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Report on the Verification of the Performance of Bt11, DAS-59122-7, MIR604, TC 1507 and GA21 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack Bt11 x DAS-59122-7 x MIR604 x TC 1507 x GA21 Maize

Validation Report

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Report on the Verification of the Performance of Bt11, 59122, MIR604, TC1507 and GA21 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack Bt11 x 59122 x MIR604 x TC1507 x GA21 Maize

6 October 2014

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) Bt11 x 59122 x MIR604 x TC1507 x GA21 maize (tolerant to herbicide products containing glufosinate ammonium/glyphosate and resistant to certain lepidopteran/coleopteran pests) and all sub-combinations of the individual events as present in the segregating progeny (except for 1507 x 59122), 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 GM stack Bt11 x 59122 x MIR604 x TC1507 x GA21 maize is SYN-BTØ11-1 × DAS-59122-7 × SYN-IR6Ø4-5 × DAS-Ø15Ø7-1 × MON-ØØØ21-9.

The GM stack Bt11 x 59122 x MIR604 x TC1507 x GA21 maize has been obtained by conventional crossing of genetically modified maize events: Bt11, 59122, MIR604, TC1507 and GA21, without any new genetic modification.

The European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF) has previously validated individually, and declared fit for purpose, the detection methods for the single events Bt11, 59122, MIR604, TC1507 and GA21 (see http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). In line with the approach defined by the European Network of GMO Laboratories (ENGL) (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EU-RL 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 Bt11 x 59122 x MIR604 x TC1507 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 Bt11 x 59122 x MIR604 x TC1507 x GA21 maize.

Noteworthy the 59122 event-specific method applied to GM stack DNA differs from the validated method for the pH value of PCR buffer (pH 8.0 instead of pH 8.3). However, this deviation does not significantly affect the performance of the method and hence the pH 8.0-method is considered equivalent to the originally validated pH 8.3 method protocol.

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

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Quality assurance

The EU-RL 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 EU-RL GMFF quality system.

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

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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 an 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 EU-RL 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, that will include the EU-RL GMFF report in its overall opinion concerning the risk assessment 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 EU-RL GMFF carries out an *in-house* verification of the performance of each event-specific method provided that the method has previously been validated by the EU-RL GMFF for the parental single-line event and that these events have been stacked by conventional crossing. These criteria are met for the GM stack Bt11 x 59122 x MIR604 x TC1507 x GA21 maize.

Upon reception of methods, samples and related data (step 1), the EU-RL 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 of the individual events.

2. Step 1 (dossier reception and acceptance)

Syngenta Crop Protection AG submitted the detection methods, data demonstrating their adequate performance, and the corresponding control samples DNA extracted from GM stack maize Bt11 x 59122 x MIR604 x TC 1507 x GA21 and from non GM maize.

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

3. Step 2 (scientific dossier 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 by the applicant for the five methods on the stack DNA. Means are the average of sixteen replicates obtained through four runs performed with ABI 7500 fast (non fast mode) sequence detection system. Percentages are expressed as GM DNA / total DNA x 100.

Table 1. Applicant values: trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) for the Bt11, 59122, MIR604, TC1507 and GA21 methods applied to GM stack Bt11 x 59122 x MIR604 x TC1507 x GA21 maize.

Bt11			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.09	0.96	5.1
RSD_r (%)	24	6.9	11
Bias (%)	6.3	6.7	2.0
59122 *			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.08	0.91	5.0
RSD_r (%)	23	11	9.6
Bias (%)	-1.3	1.1	0.0
MIR604			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.08	0.94	5.0
RSD_r (%)	16	13	12
Bias (%)	2.5	4.4	0.0
TC1507			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.08	1.1	5.8
RSD_r (%)	21	16	18
Bias (%)	-5.0	20	16
GA21			
Unknown sample GM%	**Expected value (GMO %)**		
	0.08	**0.9**	**5.0**
Mean	0.08	0.93	4.9
RSD_r (%)	10	6.5	13
Bias (%)	-3.8	3.3	-2.0

* These data were obtained by the applicant using commercial PCR buffer pH 8.3 (DAS-59122 validated method).

The EU-RL 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 calibration curves and DNA sequences were addressed to the applicant. The EU-RL GMFF verified the data and the complementary information received and considered satisfactory the clarifications provided.

The dossier was therefore moved to step 3.

4. Step 3 (EU-RL GMFF experimental testing)

In step 3 the EU-RL GMFF implemented the five methods in its own laboratory and performed a verification of their performance on DNA extracted from GM stack Bt11 x 59122 x MIR604 x TC1507 x GA21 maize.

During step 3 one of the five methods (59122) showed underperformance in repeated tests (strong inhibition, high relative bias and RSDr, slopes outside acceptable range for both the GM and the reference system).

The EU-RL GMFF requested the applicant to provide new reagents and additional control samples for the verification of 59122 method on the stacked material (March 2013). In December 2013 the EU-RL GMFF received from the applicant the reagents and control samples and a report entitled *"Identification of PCR Buffer as Key Component for Underperformance of the Event-specific Detection Method for DAS-59122-7 Transformation Event in Bt11 × DAS-59122-7 × MIR604 × TC1507 × GA21 Maize in Response to EU-RL GMFF Request Concerning the EFSA/GMO/DE/2011/99 Application"*.

ABI PCR buffer II was identified as the reason for the underperformance of the method. Significant batch-to-batch variability of ABI PCR buffer II resulting in performance variations was demonstrated. In addition, it was shown that in-house preparations of PCR buffer of identical composition as ABI PCR buffer II but with a pH of 8.0 significantly improved the performance of the method. This version of the 59122 method (version B) differs only for the pH value of the PCR buffer (pH 8.0 instead of pH 8.3) and, after the tests performed by the EU-RL GMFF, it was shown to meet the ENGL performance criteria when applied to the maize GM stack Bt11 x 59122 x MIR604 x TC1507 x GA21. In session 4.6 data concerning version B are reported. Moreover, the above-mentioned report, demonstrating the influence of PCR buffer pH, is available in Annex 1.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenised seeds of heterozygous GM stack Bt11 x 59122 x MIR604 x TC1507 x GA21 maize.
- genomic DNA extracted from homogenized seeds of non GM maize.

The EU-RL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize and genomic DNA extracted from non-GM maize in a constant amount of total maize DNA (GM DNA + non-GM DNA). The same concentrations as in the validation of the methods for the single lines were employed. Table 2 shows the five GM concentrations used in the verification of the Bt11, 59122, MIR604, TC1507 and GA21 methods when applying them to genomic DNA extracted from the GM stack Bt11 X 59122 X MIR604 X TC1507 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, 59122, MIR604, TC1507 and GA21 in Bt11 X 59122 X MIR604 X TC1507 X GA21 in the verification samples.

Bt11 GM% (GM DNA / total DNA x 100)	**59122 GM%** (GM DNA / total DNA x 100)	**MIR604 GM%** (GM DNA / total DNA x 100)	**TC1507 GM%** (GM DNA / total DNA x 100)	**GA21 GM%** (GM DNA / total DNA x 100)
0.09	0.10	0.1	0.1	0.09
0.40	0.40	0.4	0.5	0.5
0.90	0.90	0.9	0.9	0.9
5.0	2.0	2.5	2.0	5.0
8.0	4.5	6.0	5.0	8.0

The *in-house* verification followed the protocols (reagents, concentrations, primers/probe sequences) already published as validated methods for the individual Bt11, MIR604, TC1507 and GA21 events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). For event 59122 a second version of the method (version B), that differs for the pH value of the PCR buffer (pH 8.0 instead of pH 8.3) was used.

4.2 DNA extraction

A method for DNA extraction from ground maize grains and seeds was previously evaluated by the EU-RL GMFF with regard to its performance characteristics and was considered valid, i.e. fit the purpose of providing maize DNA of appropriate quality and quantity for subsequent PCR experiments. The protocol for the DNA extraction method is available at http://gmo-crl.jrc.ec.europa.eu/summaries/TC1507-DNAextrc.pdf.

Consequently, the EU-RL 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 the Bt11, MIR604 and GA21 event-specific detection methods and high mobility group gene (*hmg*) for the 59122 and TC1507 event-specific detection methods. 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, 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 GM%.

4.4 PCR methods

During the verification study, the EU-RL GMFF carried out parallel tests on DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize using the single detection methods previously validated for the respective single GM events Bt11, 59122, MIR604, TC1507 and GA21.

For detection of GM maize events Bt11, 59122, MIR604, TC1507 and GA21, DNA fragments of 68-bp, 86-bp, 76-bp, 58-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 Bt11, 59122, MIR604, TC1507 and GA21.

For quantification of GM maize events Bt11, MIR604 and GA21, a taxon-specific reference system amplifies a 135-bp fragment of *adh1 a* maize endogenous gene (Entrez Database Accession No. AY691949), using two *adh1* gene-specific primers and a *adh1* gene-specific probe labelled with VIC and TAMRA. For quantification of GM maize events 59122 and TC1507, a taxon-specific reference system amplifies a 79-bp fragment of *hmg a* maize endogenous gene, using two *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM and TAMRA.

For quantification of GM maize events TC1507 and 59122 standard curves were generated both for the TC1507 and 59122, and for the *hmg* specific system by plotting Ct values of the calibration standards against the logarithm of the DNA copy numbers and by fitting a linear regression into these data. Thereafter, the normalised Ct values of the unknown samples are measured and, by means of the regression formula, the relative amount of 1507 and 59122 DNA is estimated.
For relative quantification of events Bt11, MIR604 and GA21 in a test sample, the normalised ΔCt values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCt values against the logarithm of the amount of Bt11, MIR604 and GA21 DNA). The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of Bt11, MIR604 and GA21 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

During the verification study on DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize, the EU-RL GMFF applied a deviation from the validated 59122 event-specific method. This deviation consists in a variation of the pH value of the PCR buffer (pH 8.0 in version B instead of pH 8.3 as originally validated). All results were evaluated using EU-RL GMFF reagents and further confirmed using reagents provided by the applicant (data not shown). For the other events no deviations from the original validated methods were introduced.

4.6 Results

Tables 3, 4 and 5 present the values of the slopes of the different standard curves generated by the EU-RL 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, for GM maize events Bt11, 59122, MIR604, TC1507 and GA21.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 1507 method on GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize.

Run	TC1507			*hmg*		
	Slope	**PCR Efficiency (%)**	**Linearity (R^2)**	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**
1	-3.13	109	0.99	-3.33	100	1.00
2	-3.19	106	1.00	-3.17	107	1.00
3	-3.20	105	0.99	-3.29	101	1.00
4	-3.16	107	0.99	-3.31	100	1.00
5	-3.08	111	0.99	-3.31	100	1.00
6	-3.09	111	0.99	-3.31	100	1.00
7	-3.21	105	1.00	-3.32	100	1.00
8	-3.16	107	0.99	-3.33	100	1.00
Mean	-3.16	108	0.99	-3.30	101	1.00

Table 4*. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 59122 method on GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize.

Run	59122			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.35	99	1.00	-3.29	101	1.00
2	-3.26	103	1.00	-3.3	101	1.00
3	-3.34	99	1.00	-3.29	101	1.00
4	-3.39	97	1.00	-3.34	99	1.00
5	-3.37	98	1.00	-3.31	100	1.00
6	-3.27	102	1.00	-3.33	100	1.00
7	-3.29	102	1.00	-3.3	101	1.00
8	-3.35	99	1.00	-3.31	101	1.00
Mean	-3.33	100	1.00	-3.31	101	1.00

*Data obtained using PCR buffer at pH 8.0

Table 5. Values of slope, PCR efficiency and linearity (R^2) for the Bt11, MIR604 and GA21 method on GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize.

Run	Bt11			MIR604			GA21		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.56	91	1.00	-3.40	97	1.00	-3.35	99	1.00
2	-3.51	93	1.00	-3.37	98	1.00	-3.29	101	1.00
3	-3.37	98	1.00	-3.32	100	1.00	-3.36	98	1.00
4	-3.49	93	1.00	-3.36	99	1.00	-3.36	99	1.00
5	-3.50	93	1.00	-3.37	98	1.00	-3.40	97	1.00
6	-3.43	96	1.00	-3.31	100	1.00	-3.34	99	1.00
7	-3.43	96	1.00	-3.35	99	1.00	-3.36	98	1.00
8	-3.50	93	1.00	-3.37	98	1.00	-3.24	103	1.00
Mean	-3.47	94	1.00	-3.36	99	1.00	-3.34	99	1.00

The mean PCR efficiencies of the GM and species-specific systems were comprised between 94% and 108%. The linearity of the methods (R^2) was ≥ 0.99 for all systems in all cases. The data presented in Tables 3, 4 and 5 confirm the appropriate performance characteristics of the five methods when tested on GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize in terms of PCR efficiency and linearity.

The EU-RL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) of the five methods applied to

samples of DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize, see from Table 6 to Table 10.

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSDr %) of the Bt11 method applied to genomic DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize.

Bt11					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.40**	**0.90**	**5.0**	**8.0**
Mean	0.08	0.36	0.84	4.7	7.4
SD	0.01	0.07	0.08	0.24	0.29
RSD_r (%)	10	18	9.2	5.1	3.9
Bias (%)	-5.8	-9.0	-6.4	-5.8	-7.0

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSDr %) of the MIR604 method applied to genomic DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize.

MIR604					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**2.5**	**6.0**
Mean	0.11	0.42	0.96	2.5	6.9
SD	0.01	0.04	0.04	0.12	0.39
RSD_r (%)	10	8.6	4.2	4.6	5.6
Bias (%)	14	5.8	7.0	1.8	14

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 1507 method applied to genomic DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize.

1507					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.50**	**0.90**	**2.0**	**5.0**
Mean	0.08	0.4	0.79	1.8	5.1
SD	0.01	0.04	0.06	0.36	0.43
RSD_r (%)	13	11	8.0	20	8.5
Bias (%)	-25	-20	-13	-12	2.0

Table 9. 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 59122 X MIR604 X TC1507 X GA21 maize.

GA21					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.50**	**0.90**	**5.0**	**8.0**
Mean	0.09	0.51	0.94	5.2	8.5
SD	0.01	0.04	0.04	0.15	0.41
RSD_r (%)	7.1	8.2	3.8	2.9	4.8
Bias (%)	3.1	2.9	4.6	3.4	6.1

Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 59122 method applied to genomic DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize.

59122*					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**2.0**	**4.5**
Mean	0.08	0.33	0.81	1.7	4.2
SD	0.00	0.02	0.03	0.09	0.14
RSD_r (%)	5.9	5.5	3.3	5.5	3.4
Bias (%)	-20	-18	-10	-14	-7.5

* Bias and RSDr were evaluated using PCR buffer pH 8 (see 4.5 for details)

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 from Table 6 to Table 10, the values range from -9.0% to -5.8% for Bt11, from -20% to -7.5% for 59122, from 1.8% to 14% for MIR604, from -25% to 2.0% for TC1507, from 2.9% to 6.1% for GA21. Therefore, all the methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize.

Table 6 to Table 10 also show the relative repeatability standard deviation (RSD_r) as estimated for each GM level. According to the ENGL acceptance criteria and method performance requirements, the EU-RL GMFF requires RSD_r values to be below 25%. As the values range from 3.9% to 18% for Bt11, from 3.3% to 5.9% for 59122, from 4.2% to 10% for MIR604, from 8% to 20% for TC1507, from 2.9% to 8.2% for GA21, the five methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize.

5. Comparison of method performance on Bt11 X 59122 X MIR604 X TC1507 X GA21 and on the single events

A comparison of the performance (bias, RSDr %) of the five methods applied to GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize and on the single-line events is shown in Tables 11 to 15. The performance of the methods on the single lines was previously established 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 11. Comparison of the performance of the Bt11 detection method applied to genomic DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize and from the single line event Bt11.

Trueness and repeatability of Bt11 quantification on Bt11 X 59122 X MIR604 X TC1507 X GA21			**Trueness and repeatability of Bt11 quantification on single event Bt11***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.09	-5.8	10	**0.09**	2.2	17
0.40	-9.0	18	**0.40**	-1.9	13
0.90	-6.4	9.2	**0.90**	1.8	11
5.0	-5.8	5.1	**5.0**	-5.2	13
8.0	-7.0	3.9	**8.0**	-1.2	9.0

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx)

Table 12. Comparison of the performance of the 59122 detection method applied to genomic DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize and to genomic DNA extracted from the single line event 59122.

Trueness and repeatability of 59122 quantification on Bt11 X 59122 X MIR604 X TC1507 X GA21			**Trueness and repeatability of 59122 quantification on single event 59122***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.10	-20	5.9	**0.10**	29	18
0.40	-18	5.5	**0.40**	15	14
0.90	-10	3.3	**0.90**	9	16
2.0	-14	5.5	**2.0**	7	14
4.5	-7.4	3.4	**4.5**	-1	8.5

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx)

Table 13. Comparison of the performance of the MIR604 detection method applied to genomic DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize and from the single line event MIR604.

Trueness and repeatability of MIR604 quantification on Bt11 X 59122 X MIR604 X TC1507 X GA21			**Trueness and repeatability of MIR604 quantification on single event MIR604***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.10	14	10	**0.10**	3.6	24
0.40	5.8	8.6	**0.40**	3.1	17
0.90	6.9	4.2	**0.90**	-1	12
2.5	1.8	4.6	**2.5**	0.7	16
6.0	14	5.6	**6.0**	-3.6	14

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx)

Table 14. Comparison of the performance of the TC1507 detection method applied to genomic DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize and from the single line event TC1507.

Trueness and repeatability of TC1507 quantification on Bt11 X 59122 X MIR604 X TC1507 X GA21			**Trueness and repeatability of TC1507 quantification on single event TC1507***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.10	-25	13	**0.10**	6	18
0.50	-20	11	**0.50**	-4	12
0.90	-13	8.0	**0.90**	3.7	7.7
2.0	-12	20	**2.0**	-1.7	8.5
5.0	2.0	8.5	**5.0**	8.4	14

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx)

Table 15. Comparison of the performance of the GA21 detection method applied to genomic DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 X GA21 maize and from the single line event GA21.

Trueness and repeatability of GA21 quantification on Bt11 X 59122 X MIR604 X TC1507 X GA21			**Trueness and repeatability of GA21 quantification on single event GA21***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.09	3.1	7.1	**0.09**	-8.7	23
0.50	2.9	8.2	**0.50**	0.8	17
0.90	4.6	3.8	**0.90**	1.6	20
5.0	3.4	2.9	**5.0**	-5.6	20
8.0	6.0	4.8	**8.0**	-8.5	17

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx)

6. Conclusions

The performance of the five event-specific methods for the detection and quantification of maize events Bt11, 59122, MIR604, TC1507 and GA21, when applied to genomic DNA extracted from GM stack Bt11 X 59122 X MIR604 X TC1507 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 confirmed that the five 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 59122 X MIR604 X TC1507 X GA21 maize.

Therefore these methods, developed and validated to detect and quantify the single events, can be equally applied for the detection and quantification of the respective events combined in GM stack Bt11 X 59122 X MIR604 X TC1507 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.

2. Commission Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically

unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation (Text with EEA relevance). OJ L 102, 7.4.2004, p. 14–25.

3. European Network of GMO Laboratories: Definition of minimum performance requirements for analytical methods of GMO testing. 13 October 2008. http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf.

Annex 1

syngenta

Identification of PCR Buffer as Key Component for Underperformance of the Event-specific Detection Method for DAS-59122-7 Transformation Event in Bt11 × DAS-59122-7 × MIR604 × TC1507 × GA21 Maize in Response to EU-RL GMFF Request Concerning the EFSA/GMO/DE/2011/99 Application

Final Report

DATA REQUIREMENT(S): Not Applicable

AUTHOR: Hope Hart

COMPLETION DATE: December 10, 2013

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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

The following statement applies to submissions to the United States Environmental Protection Agency (US EPA).

No Claim of Confidentiality

No claim of confidentiality is made for any information contained in this report on the basis of its falling within the scope of Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Section 10 (d) (1) (A), (B), or (C).

Company: *Syngenta Seeds, Inc.*

Company Representative:

Katie Davis
Manager, Regulatory Affairs

December 10, 2013
Date

These data are the property of Syngenta Seeds, Inc. and, as such, are considered to be confidential for all purposes other than compliance with the regulations implementing FIFRA Section 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under any other provision of common law or statute or in any other country.

The following statement applies to submissions to regulatory agencies and other competent authorities other than the US EPA and all other viewers.

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Its submission does not constitute a waiver of any right to confidentiality that may exist in any other country.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This is not a study as defined by 40 CFR Part 160.3 and is therefore not subject to Federal Insecticide, Fungicide, and Rodenticide Act Good Laboratory Practice Standards. However, all components of this analysis were performed according to accepted scientific practices, and relevant records have been retained.

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GENERAL INFORMATION

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Records Retention

Raw data, the original copy of this report, and other relevant records are archived at Syngenta, 3054 East Cornwallis Road, Research Triangle Park, NC 27709-2257 USA.

Additional Test Sites

The analytical work reported herein was conducted at Eurofins GeneScan GmbH, Engesserstrasse 4, 79108 Freiburg, Germany.

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LIST OF ACRONYMS AND ABBREVIATIONS

CT	cycle threshold
DNA	deoxyribonucleic acid
E	amplification efficiency
ENGL	European Network of GMO Laboratories
EU-RL GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
non-GM	non-genetically modified
PCR	polymerase chain reaction
RSD_r	relative repeatability standard deviation
STDEV	standard deviation
US EPA	United States Environmental Protection Agency

1.0 EXECUTIVE SUMMARY

The combined maize trait product Bt11 × DAS-59122-7 × MIR604 × TC1507 × GA21 maize (hereafter referred to as 'Bt11 × 59122 × MIR604 × 1507 × GA21 maize') is a genetically modified (GM) maize that is produced by conventional breeding crosses of the following GM maize events: Bt11, DAS-59122-7[1] (hereafter referred to as 59122 maize), MIR604, TC1507[2] (hereafter referred to as 1507 maize) and GA21.

The following report describes the generation of additional data evaluating Applied Biosystems® GeneAmp® PCR buffer II (ABI PCR buffer II) with the quantitative polymerase chain reaction (PCR) methods to specifically detect Event 59122 maize in the hybrid Bt11 × 59122 × MIR604 × 1507 × GA21 as requested by the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) (JRC.DG.1.3- MBG/JK/en/lv). During the EU-RL GMFF in-house verification of the real-time PCR methods submitted for the quantitative detection of the maize events Bt11, 59122, MIR604, 1507, and GA21 in the hybrid Bt11 × 59122 × MIR604 × 1507 × GA21 maize, underperformance of the 59122-specific method was observed, whereas the performance of the Bt11-, MIR604-, TC1507-, and GA21-specific methods met the European Network of GMO Laboratories (ENGL) acceptance criteria. The 59122-specific method had been successfully validated in a collaborative study in 2005 for the quantitative detection of 59122 maize. In addition, the method performed according to the ENGL acceptance criteria in the in-house validation study on the quantitative detection of the events Bt11, 59122, MIR604, 1507, and GA21 in the hybrid Bt11 × 59122 × MIR604 × 1507 × GA21 maize submitted to the EU-RL GMFF (EFSA-GMO-DE-2011-99).

Extensive investigations were carried out to clarify why the 59122-specific method underperformed during the EU-RL GMFF validation study. ABI PCR buffer II was identified as a key component for the underperformance of the 59122-specific detection method in the hybrid Bt11 × 59122 × MIR604 × 1507 × GA21 maize. Significant batch-to-batch variability of ABI PCR buffer II resulting in performance variations was demonstrated. In addition, it was shown that in-house preparation of PCR buffer of identical composition as ABI PCR buffer II but with a pH of 8.0 significantly improved the performance of the 59122-specific detection method in the hybrid Bt11 × 59122 × MIR604 × 1507 × GA21 maize.

[1] Event DAS-59122-7 may be referred to as maize line 59122 in the European Union. It is normally referred to as Event DAS-59122-7 in applications submitted by Dow AgroSciences LLC to most countries other than the European Union. This applies also to the Detection Method published by the Community Reference Laboratory for Food and Feed in the EU.

[2] Event TC1507 may be referred to as maize line 1507 in the European Union. It is normally referred to as Event TC1507 in applications submitted by Dow AgroSciences LLC to most countries other than the European Union. This applies also to the Detection Method published by the Community Reference Laboratory for Food and Feed in the EU.

2.0 INTRODUCTION

The combined maize trait product Bt11 × DAS-59122-7 × MIR604 × TC1507 × GA21 maize (hereafter referred to as 'Bt11 × 59122 × MIR604 × 1507 × GA21 maize') is a genetically modified (GM) maize that is produced by conventional breeding crosses of the following GM maize events: Bt11, DAS-59122-7[1] (hereafter referred to as 59122 maize), MIR604, TC1507[2] (hereafter referred to as 1507 maize) and GA21.

The following report describes the generation of additional data evaluating Applied Biosystems® GeneAmp® PCR buffer II (ABI PCR buffer II) with the quantitative polymerase chain reaction (PCR) methods to specifically detect Event 59122 maize in the hybrid Bt11 × 59122 × MIR604 × 1507 × GA21 as requested by the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) (Reference number JRC.DG.1.3- MBG/JK/en/lv). During the EU-RL GMFF in-house verification of the real-time PCR methods submitted for the quantitative detection of the maize events Bt11, 59122, MIR604, 1507, and GA21 in the hybrid Bt11 × 59122 × MIR604 × 1507 × GA21 maize underperformance of the 59122-specific method was observed, whereas the performance of the Bt11-, MIR604-, TC1507-, and GA21-specific methods met the European Network of GMO Laboratories (ENGL) acceptance criteria. The 59122-specific method had been successfully validated in a collaborative study in 2005 for the quantitative detection of 59122 maize. In addition, the method performed in line with the ENGL acceptance criteria in the in-house validation study on the quantitative detection of the events Bt11, 59122, MIR604, 1507, and GA21in the hybrid Bt11 × 59122 × MIR604 × 1507 × GA21 maize submitted to the EU-RL GMFF (EFSA-GMO-DE-2011-99). Extensive investigations were carried out to clarify why the 59122-specific method underperformed during the EU-RL GMFF validation study.

2.1 Control of Bias Statement

Any rejected data, and the documented reasons for the rejection of those data, were retained in the study file.

2.2 Statistical Analysis Statement

Average, standard deviation, slope, intercept, R^2 coefficient, and amplification efficiency calculations were performed using Microsoft Office Excel® 2010 software.

[1] Event DAS-59122-7 may be referred to as maize line 59122 in the European Union. It is normally referred to as Event DAS-59122-7 in applications submitted by Dow AgroSciences LLC to most countries other than the European Union. This applies also to the Detection Method published by the Community Reference Laboratory for Food and Feed in the EU.

[2] Event TC1507 may be referred to as maize line 1507 in the European Union. It is normally referred to as Event TC1507 in applications submitted by Dow AgroSciences LLC to most countries other than the European Union. This applies also to the Detection Method published by the Community Reference Laboratory for Food and Feed in the EU.

2.3 Data Quality and Integrity

No circumstances occurred during the conduct of this analysis that would have adversely affected the quality or integrity of the data generated.

3.0 BATCH-TO-BATCH VARIABILITY OF COMMERCIAL PCR BUFFER II

The first indicators of batch-to-batch variability of ABI PCR buffer II came from experiments in which deoxyribonucleic acid (DNA) preparations of Bt11 × 59122 × MIR604 × 1507 × GA21 maize and non-genetically modified (non-GM) maize were tested for absence of PCR inhibitory substances. The testing was carried out with five different lots of ABI PCR buffer II and revealed inconsistent (however reproducible) results with the different lots.

3.1 Experimental Setup

In order to assess the influence of different ABI PCR buffer II production lots on the performance of the 59122-specific method, five ABI PCR buffer II lots were tested. Five different reaction mixes were prepared as described in the publicly available event-specific detection protocol (http://gmo-crl.jrc.ec.europa.eu/summaries/59122-Protocol%20Validation.pdf), differing only in the ABI PCR buffer II lot used. An experimental design generally used for the detection of DNA inhibition was applied. Subsequent fourfold serial dilutions of a DNA sample adjusted to a concentration of 40 ng/µl of 10% Bt11 × 59122 × MIR604 × 1507 × GA21 maize DNA in non-GM maize DNA were prepared. The starting DNA samples at 40 ng/µl as well as all dilutions (1:4, 1:16, 1:64, and 1:256) were analyzed in triplicates on ABI 7900HT. The data were evaluated as follows: to measure inhibition, the cycle threshold (CT) values of the four diluted samples were plotted against the logarithm of the reciprocal dilution factor and the CT value for the undiluted (40 ng/µl; 200 ng/reaction) sample was extrapolated from the equation calculated by linear regression. Subsequently, the extrapolated CT for the undiluted sample was compared with the measured one. A deviation of the measured CT value for the undiluted sample from the calculated CT value by ≥ 0.5 cycles was considered an indicator for the presence of PCR inhibitors, or unrobust performance of the PCR method.

3.2 Results and Discussion

Batch-to-batch variability of PCR buffer II was shown in experiments in which DNA preparations of Bt11 × 59122 × MIR604 × 1507 × GA21 maize and non-GM maize were tested for absence of PCR inhibitory substances (Table 1). The data revealed inconsistent results with the five different ABI PCR buffer II lots. These inconsistent results were reproducible.

TABLE 1 **Comparison of Extrapolated CT Values versus Measured CT Values with the 59122-specific Method with Various Lots of ABI PCR Buffer II with 10% Bt11 × 59122 × MIR604 × 1507 × GA21 DNA**

ABI PCR buffer II lot number	CT extrapolated	CT measured	delta CT	Result
R08583	24.8	25.0	0.2	No inhibition
S00669	24.9	25.0	0.1	No inhibition
1210039	25.0	25.7	0.7	Inhibition
R01915	24.7	25.7	1.0	Inhibition
R02959	24.9	25.4	0.5	Inhibition

The results indicated PCR inhibition in the DNA preparations tested with buffer lots 1210039, R01915, and R02959. No PCR inhibition was observed with buffer lots R08583 and S06690.

4.0 SEARCH FOR MEANS TO RESTORE ROBUST PERFORMANCE OF THE 59122-SPECIFIC PCR METHOD

In an attempt to restore robust performance of the 59122-specific PCR method, PCR buffer II lots were prepared in-house according to the recipe given by the manufacturer (500 mM potassium chloride, 100 mM Tris-HCl, pH 8.3 at room temperature). In addition, slightly modified buffer variants with different pH values were produced.

4.1 Experimental Setup

A total of six PCR buffer II variants were tested, four in-house variants, ABI PCR buffer II lot 1210039 at pH 8.3 (unadjusted), and ABI PCR buffer II lob 1210039 with the pH adjusted to 8.0 (Table 2). Reaction mixes were prepared as described in the publicly available event-specific detection protocol (http://gmo-crl.jrc.ec.europa.eu/summaries/59122-Protocol%20Validation.pdf), differing only in the variant of PCR buffer II included in the mix. An experimental set-up generally used for detection of DNA inhibition was applied. Subsequent fourfold serial dilutions of a DNA sample adjusted to a concentration of 40 ng/µl were prepared (10% Bt11 × 59122 × MIR604 × 1507 × GA21 maize DNA in non-GM maize DNA background) and analyzed using the 59122-specific PCR method. All dilutions were analyzed in triplicates on ABI 7900HT Sequence Detection System. The data were evaluated

as follows: to measure inhibition, the CT values of the four diluted samples were plotted against the logarithm of the reciprocal dilution factor and the CT value for the undiluted (40 ng/µl; 200 ng/reaction) sample was extrapolated from the equation calculated by linear regression. Subsequently, the extrapolated CT for the undiluted sample was compared with the measured one. A deviation of the measured CT value for the undiluted sample from the calculated CT value by ≥ 0.5 cycles was considered an indicator for the presence of PCR inhibitors, or unrobust performance of the PCR method. All buffers had identical salt concentrations (500 mM potassium chloride and 100 mM Tris-HCl) and differ only by pH value and manufacturer.

TABLE 2 PCR Buffer II Variants

Lot number	pH value	Source
1210039	8.3 (unadjusted)	ABI
1210039	8.0 (adjusted)	ABI; pH adjusted in-house
1	8.3	In-house
2	8.0	In-house
5	8.3	In-house
6	8.0	In-house

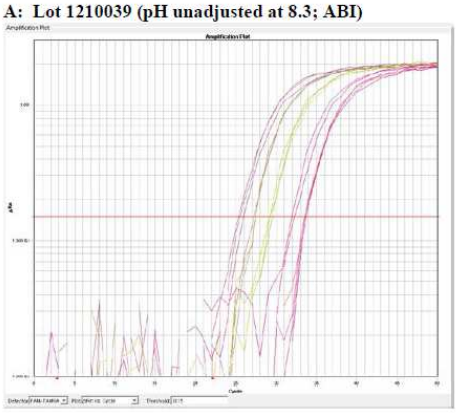
4.2 Results and Discussion

The shapes of amplification plots were compared (FIGURE), and the data were evaluated as described above (Table 3). For the 59122-specific PCR method the pH 8.0 variants show very regular and reliable amplification curves providing more uniform CT values within the duplicates. No scattering or forking effects occurred. In contrast all pH 8.3 variants show significant CT differences between replicates.

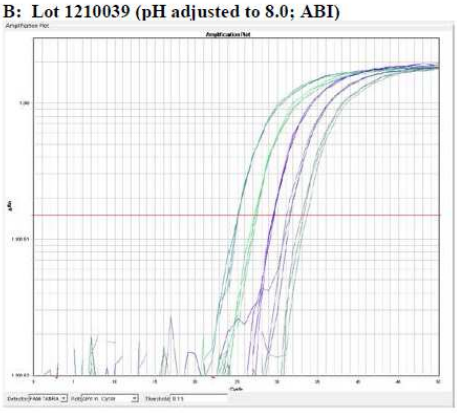
No inhibitory effects were observed for any of the three PCR buffer II variants with a pH value of 8.0 (1210039 pH adjusted to 8.0, lot 2, and lot 6). However, all three PCR buffer II variants with a pH value of 8.3 (1210039, lot 1, and lot 5) showed PCR inhibition for the same DNA sample.

FIGURE Logarithmic Amplification Plots

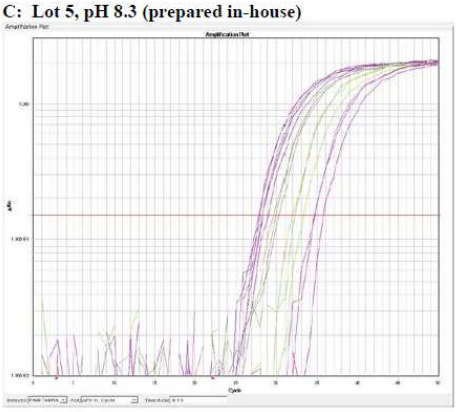
A: Lot 1210039 (pH unadjusted at 8.3; ABI)



B: Lot 1210039 (pH adjusted to 8.0; ABI)



C: Lot 5, pH 8.3 (prepared in-house)



D: Lot 6, pH 8.0 (prepared in-house)

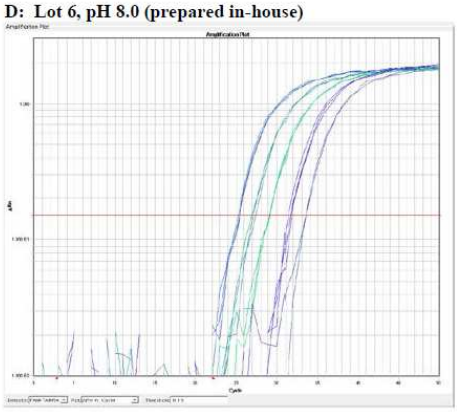


TABLE 3 Comparison of Extrapolated CT Values versus Measured CT Values

PCR buffer variant	Source	CT extrapolated	CT measured	delta CT	Result
Lot 1210039, pH 8.3 unadjusted	ABI	25.1	25.6	0.5	Inhibition
Lot 1210039, adjusted to pH 8.0	ABI	25.3	25.1	-0.2	No inhibition
Lot 1, pH 8.3	In-house	26.9	27.5	0.6	Inhibition
Lot 2, pH 8.0	In-house	25.0	25.3	0.3	No inhibition
Lot 5, pH 8.3	In-house	25.8	28.2	2.4	Inhibition
Lot 6, pH 8.0	In-house	25.1	25.2	0.1	No inhibition

5.0 ASSESSMENT OF COMMERCIAL AND IN-HOUSE PREPARED PCR BUFFER II VARIANTS

In order to generate additional data verifying the hypothesis that changing the pH of PCR buffer II from 8.3 to 8.0 improves the 59122-specific PCR method performance significantly and restores former robustness, further experiments were carried out. The quantification performance of the method was assessed.

5.1 Precision and Trueness

5.1.1 Experimental Setup

In order to assess quantification performance and determine precision and trueness according to the definitions set out in the guidance documents of the EU-RL GMFF, the following experimental design was carried out in three independent tests for each of two PCR buffer II variants (lot 1210039 pH 8.3 and lot 6 pH 8.0). Reaction mixes were prepared as described in the publicly available 59122-specific detection protocol (http://gmo-crl.jrc.ec.europa.eu/summaries/59122-Protocol%20Validation.pdf), differing only in the PCR buffer used.

Reference samples (containing 5%, 0.9%, and 0.08% Bt11 × 59122 × MIR604 × 1507 × GA21 DNA in non-GM maize DNA) were analyzed in triplicates at 200 ng genomic DNA per reaction. The quantification results were determined by calculating the mean value of triplicates. On each PCR plate for the 59122-specific system, calibration standards (standard 1 through to standard 4 and serial dilutions of Bt11 × 59122 × MIR604 × 1507 × GA21 in non-GM maize DNA) were run in triplicates for both the 59122-specific PCR method and the maize *hmg*-specific PCR method. One negative control per method was included to verify purity of the reagents. A total of eight quantification results were generated for the 5%, 0.9%, and 0.085% Bt11 × 59122 × MIR604 × 1507 × GA21 DNA mixtures with each PCR buffer II variant.

Precision (RSD_r)
Precision is defined as the standard deviation of test results obtained under repeatability conditions (*i.e.* RSD_r). Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time (*i.e.* intra-laboratory repeatability).

Acceptance criterion: RSD_r should be ≤ 25% over the whole dynamic range (0.08% to 5% Bt11 × 59122 × MIR604 × 1507 × GA21 DNA).

Trueness (Bias)
Trueness is defined as the closeness of agreement between the average value obtained from a large series of test results and the accepted reference value.

Acceptance criterion: Trueness should be within ±- 25% of the accepted reference value over the whole dynamic range (0.08% to 5% Bt11 × 59122 × MIR604 × 1507 × GA21 DNA).

5.1.2 Results and Discussion

For each of the three reference samples the mean value (MEAN), the relative deviation from the expected value (BIAS),the standard deviation (STDEV), and the relative standard deviation (RSD_r) of the quantification results were calculated in order to determine trueness and repeatability. The results are shown in Table 4 and Table 5.

TABLE 4 Quantification Results under Repeatability Conditions with ABI PCR Buffer II Lot 1210039, pH 8.3

DNA	Test 1	Test 2	Test 3	MEAN	BIAS	STDEV	RSD_r
5 %	4.04 %	5.25 %	N/A	4.19 %	**-16.2 %**	0.839	**20.0 %**
	2.93 %	4.16 %	N/A				
	4.34 %	5.28 %	N/A				
	3.20 %	4.28 %	N/A				
0.9 %	0.550 %	0.791 %	N/A	0.586 %	**-34.9 %**	0.1105	**18.9 %**
	0.465 %	0.656 %	N/A				
	0.514 %	0.671 %	N/A				
	0.491 %	0.552 %	N/A				
0.08 %	0.033 %	0.046 %	0.007 %	0.054 %	**-32.5 %**	0.0404	**74.8 %**
	0.026 %	0.031 %	0.105 %				
	0.062 %	0.123 %	N/A				

N/A = not applicable.

TABLE 5 **Quantification Results under Repeatability Conditions with PCR Buffer II Lot 6, pH8.0**

DNA	Test 1	Test 2	Test 3	MEAN	BIAS	STDEV	RSD_r
5 %	4.94 %	5.00 %	N/A	5.00 %	0.0 %	0.106	2.1 %
	4.93 %	5.15 %	N/A				
	5.03 %	5.08 %	N/A				
	5.03 %	4.80 %	N/A				
0.9 %	0.840 %	0.890 %	N/A	0.854 %	-5.1 %	0.0457	5.4 %
	0.926 %	0.829 %	N/A				
	0.806 %	0.903 %	N/A				
	0.816 %	0.820 %	N/A				
0.08 %	0.072 %	0.066 %	0.070 %	0.065 %	-18.8 %	0.0062	9.5 %
	0.064 %	0.054 %	0.072 %				
	0.060 %	0.065 %	N/A				

5.2 Amplification Efficiency

5.2.1 Experimental setup

In order to assess amplification efficiency (E) and R^2 coefficient of the *hmg*- and 59122-specific PCR methods, linear regression analysis of CT values versus log[GM concentration] was performed. The regression parameters of the standards of three independent tests including slope, intercept, and R^2 have been determined. The efficiency of the amplification was calculated using the following equation: $E = [10(-1/slope)] -1$.

Acceptance criteria:
The average value of the slope should be in the range of $-3.1 \geq slope \geq -3.6$.
The average value of R^2 should be ≥ 0.98.

5.2.2 Results and discussion

Table 6 summarizes the amplification efficiency and R^2 coefficient data for the *hmg*-specific PCR method. For the *hmg*-specific method, the average value of the slope is -2.85, and the average value of R^2 coefficient is 0.98 for Lot 1210039. The average value of the slope is -3.18, and the average value of R^2 coefficient is 1.00 for Lot 6, pH 8.0. Therefore, the efficiency and R^2 coefficient are acceptable for Lot 6, pH 8.0. The R^2 coefficient for Lot 1210039 is acceptable, but the slope is not acceptable.

TABLE 6 **Regression Parameters and PCR Efficiencies of the *hmg*-specific Regression Lines**

	Lot 1210039			
	Slope	**Intercept**	**R^2 Coefficient**	**Efficiency**
Test 1	-2.75	37.3	0.98	1.31
Test 2	-2.86	37.8	0.98	1.24
Test 3	-2.94	38.0	0.99	1.19
MEAN	**-2.85**	**37.7**	**0.98**	**1.25**
	Lot 6, pH 8.0			
	Slope	**Intercept**	**R^2 Coefficient**	**Efficiency**
Test 1	-3.14	38.4	1.00	1.08
Test 2	-3.23	38.9	1.00	1.04
Test 3	-3.16	38.8	1.00	1.07
MEAN	**-3.18**	**38.7**	**1.00**	**1.06**

Table 7 summarizes the amplification efficiency and R^2 coefficient data for the 59122-specific PCR method. For the 59122-specific method, the average value of the slope is -2.94, and the average value of R^2 coefficient is 0.99 for Lot 1210039. The average value of the slope is -3.27, and the average value of R^2 coefficient is 1.00 for Lot 6, pH 8.0. Therefore, the efficiency and R^2 coefficient are acceptable for Lot 6, pH 8.0. The R^2 coefficient for Lot 1210039 is acceptable, but the slope is not.

TABLE 7 **Regression Parameters and PCR Efficiencies of the 59122-specific Regression Lines**

	Lot 1210039			
	Slope	**Intercept**	**R^2 Coefficient**	**Efficiency**
Test 1	-2.97	37.9	0.99	1.17
Test 2	-3.01	38.2	0.99	1.15
Test 3	-2.84	37.6	1.00	1.25
MEAN	**-2.94**	**37.9**	**0.99**	**1.19**
	Lot 6, pH 8.0			
	Slope	**Intercept**	**R^2 Coefficient**	**Efficiency**
Test 1	-3.24	38.3	1.00	1.03
Test 2	-3.27	38.4	1.00	1.02
Test 3	-3.29	38.5	1.00	1.01
MEAN	**-3.27**	**38.4**	**1.00**	**1.02**

6.0 CONCLUSIONS

This report summarizes the results identifying ABI PCR buffer II ABI as a key component for underperformance of the event-specific detection method for the 59122 transformation event in the maize hybrid Bt11 × 59122 × MIR604 × 1507 × GA21. PCR experiments with the 59122-specific PCR method and different PCR buffer II variants showed significant differences in overall method performances. It was shown that in-house preparation of PCR buffer of identical composition as ABI PCR buffer II, but with a pH of 8.0, resulted in improved amplification curves revealing less variation between replicates and satisfying quantification results with the event-specific detection method for the 59122 transformation event in the maize hybrid Bt11 × 59122 × MIR604 × 1507 × GA21.

We therefore recommend to replace the original ABI PCR buffer II that has a pH of 8.3 by either in-house prepared PCR buffer II of identical salt concentrations adjusted to pH 8.0 or ABI PCR buffer II with a pH value adjusted to 8.0, in order to improve and recover the overall method performance of the 59122-specific PCR method.

7.0 REFERENCES

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Abstract

An application was submitted by Syngenta Crop Protection AG to request the authorisation of genetically modified stack (GM stack) Bt11 x 59122 x MIR604 x TC1507 x GA21 maize (tolerant to herbicide products containing glufosinate ammonium/glyphosate and resistant to certain lepidopteran/coleopteran pests) and all sub-combinations of the individual events as present in the segregating progeny (except for 1507 x 59122), 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 GM stack Bt11 x 59122 x MIR604 x TC1507 x GA21 maize is SYN-BTØ11-1 × DAS-59122-7 × SYN-IR6Ø4-5 × DAS-Ø15Ø7-1 × MON-ØØØ21-9.

The GM stack Bt11 x 59122 x MIR604 x TC1507 x GA21 maize has been obtained by conventional crossing of genetically modified maize events: Bt11, 59122, MIR604, TC1507 and GA21, without any new genetic modification.

The European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF) has previously validated individually, and declared fit for purpose, the detection methods for the single events Bt11, 59122, MIR604, TC1507 and GA21 (see http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). In line with the approach defined by the European Network of GMO Laboratories (ENGL) (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EU-RL 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 Bt11 x 59122 x MIR604 x TC1507 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 Bt11 x 59122 x MIR604 x TC1507 x GA21 maize.
Noteworthy the 59122 event-specific method applied to GM stack DNA differs from the validated method for the pH value of PCR buffer (pH 8.0 instead of pH 8.3). However, this deviation does not significantly affect the performance of the method and hence the pH 8.0-method is considered equivalent to the originally validated pH 8.3 method protocol.

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