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

European Union Reference Laboratory for Genetically Modified Food and Feed

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Contact information
Molecular Biology and Genomics Unit
Address: Joint Research Centre, Via Enrico Fermi 2749, TP 201, 21027 Ispra (VA), Italy
E-mail: eurl-gmff@jrc.ec.europa.eu
Tel.: +39 0332 78 5165
Fax: +39 0332 78 9333

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

24 June 2016

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 stack (GM stack) 3272 x Bt11 x MIR604 x GA21 maize (producing an alpha amylase that catalyses the hydrolysis of starch to soluble sugar and carrying insect resistance and tolerance of gluphosinate ammonium and glyphosate) 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 on GM Food and GM Feed. The unique identifier assigned to GM stack 3272 x Bt11 x MIR604 x GA21 maize is SYN-E3272-5 x SYN-BTØ11-1 X SYN-IR6Ø4-5 X MON-ØØØ21-9.

The GM stack 3272 x Bt11 x MIR604 x GA21 maize has been obtained by conventional crossing between the genetically modified maize events 3272, Bt11, MIR604 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 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) 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 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 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: 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.

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit (MBG)
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, 21027 Ispra (VA) – Italy
Functional mailbox: eurl-gmff@jrc.ec.europa.eu

1. Introduction

The EU legislative system [1, 2] 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) 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 3272 x Bt11 x MIR604 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, the 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 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 [3] and with regard to their documentation and reliability.

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSD_r %) calculated by the applicant for the four methods applied to 3272 x Bt11 x MIR604 x GA21 maize genomic DNA. Means are the average of sixteen replicates. Means of performance parameters for GM levels 0.9% and 5.0% were obtained through four runs performed with ABI PRISM® 7900 HT real-time PCR equipment and means of the performance parameters for the GM level 0.08% were obtained by the applicant through two additional runs with an extended calibration curve to avoid extrapolation on ABI PRISM® 7900 HT 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_r %) provided by the applicant for the 3272, Bt11, MIR604 and GA21 methods applied to GM stack 3272 x Bt11 x MIR604 x GA21 maize.

3272*			
Sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.079	0.71	4.4
RSD_r (%)	15.8	11.4	12.0
Bias (%)	-1.3	-21.1	-12.0
Bt11*			
Sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.076	0.71	4.1
RSD_r (%)	17.0	14.4	12.4
Bias (%)	-5.0	-21.1	-18.0
MIR604*			
Sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.076	0.73	4.4
RSD_r (%)	13.2	11.4	14.1
Bias (%)	-5.0	-18.9	-12.0

GA21 *			
Sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.079	0.71	4.3
RSD_r (%)	12.7	9.7	17.2
Bias (%)	-1.3	-21.1	-14.0

* 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 [(3)].

Two requests of complementary information regarding the method, the control samples and DNA sequences were 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 four 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 GA21 maize.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenised seeds of GM stack 3272 x Bt11 x MIR604 x GA21 maize (NP2673GA21/MIR604xNP2052AMY/Bt11), hemizygous for the loci, as positive control sample.
- genomic DNA extracted from homogenised seeds of conventional (non-GM) maize (NP2673xNP2052), 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 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 and GA21 methods when applying them to genomic DNA extracted from the GM stack 3272 x Bt11 x MIR604 x GA21 maize.

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

3272 GM%* [(GM DNA / total maize DNA x 100)]	**Bt11 GM%*** [(GM DNA / total maize DNA x 100)]	**MIR604 GM%*** [(GM DNA / total maize DNA x 100)]	**GA21 GM%*** [(GM DNA / total maize DNA x 100)]
0.09	0.09	0.10	0.09
0.40	0.40	0.40	0.50
0.90	0.90	0.90	0.90
5.0	5.0	2.5	5.0
8.0	8.0	6.0	8.0

* percentages 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 3272, Bt11, MIR604 and GA21 GM events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

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.

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/Bt11field_maize_DNAExtr.pdf. This was the method proposed by the applicant.

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 *alcohol dehydrogenase 1* (*adh1*). 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 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 the 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 GA21 maize using the single detection methods previously validated for the respective single GM events 3272, Bt11, MIR604 and GA21.

For detection of GM maize events 3272, Bt11, MIR604 and GA21, DNA fragments of 95-bp, 68-bp, 76-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 reporter dye at their 5′-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at their 3′-end for all four events.

For quantification of GM maize events 3272, Bt11, MIR604 and GA21, a taxon-specific reference system amplifies a 135-bp fragment of *adh1, alcohol dehydrogenase 1*, 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 the relative quantification of GM maize events 3272, Bt11, MIR604 and GA21 DNA in a test sample, the ΔCq values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCq values against the logarithm of the relative amount of 3272, Bt11, MIR604 and GA21 event DNA in the respective calibration samples. The ΔCq values of the unknown samples are measured and, by means of the regression formula, the relative amount of 3272, Bt11, MIR604 and GA21 events 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.

4.5 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 $[10^{(-1/slope)} - 1] \times 100\%$, and of the coefficient of determination (R^2) reported for all PCR systems in the eight runs, for GM maize events 3272, Bt11, MIR604 and GA21. Slope and R^2 coefficient 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 GA21 maize.

Run	3272		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.49	93	1.00
2	-3.48	94	1.00
3	-3.34	99	1.00
4	-3.46	94	1.00
5	-3.33	100	1.00
6	-3.46	95	1.00
7	-3.43	96	1.00
8	-3.44	95	1.00
Mean	-3.43	96	1.00

Table 4. Values of standard curve slope, PCR efficiency and R^2 coefficient for the Bt11 method on GM stack 3272 x Bt11 x MIR604 x GA21 maize.

Run	Bt11		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.49	93	1.00
2	-3.46	95	1.00
3	-3.41	96	1.00
4	-3.42	96	1.00
5	-3.53	92	1.00
6	-3.38	98	1.00
7	-3.47	94	1.00
8	-3.48	94	1.00
Mean	-3.46	95	1.00

Table 5. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MIR604 method on GM stack 3272 x Bt11 x MIR604 x GA21 maize.

Run	MIR604		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.30	101	1.00
2	-3.28	102	1.00
3	-3.33	100	1.00
4	-3.33	100	1.00
5	-3.26	102	1.00
6	-3.28	102	1.00
7	-3.32	100	1.00
8	-3.31	100	1.00
Mean	-3.30	101	1.00

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

Run	GA21		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.32	100	1.00
2	-3.20	105	1.00
3	-3.30	101	1.00
4	-3.13	108	1.00
5	-3.36	98	1.00
6	-3.29	102	1.00
7	-3.23	104	1.00
8	-3.35	99	1.00
Mean	-3.27	102	1.00

The mean PCR efficiencies of the ΔCq calibration curves were 96% for the 3272 system, 95% for the Bt11 sysetem, 101% for the MIR604 system and 102% for the GA21 system, 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 3272 x Bt11 x MIR604 x GA21 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 3272 x Bt11 x MIR604 x GA21 maize, see tables 7-10.

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 3272 method applied to genomic DNA extracted from GM stack 3272 x Bt11 x MIR604 x GA21 maize.

3272					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.40**	**0.90**	**5.0**	**8.0**
Mean	0.09	0.38	0.93	5.0	7.7
SD	0.01	0.02	0.04	0.21	0.21
RSD_r (%)	9.9	6.2	4.4	4.2	2.7
Bias (%)	-1.9	-4.8	3.4	-0.25	-3.4

Table 8. 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 3272 x Bt11 x MIR604 x GA21 maize.

Bt11					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.40**	**0.90**	**5.0**	**8.0**
Mean	0.09	0.38	0.91	5.0	7.6
SD	0.01	0.02	0.04	0.10	0.21
RSD_r (%)	10	5.4	4.0	2.1	2.7
Bias (%)	-4.4	-4.6	1.6	-0.55	-4.5

Table 9. 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 3272 x Bt11 x MIR604 x GA21 maize.

MIR604					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**2.5**	**6.0**
Mean	0.11	0.44	0.93	2.5	6.0
SD	0.00	0.04	0.05	0.13	0.25
RSD_r (%)	4.5	9.4	5.0	5.1	4.1
Bias (%)	5.4	11	3.2	1.8	0.45

Table 10. 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 GA21 maize.

GA21					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.50**	**0.90**	**5.0**	**8.0**
Mean	0.10	0.53	0.92	5.6	8.5
SD	0.01	0.04	0.06	0.50	0.73
RSD_r (%)	10	7.2	6.1	9.0	8.6
Bias (%)	5.9	5.7	2.1	12	6.3

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 ± 25% across the entire dynamic range. As shown in Tables 7, 8, 9 and 10, the values range from -4.8% to 3.4% for 3272, from -4.6% to 1.6% for Bt11, from 0.45% to 11% for MIR604, and from 2.1% to 12% for GA21. Therefore, the four 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 GA21 maize.

Tables 7, 8, 9 and 10 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 2.7% and 9.9% for 3272, between 2.1% and 10% for Bt11, between 4.1% and 9.4% for MIR604 and between 6.1% and 10% for GA21, the four methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack 3272 x Bt11 x MIR604 x GA21 maize.

5. Conclusions

The performance of the four event-specific methods for the detection and quantification of maize single line events 3272, Bt11, MIR604 and GA21, when applied to genomic DNA extracted from GM stack 3272 x Bt11 x MIR604 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 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 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.

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