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Report on the Verification of the Performance of Bt11, MIR162, MIR604, TC1507, 5307 and GA21 Event-specific PCR-based Methods applied to DNA Extracted from GM Stack Maize Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21

European Union Reference Laboratory for Genetically Modified Food and Feed

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Joint Research Centre

EURL European Union Reference Laboratory for GM Food & Feed

European Commission
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Institute for Health and Consumer Protection

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6 March 2015

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Syngenta Crop Protection AG to request the authorisation of genetically modified (GM stack) maize Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 (with a set of proteins and truncated proteins for protection or control against certain lepidopteran and coleopteran pests and glyphosate-tolerance) and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, and import and processing, in accordance with articles 5 and 17 of Regulation (EC) No 1829/2003 on GM Food and Feed. The unique identifier assigned to the GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize is SYN-BTØ11-1 x SYN-IR162-4 x SYN-IR6Ø4-5 x DAS-Ø15Ø7-1 x SYN-Ø53Ø7-1 x MON-ØØØ21-9.

The GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize has been obtained by conventional crossing of six genetically modified single line maize events: Bt11, MIR162, MIR604, TC1507, 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 line maize events Bt11, MIR162, MIR604, TC1507, 5307 and GA21 and has published the corresponding reports (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) the EURL GMFF therefore has carried out only an *in-house* verification of the performance of each validated method when applied to DNA extracted from the GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

The hereby reported *in-house* verification study led to the conclusion that the individual methods meet the ENGL requirements also when applied to DNA extracted from the GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

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

Quality assurance

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

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

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

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

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Content

1. Introduction

The EU legislative system [1, 2] for genetically modified food and feed provides that any GMO for food and feed use shall undergo 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 (GM 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 of 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, which 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) the EURL GMFF carries out an *in-house* verification of the performance of each event-specific method 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 Bt11 x MIR162 x MIR604 x TC1507 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 (EC) No 641/2004 (Annex I).

The results of the *in-house* verification study were evaluated with reference to ENGL method performance requirements [3] and to the validation results on the individual events.

2. Step 1 (dossier reception and acceptance)

Syngenta Crop Protection AG submitted the detection methods and the corresponding control DNA samples extracted from the GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 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 [3] and with regard to their documentation and reliability.

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSDr %) calculated on the basis of the data provided by the applicant for the six methods on the stack

DNA. Means are the average of sixteen replicates obtained through four runs (five runs for TC1507) on ABI 7900 HT. Percentages are expressed as copy number of GM DNA / copy number of non-GM DNA × 100.

Table 1. Estimates of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSD_r %) provided by the applicant for the Bt11, MIR162, MIR604, TC1507, 5307 and GA21 methods applied to GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

Bt11			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.075	0.79	4.4
RSD_r (%)	22.1	5.8	10.0
Bias (%)	-6.3	-12.2	-12.0
MIR162			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.084	0.82	4.6
RSD_r (%)	22.4	11.6	10.9
Bias (%)	5.0	-8.9	-8.0
MIR604			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.077	0.81	4.6
RSD_r (%)	19.9	8.3	8.5
Bias (%)	-3.8	-10.0	-8.0
TC1507			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.084	0.96	5.2
RSD_r (%)	14.6	7.0	5.8
Bias (%)	5.0	6.7	4.0
5307			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.087	0.88	4.7
RSD_r (%)	14.9	6.1	6.0
Bias (%)	8.7	-2.2	-6.0
GA21			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.081	0.85	4.6
RSD_r (%)	15.8	6.8	7.0
Bias (%)	1.3	-5.6	-8.0

The EURL GMFF verified the data and concluded that they were reliable and seemed to confirm that the methods meet the ENGL acceptance criteria [3].

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 DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from seeds of GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize;
- genomic DNA extracted from seeds of non-GM maize.

The EURL GMFF prepared test samples of different GM concentrations by mixing genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize with genomic DNA extracted from non-GM maize in a constant amount of total maize DNA. The same concentrations as used in the validation of the methods for the parent single lines were achieved. Table 2 shows the five GM concentrations used in the verification of the Bt11, MIR162, MIR604, TC1507, 5307 and GA21 methods when applying them to genomic DNA extracted from the GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize. These are the same concentrations used in the validation of these methods for the parental single line GMOs.

Table 2. Percentage of Bt11, MIR162, MIR604, TC1507, 5307 and GA21 in Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 in the verification samples.

Bt11 GM % (GM DNA / total DNA x 100)	**MIR162 GM %** (GM DNA / total DNA x 100)	**MIR604 GM %** (GM DNA / total DNA x 100)
0.09	0.1	0.1
0.4	0.4	0.4
0.9	0.9	0.9
5.0	2.0	2.5
8.0	5.0	6.0
TC1507 GM % (GM DNA / total DNA x 100)	**5307 GM %** (GM DNA / total DNA x 100)	**GA21 GM %** (GM DNA / total DNA x 100)
0.1	0.04	0.09
0.5	0.1	0.5
0.9	0.9	0.9
2.0	2.5	5.0
5.0	5.0	8.0

The protocols (reagents, concentrations, primers/probes sequences) described by the applicant were implemented precisely in the EURL GMFF laboratory. The *in-house* verification followed the protocols already published as validated methods for the individual Bt11, MIR162, MIR604, TC1507, 5307 and GA21 single events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx), with the small modifications described in section 4.5 (Deviations from the validated methods).

4.2 DNA extraction

A method for DNA extraction from maize seeds was previously evaluated by the EURL GMFF with regards to its performance characteristics and was considered valid i.e. fit for the purpose of providing maize DNA of appropriate quality and amount for being used in subsequent PCR experiments. The protocol for the DNA extraction method is available at http://gmo-crl.jrc.ec.europa.eu/summaries/TCTC1507-DNAextrc.pdf.

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

4.3 Experimental design

Eight PCR runs for each method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the reference system *Alcohol dehydrogenase 1* gene (*Adh1*) for Bt11, MIR162, MIR604, 5307 and GA21 methods and the reference system *high mobility group* gene (*hmg*) for TC1507 method. Five GM levels were examined per run, for each GM level in duplicate. PCR analysis was performed in triplicate for all samples. In total, for each method (Bt11, MIR162, MIR604, TC1507, 5307 and GA21), the quantification of the five GM levels was calculated as an average of sixteen replicates per GM level (8 runs x 2 replicated levels per run). An Excel spreadsheet was used for determination of GM%.

4.4 PCR methods

During the verification study, the EURL GMFF carried out tests on DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize, using the methods previously validated for the respective single-line GM maize events Bt11, MIR162, MIR604, TC1507, 5307 and GA21.

For the detection of GM maize events Bt11, MIR162, MIR604, TC1507, 5307 and GA21, DNA fragments of 68-bp, 92-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) is used as reporter dye at its 5'-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3'-end.

For quantification of GM maize events Bt11, MIR162, MIR604, 5307 and GA21, a taxon-specific reference system amplifies a 135-bp fragment of *Alcohol dehydrogenase 1* (*Adh1*) maize endogenous gene (GenBank X04050), using two *Adh1* gene-specific primers and an *Adh1* gene-specific probe labelled with VIC and TAMRA.

For quantification of GM maize event TC1507, a taxon-specific reference system amplifies a 79-bp fragment of *high mobility group* (*hmg*) maize endogenous gene (GenBank AJ131373.1), using two *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM and TAMRA.

For relative quantification of GM maize events Bt11, MIR162, MIR604, 5307 and GA21 DNA, respectively in a separate test sample, the ΔCt values of calibration samples are used to calculate, by linear regression, standard curves (plotting ΔCt values against the logarithm of the amounts of Bt11, MIR162, MIR604, 5307 and GA21 events DNA, respectively). The ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of Bt11, MIR162, MIR604, 5307 and GA21 DNA, respectively, is estimated.

For relative quantification of GM maize event TC1507 DNA, standard curves are generated both for the TC1507 and the *hmg* specific system by plotting Ct values of the calibration standards against the logarithm of the target DNA copy numbers and by fitting a linear regression into these data. The Ct values of the unknown samples are measured and, by means of the regression formula, the relative amount of TC1507 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.

4.5 Deviations from the validated methods

As indicated in the validated methods for Bt11, MIR162, MIR604, 5307 and GA21 (single events), sulforhodamine was added to the Sigma JumpStart *Taq* ReadyMix. The sulforhodamine concentration in the supplemented 2x Sigma JumpStart *Taq* ReadyMix specified in the validated protocols for the MIR604 and GA21 event-specific methods (300 nM) was doubled by the applicant and by the EURL GMFF in order to get passive reference fluorescence values clearly above the background for the ABI 7900 HT sequence detection system used in the testing.

The sulforhodamine concentration (600 nM) in the supplemented 2x Sigma JumpStart *Taq* ReadyMix used by the applicant and by the EURL GMFF for the detection of MIR604 and GA21 events in stack maize Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 corresponds to the one specified in the Bt11 and MIR162 protocols.

Moreover, in the method for TC1507 the applicant and the EURL GMFF replaced the ROX Passive Reference Dye produced by Applied Biosystems with the ROX produced by Invitrogen.

4.6 Results

Tables 3, 4, 5, 6, 7 and 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 $[10^{(-1/slope)} - 1] \times 100$, and of the R^2 (expressing the linearity of the regression) reported for all PCR systems in the eight runs.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the Bt11 method on GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

Run	Bt11		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.12	109	0.99
2	-3.34	99	1.00
3	-3.42	96	1.00
4	-3.21	105	0.99
5	-3.10	110	0.99
6	-3.40	97	1.00
7	-3.36	98	1.00
8	-3.46	94	1.00
Mean	-3.30	101	1.00

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MIR162 method on GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

Run	MIR162		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.25	103	1.00
2	-3.56	91	1.00
3	-3.46	95	0.99
4	-3.47	94	0.99
5	-3.41	97	0.99
6	-3.40	97	1.00
7	-3.41	96	1.00
8	-3.41	96	1.00
Mean	-3.42	96	1.00

Table 5. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MIR604 method on GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

Run	MIR604		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.37	98	1.00
2	-3.31	100	1.00
3	-3.35	99	1.00
4	-3.35	99	1.00
5	-3.22	104	1.00
6	-3.24	104	1.00
7	-3.35	99	1.00
8	-3.38	98	1.00
Mean	-3.32	100	1.00

Table 6. Values of standard curve slope, PCR efficiency and linearity (R^2) for the TC1507 method on GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

Run	TC1507			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.20	105	0.99	-3.18	106	1.00
2	-3.29	101	0.98	-3.19	106	1.00
3	-3.24	104	0.99	-3.31	100	0.99
4	-3.10	110	1.00	-3.21	105	0.99
5	-3.23	104	0.98	-3.28	102	0.99
6	-3.27	102	1.00	-3.19	106	0.99
7	-3.23	104	0.99	-3.23	104	1.00
8	-3.26	102	0.99	-3.16	107	0.99
Mean	-3.23	104	0.99	-3.22	105	0.99

Table 7. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 5307 method on GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

Run	5307		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.38	98	1.00
2	-3.28	102	1.00
3	-3.34	99	1.00
4	-3.39	97	1.00
5	-3.38	98	1.00
6	-3.45	95	1.00
7	-3.32	100	1.00
8	-3.43	96	1.00
Mean	-3.37	98	1.00

Table 8. Values of standard curve slope, PCR efficiency and linearity (R^2) for the GA21 method on GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

Run	GA21		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.25	103	1.00
2	-3.34	99	1.00
3	-3.37	98	0.99
4	-3.25	103	1.00
5	-3.29	101	1.00
6	-3.34	99	1.00
7	-3.26	103	1.00
8	-3.29	101	1.00
Mean	-3.30	101	1.00

The mean PCR efficiencies of the calibration curves for each of the five ΔCt event-specific methods were above 90% (101%, 96%, 100%, 98% and 101% for Bt11, MIR162, MIR604, 5307 and GA21 respectively). The mean PCR efficiencies of the calibration curve for the TC1507 event-specific assay was 104% and the mean PCR efficiency of the *hmg* reference gene assay for the TC1507 method was 105%. The linearity of the ΔCt methods (R^2) was 1.00 for Bt11, MIR162, MIR604, 5307 and GA21, and 0.99 for the event-specific and the *hmg* reference gene assays for TC1507. The data generated by the EURL GMFF and presented in Tables 3, 4, 5, 6, 7 and 8 are in line with the data presented by the applicant and confirm the appropriate performance characteristics of the six methods when tested on DNA extracted from the GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize in terms of PCR efficiency and linearity.

The EURL GMFF also assessed the values of trueness and precision (expressed as bias and RSDr %, relative repeatability standard deviation), of the six methods applied to samples of genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize, see Tables 9, 10, 11, 12, 13 and 14.

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the Bt11 method applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

Bt11					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.4**	**0.9**	**5.0**	**8.0**
Mean	0.10	0.40	0.93	5.5	9.1
SD	0.01	0.07	0.10	0.65	1.1
RSD_r (%)	12	18	10	12	12
Bias (%)	7.8	0.0	3.5	11	13

Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MIR162 method applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

MIR162					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.4**	**0.9**	**2.0**	**5.0**
Mean	0.12	0.43	0.89	1.9	5.1
SD	0.02	0.08	0.16	0.27	0.87
RSD_r (%)	13	20	18	14	17
Bias (%)	23	6.7	-1.2	-6.4	2.7

Table 11. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MIR604 method applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

MIR604					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.4**	**0.9**	**2.5**	**6.0**
Mean	0.11	0.42	0.98	2.6	6.0
SD	0.02	0.03	0.04	0.12	0.34
RSD_r (%)	14	7.8	4.6	4.5	5.7
Bias (%)	6.5	5.1	9.1	5.3	0.54

Table 12. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the TC1507 method applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

TC1507					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**0.9**	**2.0**	**5.0**
Mean	0.10	0.44	0.97	1.83	5.8
SD	0.02	0.07	0.10	0.16	0.56
RSD_r (%)	23	17	11	8.9	9.7
Bias (%)	-0.32	-12	8.1	-8.4	15

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 Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

5307					
Unknown sample GM%	**Expected value (GMO%)**				
	0.04	**0.1**	**0.9**	**2.5**	**5.0**
Mean	0.04	0.11	0.93	2.6	5.3
SD	0.01	0.01	0.12	0.27	0.43
RSD_r (%)	15	10	12	10	8.1
Bias (%)	-3.7	5.3	3.9	5.1	6.0

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 Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

GA21					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.5**	**0.9**	**5.0**	**8.0**
Mean	0.10	0.51	0.95	5.1	7.8
SD	0.01	0.05	0.06	0.32	0.67
RSD_r (%)	14	9.2	6.7	6.3	8.7
Bias (%)	6.4	2.5	5.3	2.6	-2.9

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 ± 25% across the entire dynamic range. As shown in Tables 9, 10, 11, 12, 13 and 14, the values range from -0.05% to 13% for Bt11, from -6.4% to 23% for MIR162, from 0.54% to 9.1% for MIR604, from -12% to 15% for TC1507, from -3.7% to 6.0% for 5307 and from -2.9% to 6.4% for GA21. Therefore, the six methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

Tables 9, 10, 11, 12, 13 and 14 also show the relative repeatability standard deviation (RSD_r) for each GM level. According to the ENGL acceptance criteria and method performance requirements, RSD_r values should be below 25%. As the values range between 10% to 18% for Bt11, between 13% to 20% for MIR162, between 4.5% to 14% for MIR604, between 8.9% to 23% for TC1507, between 8.1% to 15% for 5307 and between 6.3% to 14% for GA21, the six methods satisfy this requirement throughout their respective dynamic ranges also when applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

5. Comparison of method performance when applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize and to DNA extracted from the single-line GM events

An indicative comparison of the performance (bias, RSDr %) of the two methods applied to GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize and to the single-line events is shown in Tables 15, 16, 17, 18, 19 and 20. The performance of the methods on the single lines was previously validated through international collaborative trials (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

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

Table 15. Qualitative comparison of the performance of the Bt11 detection method applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize and to genomic DNA extracted from the single-line event Bt11.

Trueness and repeatability of Bt11 quantification on GM-Stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize			**Trueness and repeatability of Bt11 quantification on single event Bt11***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.09	7.8	12	**0.09**	2.2	17
0.4	0.0	18	**0.4**	-1.9	13
0.9	3.5	10	**0.9**	1.8	11
5.0	11	12	**5.0**	-5.2	13
8.0	13	12	**8.0**	-1.2	9.0

*Data taken from original method validation (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 16. Qualitative comparison of the performance of the MIR162 detection method applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize and to genomic DNA extracted from the single-line event MIR162.

Trueness and repeatability of MIR162 quantification on Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21			**Trueness and repeatability of MIR162 quantification on single event MIR162***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	23	13	**0.1**	0.2	13
0.4	6.7	20	**0.4**	2.9	12
0.9	-1.2	18	**0.9**	-1.7	12
2.0	-6.4	14	**2.0**	1.4	10
5.0	2.7	17	**5.0**	4.3	8

*Data taken from original method validation (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 17. Qualitative comparison of the performance of the MIR604 detection method applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize and to genomic DNA extracted from the single-line event MIR604.

Trueness and repeatability of MIR604 quantification on Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21			**Trueness and repeatability of MIR604 quantification on single event MIR604***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	6.5	14	**0.1**	3.6	24
0.4	5.1	7.8	**0.4**	3.1	17
0.9	9.1	4.6	**0.9**	-1.0	12
2.5	5.3	4.5	**2.5**	0.7	16
6.0	0.54	5.7	**6.0**	-3.6	14

*Data taken from original method validation (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 18. Qualitative comparison of the performance of the TC1507 detection method applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize and to genomic DNA extracted from the single-line event TC1507.

Trueness and repeatability of TC1507 quantification on Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21			**Trueness and repeatability of TC1507 quantification on single event TC1507***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.1	-0.32	23	**0.1**	6.0	18
0.5	-12	17	**0.5**	-4.0	12
0.9	8.1	11	**0.9**	3.7	7.7
2.0	-8.4	8.9	**2.0**	-1.7	8.5
5.0	15	9.7	**5.0**	8.4	14

*Data taken from original method validation (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 19. Qualitative comparison of the performance of the 5307 detection method applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize and to genomic DNA extracted from the single-line event 5307.

Trueness and repeatability of 5307 quantification on Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21			**Trueness and repeatability of 5307 quantification on single event 5307***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.04	-3.7	15	**0.04**	11	13
0.1	5.3	10	**0.1**	7.2	9.7
0.9	3.9	12	**0.9**	3.8	8.8
2.5	5.1	10	**2.5**	6.6	4.7
5.0	6.0	8.1	**5.0**	4.5	9.6

*Data taken from original method validation (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 20. Qualitative comparison of the performance of the GA21 detection method applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize and to genomic DNA extracted from the single-line event GA21.

Trueness and repeatability of GA21 quantification on Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21			**Trueness and repeatability of GA21 quantification on single event GA21***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.09	6.4	14	**0.09**	-8.7	23
0.5	2.5	9.2	**0.5**	0.8	17
0.9	5.3	6.7	**0.9**	1.6	20
5.0	2.6	6.3	**5.0**	-5.6	20
8.0	-2.9	8.7	**8.0**	-8.5	17

*Data taken from original method validation (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

6. Conclusions

The performance of the six event-specific methods for the detection and quantification of maize events Bt11, MIR162, MIR604, TC1507, 5307 and GA21, when applied to genomic DNA extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant.

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

In conclusion, the verification study carried out by the EURL GMFF confirmed that the six methods are capable to detect, identify and quantify each of the GM events when applied to genomic DNA of suitable quality, extracted from GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

Therefore these methods, originally developed and validated to detect and quantify the events in the single event parental GMOs, can be equally applied for the detection and quantification of the respective events combined in GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

7. 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.

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Title: **Report on the Verification of the Performance of Bt11, MIR162, MIR604, TC1507, 5307 and GA21 Event-specific PCR-based Methods applied to DNA Extracted from GM Stack Maize Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21**

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Abstract

An application was submitted by Syngenta Crop Protection AG to request the authorisation of genetically modified (GM stack) maize Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 (with a set of proteins and truncated proteins for protection or control against certain lepidopteran and coleopteran pests and glyphosate-tolerance) and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, and import and processing, in accordance with articles 5 and 17 of Regulation (EC) No 1829/2003 on GM Food and Feed. The unique identifier assigned to the GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize is SYN-BTØ11-1 x SYN-IR162-4 x SYN-IR6Ø4-5 x DAS-Ø15Ø7-1 x SYN-Ø53Ø7-1 x MON-ØØØ21-9.

The GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize has been obtained by conventional crossing of six genetically modified single line maize events: Bt11, MIR162, MIR604, TC1507, 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 line maize events Bt11, MIR162, MIR604, TC1507, 5307 and GA21 and has published the corresponding reports (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) the EURL GMFF therefore has carried out only an in-house verification of the performance of each validated method when applied to DNA extracted from the GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

The hereby reported in-house verification study led to the conclusion that the individual methods meet the ENGL requirements also when applied to DNA extracted from the GM stack Bt11 x MIR162 x MIR604 x TC1507 x 5307 x GA21 maize.

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