Report on the Verification of the Performance of Bt11 and MIR604 Event-specific Methods on the Maize Event Bt11 x MIR604 Using Real-Time PCR

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Executive Summary

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, has carried out a verification study to assess the performance of two quantitative event-specific methods on the maize event Bt11 x MIR604 (unique identifier SYN-BTØ11-1 x SYN-IR6Ø4-5) which combines the Bt11 and MIR604 transformation events. The two methods have been validated individually on single-trait events, to detect and quantify each event in maize samples. This study was conducted according to internationally accepted guidelines [1, 2].

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Syngenta Seeds S.A.S. provided the detection methods and the control samples: genomic DNA extracted from homogenised seeds of Bt11 x MIR604 maize (NP2276Bt11/NP2391MIR604), genomic DNA extracted from homogenised seeds of non-GM maize (NP2276/NP2391) and flour ground from seeds of NP2276Bt11/NP2391MIR604 and from seed of NP2276/NP2391. The EURL-GMFF prepared the verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house verification study were evaluated with reference to the European Network of GMO Laboratories (ENGL) method performance requirements (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and to the validation results on the individual parental events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results of this EURL-GMFF verification study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/.

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Report on Steps 1-3 of the Validation Process

Syngenta Seeds S.A.S. submitted the detection methods and control samples of the maize event Bt11 x MIR604 (unique identifier SYN-BTØ11-1 x SYN-IR6Ø4-5) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "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" and according to its operational procedures ("Description of the EURL-GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/doc/Description%20CRL%20validation%20process.pdf).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requir_Analyt_methods_131008.pdf) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed and one request of complementary information was addressed to the applicant. Upon reception of the complementary information, the scientific assessment of the detection method for the Bt11 x MIR604 maize was positively concluded in May 2008.

The event-specific detection methods for the two maize lines hosting the single events Bt11 and MIR604 were validated by the EURL-GMFF following the conclusion of the respective international collaborative studies and the publication of the validation reports (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). Hence, the detection methods applied on the maize event Bt11 x MIR604 did not undergo a full validation process. The EURL-GMFF performed a verification of the detection methods to confirm that they exhibit a comparable performance on samples of event Bt11 x MIR604 combining the two traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In May 2008, the EURL-GMFF concluded the experimental verification of the method characteristics (step 3, experimental testing of the samples and methods) by quantifying, with each specific method, five blind GM-levels within the range 0.09%-8% and 0.1%-6% for Bt11 and MIR604 respectively, on a DNA/DNA ratio. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were mostly within the limits established by the ENGL.

A Technical Report summarising the results of tests carried out by the EURL-GMFF (step 3) is available on request.

Content

1. Introduction

Syngenta Seeds S.A.S. submitted the detection methods for Bt11 and MIR604 and the control samples of the maize event Bt11 x MIR604 (unique identifier SYN-BTØ11-1 x SYN-IR6Ø4-5) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed, established by Regulation (EC) 1829/2003, carried out a verification of the two event-specific methods for the detection and quantification of Bt11 and MIR604 in the Bt11 x MIR604 maize event combining the two traits. The single methods had been previously validated by international collaborative studies on the single-trait maize events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Upon reception of methods, samples and related data (step 1), the EURL-GMFF carried out the assessment of the documentation (step 2) and the verification of the methods (step 3) according to the requirements of Regulation (EC) 641/2004 and following EURL-GMFF operational procedures. The EURL-GMFF method verification was concluded in May 2008.

A method for DNA extraction from maize seeds, submitted by the applicant, was evaluated by the EURL-GMFF to confirm its performance characteristics. The protocol for DNA extraction is available at http://gmo-crl.jrc.ec.europa.eu/.

The procedure of the in-house verification consisted of a quantitative real-time Polymerase Chain Reaction (PCR). The methodology consists of two event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of events Bt11 and MIR604 DNA to total maize DNA in the Bt11 x MIR604 maize event. The procedures were simplex systems, in which the events Bt11 and MIR604 were quantified in reference to the maize *adh1* (alcohol dehydrogenase-1) taxon-specific endogenous gene.

The study was carried out in accordance to the following internationally accepted guidelines:

- ✓ ISO 5725:1994 (1)
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (2).

2. Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenised seeds of BT11 x MIR604 maize (NP2276Bt11/NP2391MIR604),
- genomic DNA extracted from homogenised seeds of non-GM maize (NP2276/NP2391),

in accordance to the provisions of Regulation (EC) No 1829/2003, Art 2.11 ["control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)].

Samples containing mixtures of BT11 x MIR604 DNA and non-GM maize genomic DNA at different GMO contents were prepared in a constant amount of total maize DNA.

The validated methods for the individual Bt11 and MIR604 events were applied in the verification as published and available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

In table 1 are indicated the five GM contents used in the verification of the Bt11 and MIR604 methods.

Table 1. Bt11 and MIR604 GM contents in maize event Bt11 x MIR604

Bt11 GM % (GM DNA / Non-GM DNA *100)	**MIR604 GM %** (GM DNA / Non-GM DNA *100)
0.09	0.10
0.40	0.40
0.90	0.90
5.00	2.50
8.00	6.00

3. Experimental design

Eight runs for each event-specific method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the target taxon-specific assay (*adh1*). Five GM contents per run were examined and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. In total, for each method (Bt11 and MIR604), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level.

4. Method

Description of the operational steps

To detect Bt11 and MIR604 events in maize event Bt11 x MIR604, two specific fragments of 68-bp and 76-bp respectively, corresponding to the integration regions of the construct into the plant genome, were amplified using specific primers.

For relative quantification of events Bt11 and MIR604 DNA, is employed a maize-specific target taxon system which amplifies a 135-bp fragment of the maize gene *adh1* (alcohol dehydrogenase 1), using *adh1* specific primers and an *adh1* specific probe labelled with VIC and TAMRA.

Standard curves are generated for each GM specific system (Bt11 or MIR604), by plotting the ΔCt values of the calibration samples against the logarithm of the amount of events Bt11 or MIR604 DNA, and by fitting a linear regression into these data. Thereafter, the relative amount of event Bt11 or MIR604 DNA is estimated by means of the regression function from the normalised ΔCt values of the unknown samples.

Detailed information on standard curve samples preparation is available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

5. Deviations reported

The Sigma JumpStart Taq Ready Mix was supplemented with 600 nM sulforhodamine for all PCR reactions (Bt11, MIR604 and Adh1 specific assays), i.e. the final concentration of sulforhodamine in each PCR reaction was 300 nM.

6. Summary of results

PCR efficiency and R^2

The values of the slopes of the standard curves, the PCR efficiency and the R^2 (expressing the linearity of the regression) are presented for Bt11 and MIR604 methods respectively in Tables 2 and 3. The data are reported for the eight runs. The PCR efficiency was calculated using the formula [10^(-1/slope)-1]*100.

Table 2. Values of standard curve slope, PCR efficiency and R^2 of Bt11 method (on event Bt11 x MIR604).

Run	Bt11		
	Slope	PCR Efficiency (%)	R^2
1	-3.46	95	1.00
2	-3.27	102	1.00
3	-3.44	95	1.00
4	-3.37	98	1.00
5	-3.33	100	1.00
6	-3.29	101	1.00
7	-3.42	96	1.00
8	-3.35	99	1.00
Mean	-3.37	98	1.00

Table 3. Values of standard curve slope, PCR efficiency and R^2 of MIR604 method (on event Bt11 x MIR604).

Run	MIR604		
	Slope	PCR Efficiency (%)	R^2
1	-3.12	109	1.00
2	-3.23	104	1.00
3	-3.20	105	1.00
4	-3.25	103	1.00
5	-3.29	101	1.00
6	-3.28	102	1.00
7	-3.41	97	1.00
8	-3.24	103	1.00
Mean	-3.25	103	1.00

The mean PCR efficiencies for the Bt11 and MIR604 methods were respectively 98% and 103%. The R^2 was 1.00 for both methods. Overall, the data reported in table 2 and 3

confirmed the appropriate performance characteristics of the two methods tested on Bt11 x MIR604 maize samples.

7. Method performance requirements

The results of the verification study for the Bt11 and MIR604 detection methods applied to event Bt11 x MIR604 maize DNA are reported in tables 4 and 5, respectively. Results were evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by the EURL-GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), see also Annex 1). In addition, tables 4 and 5 report estimates the trueness and the relative repeatability standard deviation for each GM content for both methods.

Table 4. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSDr %) of the Bt11 method on event Bt11 x MIR604 maize DNA.

Bt11					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.4**	**0.9**	**5.0**	**8.0**
Mean	0.09	0.39	0.97	5.41	8.76
SD	0.01	0.05	0.09	0.39	0.95
RSDr (%)	14	12	9.6	7.2	11
Bias (%)	5.5	-2.1	7.6	8.3	9.5

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the MIR604 method on event Bt11 x MIR604 maize DNA.

MIR604					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.4**	**0.9**	**2.5**	**6.0**
Mean	0.09	0.40	0.95	2.54	6.80
SD	0.02	0.03	0.10	0.18	0.60
RSDr (%)	21	8.4	10	7.0	8.8
Bias (%)	-8.2	0.2	5.5	1.8	13

The *trueness* of the method is estimated using the measures of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method, measured as bias from the accepted value, should be ± 25% across the entire dynamic range. As shown in Tables 4 and 5, both methods satisfied the above requirement throughout their respective dynamic ranges; in fact, the highest bias was 9.5% and 13% for the Bt11 and MIR604 methods, respectively.

Tables 4 and 5 further document the *relative repeatability standard deviation* (RSD_r) as estimated for each GM content. In order to accept methods for collaborative trial evaluation, the EURL-GMFF requires that RSD_r values are below 25%, as indicated by ENGL (Definition of

Minimum Performance Requirements for Analytical Methods of GMO Testing" [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm]). As it can be observed from the values reported in Tables 4 and 5, the two methods satisfied this requirement throughout their respective dynamic ranges; in fact, the highest RSDr was 14% and 21% for the Bt11 and MIR604 methods, respectively.

8. Comparison of method performance between event Bt11 x MIR604 and the single trait events

An indicative comparison of the two method performances on the maize event Bt11 x MIR604 and on the single trait events is shown in tables 6 and 7. The performance of the methods on the single lines was previously assessed though international collaborative trials.

Table 6. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the Bt11 detection method on events Bt11 x MIR604 and Bt11.

Trueness and repeatability of Bt11 quantification on Bt11 x MIR604			**Trueness and repeatability of Bt11 quantification on single event Bt11***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.09	5.5	14	**0.09**	2.2	17
0.4	-2.1	12	**0.4**	-1.9	13
0.9	7.6	10	**0.9**	1.8	11
5.0	8.3	7	**5.0**	-5.2	13
8.0	9.5	11	**8.0**	-1.2	9

*method validated in collaborative trial (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 7. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the MIR604 detection method on events Bt11 x MIR604 and MIR604.

Trueness and repeatability of MIR604 quantification on Bt11 x MIR604			**Trueness and repeatability of MIR604 quantification on single event MIR604***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.1	-8.2	21	**0.1**	3.6	24
0.4	0.2	8	**0.4**	3.1	17
0.9	5.5	10	**0.9**	-1.0	12
2.5	1.8	7	**2.5**	0.7	16
6.0	13	9	**6.0**	-3.6	14

*method validated in collaborative trial (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

The Bt11 and MIR604 event-specific methods (tables 6 and 7), showed slightly higher or very similar trueness values when were applied to the event Bt11 x MIR604, in comparison to the

single trait events. In all cases, the trueness was within the acceptance value of ± 25% as set by ENGL; in fact the highest values of bias are 9.5% and 13% for Bt11 and MIR604 methods, respectively.

For relative repeatability standard deviation (RSDr %), the Bt11 and MIR604 event-specific methods (Table 6 and 7) showed slightly lower or very similar values when applied to event Bt11 x MIR604, compared to the single events. In all cases, the results were below the established ENGL acceptance level of 25%; in fact, the highest values of RSDr were 14% and 21% for Bt11 and MIR604 methods, respectively.

Therefore, Bt11 and MIR604 detection methods developed to detect and quantify the single events can be equally applied for the quantification of the respective events combined in event Bt11 x MIR604, as demonstrated by the verification.

9. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events Bt11 and MIR604 combined in maize event Bt11 x MIR604 have been evaluated with respect to the method acceptance criteria and the method performance requirements recommended by the ENGL (as detailed under http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), and to the validation results obtained for the single trait events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results obtained during the present verification study indicate that the analytical modules of the methods submitted by the applicant comply with ENGL performance criteria. The methods are therefore applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The EURL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32232) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)].

11. References

1. Horwitz, W., 1995, Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67: 331-343.

2. International Standard (ISO) 5725:1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization.

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: The description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: The practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative

requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: The closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: The accuracy should be within ± 25% of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: The rate of amplification that leads to a theoretical slope of –3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $[10^{(-1/slope)}] - 1$

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of (- 3.1 ≥ slope ≥ - 3.6)

R^2 Coefficient

Definition: The R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R^2 should be ≥ 0.98.

Repeatability Standard Deviation (RSD_r)

Definition: The standard deviation of test results obtained under repeatability conditions. 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.

Acceptance Criterion: The relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

Limit of Quantitation (LOQ)

Definition: The limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than $1/10^{th}$ of the value of the target concentration with an RSD_r ≤ 25%. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

Acceptance Criterion: LOD should be less than $1/20^{th}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq$ 5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance Criterion: The response of an assay with respect to these small variations should not deviate more than $\pm$ 30%. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements

Dynamic Range

Definition: In the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD_R)

Definition: The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An RSD_R < 50 % is acceptable for concentrations below 0.2%.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within $\pm$ 25% of the accepted reference value over the whole dynamic range.