DAS-40278-9, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events MON89034, 1507, NK603, MIR162 and DAS-40278-9 (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 MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 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 MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 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 MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 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)

DowAgroSciences Ltd 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 MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 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 RSD<sub>r</sub> %) calculated by the applicant for the five methods applied to MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize genomic DNA. Means are the average of sixteen replicates obtained through five runs for event MON89034, or eight runs for events 1507, NK603, MIR162 and DAS-40278-9. The runs were performed with ABI 7500 real-time PCR equipment for events MON89034, 1507, MIR162 and DAS-40278-9 and with ABI 7900HT real-time PCR equipment for event NK603. Individual GM-values were obtained by averaging triplicates. 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  $\leq 1$ , 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, RSD<sub>r</sub> %) provided by the applicant for the MON89034, 1507, NK603, MIR162 and DAS-40278-9 methods applied to GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

MON89034 *				
Sample GM %	Expected value (GMO %)			
	5.00	0.900	0.100	0.085
Mean	4.95	0.843	0.083	0.077
RSD <sub>r</sub> (%)	2.6	3.2	9.0	11.7
Bias (%)	-1.0	-6.3	-17	-9.4
1507 *				
Sample GM %	Expected value (GMO %)			
	5.00	0.900	0.100	0.085
Mean	5.38	0.888	0.095	0.079
RSD <sub>r</sub> (%)	9.1	7.7	17.5	18.6
Bias (%)	7.6	-1.3	-5.0	-7.1
NK603 *				
Sample GM %	Expected value (GMO %)			
	5.00	0.900	0.100	0.085
Mean	4.94	0.825	0.082	0.068
RSD <sub>r</sub> (%)	6.7	6.0	13.5	13.3
Bias (%)	-1.2	-8.3	-18.0	-20.0

<b>MIR162 *</b>				
<b>Sample GM %</b>	<b>Expected value (GMO %)</b>			
	<b>5.00</b>	<b>0.900</b>	<b>0.100</b>	<b>0.085</b>
<b>Mean</b>	4.84	0.949	0.102	0.091
<b>RSD<sub>r</sub> (%)</b>	11.0	12.1	14.7	13.8
<b>Bias (%)</b>	-3.2	5.4	2.0	7.1
<b>DAS-40278-9 *</b>				
<b>Sample GM %</b>	<b>Expected value (GMO %)</b>			
	<b>5.00</b>	<b>0.900</b>	<b>0.100</b>	<b>0.085</b>
<b>Mean</b>	5.28	0.937	0.096	0.085
<b>RSD<sub>r</sub> (%)</b>	7.0	6.5	17.8	12.8
<b>Bias (%)</b>	5.6	4.1	-4.0	0.0

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

The applicant applied the following modifications to the validated methods:

- For MON 89034 the validated method was verified in combination with the taxon-specific reference system *hmg* described in the validation protocol of MON 87460 ([http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27\\_MON87460\\_validated\\_Method.pdf](http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27_MON87460_validated_Method.pdf)). The EURL GMFF had already approved the substitution of the taxon-specific method module in the verification study for other GM events (EURL-VL-01/11 <http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-1507-59122-MON810-NK603%20.pdf>).
- For 1507 the single component reaction mix used for the validated 1507 method was replaced by the Sso Advanced™ Universal Probes Supermix (Bio-Rad) supplemented with Bovine Serum Albumin. The EURL GMFF had already approved the modification of the reaction conditions through the verification study EURL-VL-04/15 (<http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-04-15-VR.pdf>).
- For NK603 the validated method for NK603 was verified in a final reaction volume of 25 µL in combination with the taxon-specific reference system *hmg*, according to the reaction conditions described in the validation protocol of MON 87460 ([http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27\\_MON87460\\_validated\\_Method.pdf](http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27_MON87460_validated_Method.pdf)). The EURL GMFF had already approved the modification of the reaction conditions through the verification study EURL-VL-01/11 (<http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-1507-59122-MON810-NK603%20.pdf>).

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>.

One request of complementary information regarding the control samples and DNA sequences was addressed to 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 three.



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

In step 3 the EURL GMFF implemented the five methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

### 4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA of GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize, hemizygous for the loci, as positive control sample.
- genomic DNA of a conventional (non-GM) maize near isogenic line that has a comparable genetic background, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 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 MON89034, 1507, NK603, MIR162 and DAS-40278-9 methods when applying them to genomic DNA extracted from the GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

Table 2. Percentage (GM %) of MON89034, 1507, NK603, MIR162 and DAS-40278-9 in MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 stack genomic DNA contained in the verification samples.

<b>MON89034 GM %*</b> [(GM DNA / total maize DNA x 100)]	<b>1507 GM %*</b> [(GM DNA / total maize DNA x 100)]	<b>NK603 GM %*</b> [(GM DNA / total maize DNA x 100)]	<b>MIR162 GM %*</b> [(GM DNA / total maize DNA x 100)]	<b>DAS-40278-9 GM %*</b> [(GM DNA / total maize DNA x 100)]
0.09	0.10	0.10	0.10	0.10
0.40	0.50	0.50	0.40	0.40
0.90	0.90	0.90	0.90	0.90
4.0	2.0	2.0	2.0	2.0
8.0	5.0	5.0	5.0	5.0

\* percentage expressed in copy number ratio.

The protocols described by the applicant were implemented precisely in the EURL GMFF laboratory and were in accordance with the protocols already published for the individual MIR162 and DAS-40278-9 GM events (available at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). The protocols for MON 89034, 1507 and NK603 were implemented with the modifications described 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 quantitative real-time PCR methods developed for MON89034, 1507, NK603, MIR162 and DAS-40278-9 *"utilize real-time TaqMan PCR methodology; the methods for event-specific quantification of MON89034, 1507, NK603, MIR162, and DAS-40278-9 in the combined trait MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 were in-house validated using DNA derived from homogenized maize seeds, however, in principle, the methods can be applied to any sample from which sufficient quantities of inhibition-free genomic maize DNA can be purified"*.

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/TC1507-DNAextrc.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 *hmg*, *high mobility group*, or *adh1*, *alcohol dehydrogenase 1*. 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, 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 %.

## 4.4 PCR methods

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize using the single detection methods previously validated for the respective single GM events MON89034, 1507, NK603, MIR162 and DAS-40278-9.

For detection of GM maize events MON89034, 1507, NK603, MIR162 and DAS-40278-9, DNA fragments of 77-bp, 58-bp, 108-bp, 92-bp and 98-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 MGB-NFQ (minor groove binder 3' nonfluorescent Quencher) as a quencher dye at their 3'-end for MON89034, and FAM and TAMRA (carboxytetramethylrhodamine) for 1507, NK603, MIR162 and DAS-40278-9 events.

For quantification of GM maize events MON89034, 1507, NK603 and DAS-40278-9, 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 TAMRA. For quantification of GM event MIR162, a taxon-specific reference system amplifies a 135-bp fragment of *alcohol dehydrogenase 1 (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 MON89034, 1507, NK603 and DAS-40278-9 standard curves are generated both for the MON89034, 1507, NK603 and DAS-40278-9 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 MON89034, 1507, NK603 and DAS-40278-9 DNA is estimated.

For relative quantification of GM maize event MIR162 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 MIR162 event DNA). The  $\Delta Cq$  values of the unknown samples are measured and, by means of the regression formula, the relative amount of MIR162 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>.

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

No deviations from the original validated methods were introduced for MIR162 and DAS-40278-9 events.

The validated method for MON89034 was verified in combination with the taxon-specific reference system *hmg*, according to the reaction conditions described in the validation protocol of MON 87460 ([http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27\\_MON87460\\_validated\\_Method.pdf](http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27_MON87460_validated_Method.pdf)). The EURL GMFF had already approved the modification of the reaction conditions with the verification study EURL-VL-04/13 ([http://gmo-crl.jrc.ec.europa.eu/summaries/JRC102290\\_EURL-VL-04-13-VR.pdf](http://gmo-crl.jrc.ec.europa.eu/summaries/JRC102290_EURL-VL-04-13-VR.pdf)).

The 1507 method was verified according to the reaction conditions using the method modified for the substitution of the 1507 method's 10 x PCR buffer with the Bio-Rad SsoAdvanced™ Universal Probes Supermix, as verified in the context of the maize stacked event verification (bridging study) EURL-VL-04/15VR (<http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-04-15-VR.pdf>), see <http://gmo-crl.jrc.ec.europa.eu/summaries/TC1507-WEB-Protocol-Validation-VERSION-B.pdf> for a detailed description of the modified method.

The validated method for NK603 was verified in a final reaction volume of 25 µl in combination with the taxon-specific reference system *hmg*, according to the reaction conditions described in the validation protocol of MON 87460 ([http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27\\_MON87460\\_validated\\_Method.pdf](http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27_MON87460_validated_Method.pdf)). The EURL GMFF had already approved the modification of the reaction conditions with the verification study EURL-VL-01/11 (<http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-1507-59122-MON810-NK603%20.pdf>).

### **4.5 Results**

Tables 3-7 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 MON89034, 1507, NK603, MIR162 and DAS-40278-9. Slope and  $R^2$  values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the MON89034 method on GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

Run	MON89034			<i>hmg</i>		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.46	94	1.00	-3.41	97	1.00
2	-3.52	92	1.00	-3.40	97	1.00
3	-3.36	98	1.00	-3.38	98	1.00
4	-3.48	94	1.00	-3.37	98	1.00
5	-3.39	97	1.00	-3.44	95	1.00
6	-3.37	98	1.00	-3.37	98	1.00
7	-3.39	97	1.00	-3.36	99	1.00
8	-3.39	97	1.00	-3.39	97	1.00
Mean	-3.42	96	1.00	-3.39	97	1.00

Table 4. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the 1507 method on GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

Run	1507			<i>hmg</i>		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.52	92	1.00	-3.48	94	1.00
2	-3.42	96	1.00	-3.47	94	1.00
3	-3.35	99	1.00	-3.50	93	1.00
4	-3.47	94	1.00	-3.43	96	1.00
5	-3.41	96	1.00	-3.52	92	1.00
6	-3.39	97	1.00	-3.41	96	1.00
7	-3.38	98	1.00	-3.46	95	1.00
8	-3.43	96	1.00	-3.43	96	1.00
Mean	-3.42	96	1.00	-3.46	94	1.00

Table 5. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the NK603 method on GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

Run	NK603			hmg		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.49	94	1.00	-3.33	100	1.00
2	-3.41	96	1.00	-3.36	98	1.00
3	-3.48	94	1.00	-3.32	100	1.00
4	-3.42	96	1.00	-3.33	100	1.00
5	-3.34	99	1.00	-3.36	98	1.00
6	-3.46	94	0.99	-3.35	99	1.00
7	-3.47	94	1.00	-3.39	97	1.00
8	-3.48	94	1.00	-3.35	99	1.00
Mean	-3.45	95	1.00	-3.35	99	1.00

Table 6. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the MIR162 method on GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

Run	MIR162		
	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.31	101	1.00
2	-3.36	98	1.00
3	-3.39	97	1.00
4	-3.41	96	1.00
5	-3.27	102	1.00
6	-3.37	98	1.00
7	-3.33	100	1.00
8	-3.39	97	1.00
Mean	-3.35	99	1.00

Table 7. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the DAS-40278-9 method on GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

Run	DAS-40278-9			<i>hmg</i>		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.31	100	1.00	-3.44	95	1.00
2	-3.40	97	1.00	-3.43	96	1.00
3	-3.44	95	1.00	-3.39	97	1.00
4	-3.45	95	1.00	-3.43	96	1.00
5	-3.50	93	1.00	-3.43	96	1.00
6	-3.43	96	1.00	-3.42	96	1.00
7	-3.53	92	1.00	-3.43	96	1.00
8	-3.51	93	1.00	-3.43	96	1.00
Mean	-3.45	95	1.00	-3.43	96	1.00

The mean PCR efficiencies of the GM and species-specific systems were above 90 % (96 % and 97 % for MON89034 and *hmg* systems; 96 % and 94 % for 1507 and *hmg* systems; 95 % and 99 % for NK603 and *hmg* systems; 99 % for MIR162 and *adh1* systems, and 95 % and 96 % for DAS-40278-9 and *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 3-7 confirm the appropriate performance characteristics of the five methods when tested on GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 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 five methods applied to samples of DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize see Tables 8-12.

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD<sub>r</sub> %) of the MON89034 method applied to genomic DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

<b>MON89034</b>					
<b>Unknown sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>8.0</b>	<b>4.0</b>	<b>0.90</b>	<b>0.40</b>	<b>0.09</b>
<b>Mean</b>	8.1	4.0	0.92	0.40	0.09
SD	0.25	0.17	0.04	0.03	0.01
RSD <sub>r</sub> (%)	3.1	4.4	4.4	7.7	13
Bias (%)	0.85	-1.0	2.5	0.59	-4.1

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD<sub>r</sub> %) of the 1507 method applied to genomic DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 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>	5.1	2.2	0.98	0.57	0.12
SD	0.30	0.15	0.05	0.05	0.02
RSD <sub>r</sub> (%)	5.9	6.6	5.3	9.2	12
Bias (%)	1.5	12	8.8	14	23

Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD<sub>r</sub> %) of the NK603 method applied to genomic DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

<b>NK603</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>	5.0	2.2	0.96	0.53	0.11
SD	0.13	0.10	0.04	0.03	0.01
RSD <sub>r</sub> (%)	2.6	4.8	4.6	4.9	8.1
Bias (%)	0.31	8.1	6.7	5.5	9.6



Table 11. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD<sub>r</sub> %) of the MIR162 method applied to genomic DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

<b>MIR162</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>	4.8	2.0	0.89	0.38	0.11
SD	0.21	0.11	0.05	0.02	0.01
RSD <sub>r</sub> (%)	4.3	5.4	5.1	6.3	11
Bias (%)	-3.3	0.55	-1.1	-4.5	6.7

Table 12. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD<sub>r</sub> %) of the DAS-40278-9 method applied to genomic DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

<b>DAS-40278-9</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>	5.2	2.2	0.95	0.41	0.11
SD	0.12	0.06	0.04	0.03	0.01
RSD <sub>r</sub> (%)	2.2	2.7	4.4	7.5	6.6
Bias (%)	3.3	11	6.1	3.3	13

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 8-12, the values range from -4.1 % to 2.5 % for MON89034, from 1.5 % to 23 % for 1507, from 0.31 % to 9.6 % for NK603, from -4.5 % to 6.7 % for MIR162 and from 3.3 % to 13 % for DAS-40278-9. Therefore, the five methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

Tables 8-12 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 3.1 % and 13 % for MON89034, between 5.3 % and 12 % for 1507, between 2.6 % and 8.1 % for NK603, between 4.3 % and 11 % for MIR162 and between 2.2 % and 7.5 % for DAS-40278-9, the five methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize.

## 5. Conclusions

The performance of the five event-specific methods for the detection and quantification of maize single line events MON89034, 1507, NK603, MIR162 and DAS-40278-9, when applied to genomic DNA extracted from GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 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 MON89034, 1507, NK603, MIR162 and DAS-40278-9, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack MON89034 x 1507 x NK603 x MIR162 x DAS-40278-9 maize or any of its sub-combinations, supposed that sufficient genomic DNA of appropriate quality is available.

## 6. References

1. Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed (Text with EEA relevance). OJ L 268, 18.10.2003, p. 1–23.
2. Regulation (EU) No 503/2013 of 3 April 2013 "on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006".
3. European Network of GMO Laboratories (ENGL), 'Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing', 2015. [http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020\\_10\\_2015.pdf](http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf).

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