



# JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of  
3272, Bt11, MIR604, 1507, 5307 and GA21  
event-specific PCR-based Methods applied to DNA  
extracted from GM Stack 3272 x Bt11 x MIR604 x  
1507 x 5307 x GA21 maize

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# **Report on the Verification of the Performance of 3272, Bt11, MIR604, 1507, 5307 and GA21 event-specific PCR-based Methods applied to DNA extracted from GM Stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize**

**12/5/2020**

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

## **Executive Summary**

An application was submitted by Syngenta Crop Protection NV/SA to request the authorisation of genetically modified stack (GM stack) 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize (traits to enhance ethanol production and to control certain insect pests and weeds) 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 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize is SYN-E3272-5 x SYN-BTØ11-1 x SYN-IR6Ø4-5 x DAS-Ø15Ø7-1 x SYN-Ø53Ø7-1 x MON-ØØØ21-9.

The GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize was obtained by conventional crossing between the genetically modified maize events: 3272, Bt11, MIR604, 1507, 5307 and GA21, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events 3272, Bt11, MIR604, 1507, 5307 and GA21 (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 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 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 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 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.

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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 combined by conventional crossing. These criteria are met for the GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 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)

Syngenta Crop Protection NV/SA 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 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 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 six methods applied to 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize genomic DNA. Means are the average of sixteen replicates obtained through four runs (five runs for the 5 % 1507 sample) performed with Applied Biosystems 7900HT Fast Real-Time PCR System real-time PCR equipment. 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 3272, Bt11, MIR604, 1507, 5307 and GA21 methods applied to GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

3272 *			
Sample GM%	Expected value (GMO %)		
	5	0.9	0.08
Mean	4.96	0.951	0.089
RSD <sub>r</sub> (%)	13.1	14.9	14.5
Bias (%)	-0.8	5.7	11.3
Bt11 *			
Sample GM %	Expected value (GMO %)		
	5	0.9	0.08
Mean	4.92	0.904	0.089
RSD <sub>r</sub> (%)	11.9	9.0	16.7
Bias (%)	-1.6	0.4	11.3
MIR604 *			
Sample GM %	Expected value (GMO %)		
	5	0.9	0.08
Mean	4.79	0.893	0.080
RSD <sub>r</sub> (%)	11.0	14.0	11.1
Bias (%)	-4.2	-0.8	0.0

<b>1507 *</b>			
<b>Sample GM %</b>	<b>Expected value (GMO %)</b>		
	<b>5</b>	<b>0.9</b>	<b>0.08</b>
<b>Mean</b>	4.51	0.746	0.061
RSD <sub>r</sub> (%)	8.3	12.4	20.7
Bias (%)	-9.8	-17.1	-23.8
<b>5307 *</b>			
<b>Sample GM %</b>	<b>Expected value (GMO %)</b>		
	<b>5</b>	<b>0.9</b>	<b>0.08</b>
<b>Mean</b>	4.85	0.868	0.084
RSD <sub>r</sub> (%)	8.1	7.7	11.5
Bias (%)	-3.0	-3.6	5.0
<b>GA21 *</b>			
<b>Sample GM %</b>	<b>Expected value (GMO %)</b>		
	<b>5</b>	<b>0.9</b>	<b>0.08</b>
<b>Mean</b>	4.71	0.864	0.083
RSD <sub>r</sub> (%)	10.7	12.2	16.3
Bias (%)	-5.8	-4.0	3.8

\* 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>.

One request of complementary information regarding the method, the control samples and the 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 3.

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

In step 3 the EURL GMFF implemented the six methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

### 4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA prepared from homogenised seeds of GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize, hemizygous for the loci, as positive control sample;



- genomic DNA prepared from homogenised seeds of conventional (non-GM) maize whose genetic background is comparable to the one of 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 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 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 3272, Bt11, MIR604, 1507, 5307 and GA21 methods when applying them to genomic DNA extracted from the GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

Table 2. Percentage (GM %) of 3272, Bt11, MIR604, 1507, 5307 and GA21 in 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 stack genomic DNA contained in the verification samples.

<b>3272 GM %*</b> [[GM DNA / total maize DNA x 100]]	<b>Bt11 GM %*</b> [[GM DNA / total maize DNA x 100]]	<b>MIR604 GM %*</b> [[GM DNA / total maize DNA x 100]]
0.09	0.09	0.10
0.40	0.40	0.40
0.90	0.90	0.90
5.0	5.0	2.5
8.0	8.0	6.0
<b>1507 GM %*</b> [[GM DNA / total maize DNA x 100]]	<b>5307 GM %*</b> [[GM DNA / total maize DNA x 100]]	<b>GA21 GM %*</b> [[GM DNA / total maize DNA x 100]]
0.10	0.04	0.09
0.50	0.10	0.50
0.90	0.90	0.90
2.0	2.5	5.0
5.0	5.0	8.0

\* percentage expressed in copy number ratio.

The applicant introduced the deviations to the protocols described in §4.4.1. The EURL GMFF implemented the protocols already published for the individual 3272, Bt11, MIR604, 1507, 5307 and GA21 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 3272, Bt11, MIR604, 1507, 5307 and GA21 depends on the isolation of sufficient quantity and quality of purified DNA. The DNA extraction method provided is intended for extraction of genomic DNA from seeds, grain and flour, which results in primarily high molecular weight DNA. The applicant also informed the EURL GMFF that many types of grain processing would denature DNA, resulting in degradation, reduction, or even elimination of DNA from a given type of processed material. In addition, different manufacturing processes for the same product can lead to various degrees of degradation of DNA. For example, DDGS (Distillers Dried Grains with Solubles) from different plants have variable amounts of DNA present after processing. Finally, to ensure high quality results, all DNA samples used for event-specific real-time PCR should be monitored for intactness and concentration prior to running any analysis.

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/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 *adh1*, alcohol dehydrogenase 1 gene for 3272, Bt11, MIR604, 5307 and GA21 events, and *hmg*, high mobility group gene for 1507. 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 3272, Bt11, MIR604, 1507, 5307 and GA21, 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 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize using the single detection methods previously validated for the respective single GM events 3272, Bt11, MIR604, 1507, 5307 and GA21.

For detection of GM maize events 3272, Bt11, MIR604, 1507, 5307 and GA21, DNA fragments of 95-bp, 68-bp, 76-bp, 58-bp, 107-bp and 101-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 a

reporter at its 5'-end, and TAMRA (carboxytetramethylrhodamine) as a quencher dye at their 3'-end for all six events.

For quantification of GM maize events 3272, Bt11, MIR604, 5307 and GA21, a taxon-specific reference system amplifies a 135-bp fragment of *adh1*, alcohol dehydrogenase 1 gene, a maize endogenous gene (GenBank X04050), using two *adh1* gene-specific primers and a gene-specific probe labelled with VIC as reporter and TAMRA as quencher dye. For quantification of GM maize event 1507, a taxon-specific reference system amplifies a 79-bp fragment of *hmg*, high mobility group gene, another maize endogenous gene (GenBank AJ131373.1), using two *hmg* gene-specific primers and a gene-specific probe labelled with FAM and TAMRA.

For relative quantification of GM maize events 3272, Bt11, MIR604, 5307 and GA21 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 3272, Bt11, MIR604, 5307 and GA21 events DNA). The  $\Delta C_q$  values of the unknown samples are measured and, by means of the regression formula, the relative amount of 3272, Bt11, MIR604, 5307 and GA21 events is estimated.

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

The following deviations from the original validated methods were introduced by the applicant:

- the sulforhodamine concentration specified in GA21 and MIR604 validated methods was doubled in order to get passive reference fluorescence values clearly above the background for the ABI 7900HT real-time PCR system used in the testing. The new sulforhodamine concentration corresponds to the one specified in Bt11 and 3272 event-specific methods;
- the Applied Biosystems ROX used in the 1507 validated method had to be replaced by ROX Reference Dye (50X) produced by Life Technologies; the ROX volume per reaction was adjusted in order to reach a comparable final ROX concentration;
- an extended calibration curve (10 % to 0.07 %) was used for 3272, Bt11, MIR604, 5307, and GA21 event-specific methods since the 0.08 % 3272 × Bt11 × MIR604 × 1507 × 5307 × GA21 sample was located outside the linear working range of the calibration curves in the validated methods.

The following deviations from the original validated methods were introduced by the EURL GMFF:

- the sulforhodamine concentration specified in the protocols for the GA21 and MIR604 methods was doubled in order to get passive reference fluorescence values clearly above the background; this sulforhodamine concentration used corresponds to the one specified in Bt11 and 3272 event-specific methods;
- the 1507 method verification was done using the method modified for the substitution of the 10x 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 <sup>(4)</sup> (see Annex I for a detailed description of the modified method).

## 4.5 Results

Tables from 3 to 8 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 3272, Bt11, MIR604, 1507, 5307 and GA21. Slope values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the 3272 method on GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

Run	3272		
	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.34	99	1.00
2	-3.30	101	1.00
3	-3.35	99	1.00
4	-3.21	105	1.00
5	-3.22	104	1.00
6	-3.39	97	1.00
7	-3.30	101	1.00
8	-3.26	103	1.00
Mean	-3.30	101	1.00

Table 4. Values of standard curve slope, PCR efficiency and R<sup>2</sup> coefficient for the Bt11 method on GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

Run	Bt11		
	Slope	PCR Efficiency (%)	R <sup>2</sup> coefficient
1	-3.30	101	1.00
2	-3.23	104	1.00
3	-3.26	102	1.00
4	-3.28	102	1.00
5	-3.20	105	1.00
6	-3.28	102	1.00
7	-3.17	107	0.99
8	-3.29	101	1.00
Mean	-3.25	103	1.00

Table 5. Values of standard curve slope, PCR efficiency and R<sup>2</sup> coefficient for the MIR604 method on GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

Run	MIR604		
	Slope	PCR Efficiency (%)	R <sup>2</sup> coefficient
1	-3.30	101	1.00
2	-3.22	105	1.00
3	-3.25	103	1.00
4	-3.17	107	1.00
5	-3.43	96	1.00
6	-3.19	106	1.00
7	-3.26	103	1.00
8	-3.33	100	1.00
Mean	-3.27	102	1.00

Table 6. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the 1507 method on GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

Run	1507			hmg		
	Slope	PCR Efficiency (%)	$R^2$ coefficient	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.53	92	1.00	-3.53	92	1.00
2	-3.52	92	1.00	-3.55	91	1.00
3	-3.60	90	1.00	-3.53	92	1.00
4	-3.70	86	1.00	-3.52	92	1.00
5	-3.56	91	1.00	-3.50	93	1.00
6	-3.57	91	1.00	-3.50	93	1.00
7	-3.59	90	1.00	-3.51	93	1.00
8	-3.67	87	1.00	-3.55	91	1.00
Mean	-3.59	90	1.00	-3.52	92	1.00

Table 7. Values of standard curve slope, PCR efficiency and  $R^2$  coefficient for the 5307 method on GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

Run	5307		
	Slope	PCR Efficiency (%)	$R^2$ coefficient
1	-3.44	95	1.00
2	-3.36	99	1.00
3	-3.36	98	1.00
4	-3.44	95	1.00
5	-3.38	98	1.00
6	-3.35	99	1.00
7	-3.41	97	1.00
8	-3.36	98	1.00
Mean	-3.39	97	1.00

Table 8. Values of standard curve slope, PCR efficiency and R<sup>2</sup> coefficient for the GA21 method on GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

Run	GA21		
	Slope	PCR Efficiency (%)	R <sup>2</sup> coefficient
1	-3.31	101	1.00
2	-3.27	102	1.00
3	-3.36	98	1.00
4	-3.21	105	1.00
5	-3.39	97	1.00
6	-3.30	101	1.00
7	-3.36	99	1.00
8	-3.35	99	1.00
Mean	-3.32	100	1.00

The mean PCR efficiencies of the GM and species-specific systems were at or above 90 % (101 % for 3272 system, 103 % for Bt11, 102 % for MIR604, 90 % for 1507 and 92 % for *hmg*, 97 % for 5307, and 100 % for GA21, respectively). The mean R<sup>2</sup> coefficient of the methods was 1.00 for all systems in all cases. The data presented in Tables 3-8 confirm the appropriate performance characteristics of the six methods when tested on GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize in terms of PCR efficiency and R<sup>2</sup> coefficient.

The EURL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSD<sub>r</sub> %) of the six methods applied to samples of DNA extracted from GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize see Tables 9-14.

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD<sub>r</sub> %) of the 3272 method applied to genomic DNA extracted from GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

3272					
Unknown sample GM %	Expected value (GMO %)				
	8.0	5.0	0.90	0.40	0.09
Mean	8.3	5.1	0.94	0.40	0.09
SD	0.79	0.43	0.07	0.04	0.01
RSD <sub>r</sub> (%)	9.5	8.4	6.9	11	12
Bias (%)	3.5	2.8	4.5	-0.75	-1.6

Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD<sub>r</sub> %) of the Bt11 method applied to genomic DNA extracted from GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

<b>Bt11</b>					
<b>Unknown sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>8.0</b>	<b>5.0</b>	<b>0.90</b>	<b>0.40</b>	<b>0.09</b>
<b>Mean</b>	8.5	5.3	0.87	0.39	0.09
SD	0.83	0.67	0.07	0.05	0.01
RSD <sub>r</sub> (%)	9.7	13	7.7	12	14
Bias (%)	6.8	5.9	-3.3	-3.4	-3.7

Table 11. 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 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 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>	6.1	2.5	0.90	0.39	0.10
SD	0.74	0.26	0.08	0.03	0.01
RSD <sub>r</sub> (%)	12	10	8.4	7.2	10
Bias (%)	1.0	1.1	0.09	-2.5	3.8

Table 12. 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 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 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>	5.0	2.0	0.99	0.53	0.12
SD	0.30	0.20	0.11	0.09	0.03
RSD <sub>r</sub> (%)	6.0	10	11	17	22
Bias (%)	0.29	2.0	10	5.2	16



Table 13. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ( $RSD_r$  %) of the 5307 method applied to genomic DNA extracted from GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

<b>5307</b>					
<b>Unknown sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>5.0</b>	<b>2.5</b>	<b>0.90</b>	<b>0.10</b>	<b>0.04</b>
<b>Mean</b>	5.1	2.6	0.93	0.10	0.04
SD	0.28	0.12	0.04	0.01	0.01
$RSD_r$ (%)	5.5	4.6	4.0	9.2	13
Bias (%)	1.8	3.8	2.8	0.23	11

Table 14. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ( $RSD_r$  %) of the GA21 method applied to genomic DNA extracted from GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

<b>GA21</b>					
<b>Unknown sample GM %</b>	<b>Expected value (GMO %)</b>				
	<b>8.0</b>	<b>5.0</b>	<b>0.90</b>	<b>0.50</b>	<b>0.09</b>
<b>Mean</b>	7.9	4.9	0.92	0.53	0.09
SD	0.75	0.46	0.08	0.06	0.01
$RSD_r$ (%)	9.5	9.4	8.3	11	14
Bias (%)	-0.66	-2.7	1.8	6.8	3.5

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 9-14, the values range from -1.6 % to 4.5 % for 3272, from -3.7 % to 6.8 % for Bt11, from -2.5 % to 3.8 % for MIR604, from 0.29 % to 16 % for 1507, from 0.23 % to 11 % for 5307 and from -2.7 % to 6.8 % for GA21. Therefore, the six methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

Tables 9-14 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 6.9 % and 12 % for 3272, between 7.7 % and 14 % for Bt11, between 7.2 % and 12 % for MIR604, between 6.0 % and 22 % for 1507, between 4.0 % and 13 % for 5307 and between 8.3 % and 14 % for GA21, the six methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize.

## 5. Conclusions

The performance of the six event-specific methods for the detection and quantification of maize single line events 3272, Bt11, MIR604, 1507, 5307 and GA21, when applied to genomic DNA extracted from GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 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 3272, Bt11, MIR604, 1507, 5307 and GA21, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 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).
4. 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, <http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-04-15-VR.pdf>

## Annex 1: modified 1507 real-time PCR method

The verification study for 1507 method, applied to GM stack 3272 x Bt11 x MIR604 x 1507 x 5307 x GA21 maize, was performed by the EURL GMFF using a modified 1507 method already verified in the context of the maize stacked event verification (bridging study) EURL-VL-04/15VR (1507 x MON 810 x MIR 162 x NK603 maize <sup>(4)</sup>). The real-time PCR reaction mixes (Tables 1 and 2) and the cycling conditions (Table 3) for the modified 1507 method are described below.

Table 1. Amplification reaction mix in the final volume/concentration per reaction well for the 1507 assay.

Component	Final concentration	µL/reaction
SsoAdvanced™ Universal Probes Supermix (2x)	1x	12.5
Primer MaiY-F1 (10 µM)	300 nM	0.75
Primer MaiY-R3 (10 µM)	300 nM	0.75
Probe MaiY-S1 (10 µM)	150 nM	0.38
30 % Bovine Serum Albumin	0.08 %	0.068
Nuclease free water	-	5.55
DNA (max 200 ng)	-	5
Total reaction volume:		25 µL

Table 2. Amplification reaction mix in the final volume/concentration per reaction well for the maize *hmg* assay.

Component	Final concentration	µL/reaction
SsoAdvanced™ Universal Probes Supermix (2x)	1x	12.5
Primer MaiJ-F1 (10 µM)	300 nM	0.75
Primer mhmg-rev (10 µM)	300 nM	0.75
Probe mhmg (10 µM)	180 nM	0.45
30 % Bovine Serum Albumin	0.08 %	0.068
Nuclease free water	-	5.48
DNA (max 200 ng)	-	5
Total reaction volume:		25 µL

Table 3. Cycling program for the modified 1507/*hmg* assays.

Step	Stage	T (°C)	Time (s)	Acquisition	Cycles
1	Initial denaturation	95	180	No	1
2	Amplification	95	15	No	45
		60	30	Yes	

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