Report on the Verification of the Performance of Bt11 and GA21 Event-specific Methods on the Maize Event Bt11 x GA21 Using Real-Time PCR

7 November 2008

Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit

Executive Summary

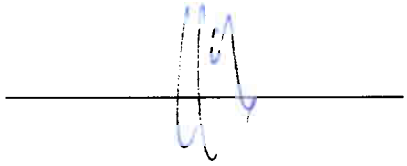
The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, has carried out an in-house verification study to assess the performance of two quantitative event-specific methods on the maize event Bt11 x GA21 (unique identifier SYN-BTØ11-1 x MON-ØØØ21-9) which combines the Bt11 and GA21 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 GA21 maize (NP982Bt11/NP2673GA21), genomic DNA extracted from homogenised seeds of non-GM maize (NP982/NP2673) and flour ground from seed of NP982Bt11/NP2673GA21 and from seed of NP982/NP2673. The JRC prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

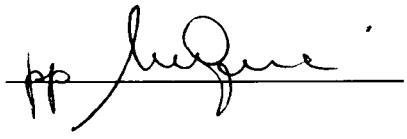
The results of the in-house verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and to the validation results on the individual parental events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results of this CRL-GMFF in-house verification studies are made publicly available at http://gmo-crl.jrc.ec.europa.eu/.

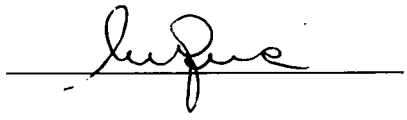
Drafted by:
C. Delobel



Report Verification Team:
1) M. Ermolli

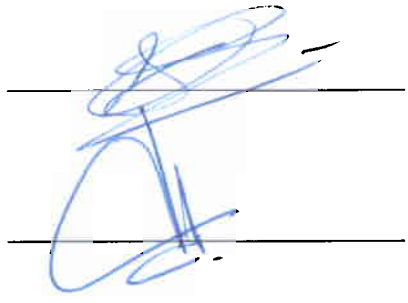


2) M. Querci



Scientific and technical approval:
M. Mazzara

Compliance with CRL Quality System:
S. Cordeil



Authorisation to publish:
G. Van den Eede

Address of contact laboratory:
European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 2749, I-21027 Ispra (VA)
Italy

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 GA21 (unique identifier SYN-BTØ11-1 x MON-ØØØ21-9) 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 Community Reference Laboratory for GM Food and Feed (CRL-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 CRL-GMFF Validation Process", http://gmo-crl.jrc.it/guidancedocs.htm).

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.it/doc/Method%20requirements.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 GA21 maize was positively concluded in April 2008.

The event-specific detection methods for the two maize lines hosting the single events Bt11 and GA21 were validated by the CRL-GMFF following the conclusion of the respective international collaborative studies and the publication of the validation reports (http://gmo-crl.jrc.it/statusofdoss.htm). Hence, the detection methods applied on the maize event Bt11 x GA21 did not undergo a full validation process. The CRL-GMFF performed an in-house verification of the detection methods to verify that they exhibit a comparable performance on samples of event Bt11 x GA21 combining the two traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In April 2008, the CRL-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%, 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 CRL-GMFF (step 3) is available on request.

Content

1. Introduction

Syngenta Seeds S.A.S. submitted the detection methods for Bt11 and GA21 and the control samples of the maize event Bt11 x GA21 (unique identifier SYN-BTØ11-1 x MON-ØØØ21-9) 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 Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed, established by Regulation (EC) 1829/2003, carried out an in-house verification of the two event-specific methods for the detection and quantification of Bt11 and GA21 in the Bt11 x GA21 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 CRL-GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the methods (step 3) according to the requirements of Regulation (EC) 641/2004 and following CRL-GMFF operational procedures. The CRL-GMFF method verification was concluded in April 2008.

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

The operational procedure of the in-house verification included the following module:

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology consists of two event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of events Bt11 and GA21 DNA to total maize DNA in the Bt11 x GA21 maize event. The procedures are simplex systems, in which the events Bt11 and GA21 were quantified in reference to the maize *adh1* (Alcohol dehydrogenase-1) 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

For the verification of the quantitative event-specific methods, control samples consisting of:

- genomic DNA extracted from homogenised seeds of BT11 x GA21 maize (NP982Bt11/NP2673GA21),
- genomic DNA extracted from homogenised seeds of non-GM maize (NP982/NP2673),

were provided by the applicant, 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 100% BT11 x GA21 and non GM maize genomic DNA at different GMO concentrations were prepared by the CRL-GMFF in a constant amount of total maize DNA, using the control samples provided.

The protocols (reagents, concentrations, primer/probe sequences) followed in the in-house verification are those already published as validated methods for the individual Bt11 and GA21 events and available at http://gmo-crl.jrc.it/statusofdoss.htm.

Table 1 shows the five GM levels of unknown samples used in the verification of the Bt11 and GA21 methods.

Table 1. Bt11 and GA21 GM contents in maize event Bt11 x GA21

Bt11 GM % (GM DNA / Non-GM DNA x 100)	**GA21 GM %** (GM DNA / Non-GM DNA x 100)
0.09	0.09
0.40	0.50
0.90	0.90
5.00	5.00
8.00	8.00

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 reference system (*Adh1*). Five GM levels 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 GA21), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level. An Excel spreadsheet was used for determination of GM%.

4. Method

Description of the operational steps

For specific detection of events Bt11 and GA21 in maize event Bt11 x GA21, two specific fragments of the integration regions of the constructs inserted into the plant genome, of 68-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 is used as reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For relative quantification of events Bt11 and GA21 DNA, a maize-specific reference system which amplifies a 135-bp fragment of the maize endogenous gene *adh1* (alcohol dehydrogenase 1), using two *adh1* gene-specific primers and an *adh1* gene-specific probe labelled with VIC and TAMRA, was used.

Standard curves are generated for each GM specific system (Bt11 or GA21), by plotting ΔCt values of the calibration standards against the logarithm of the amount of events BT11 or GA21 DNA, and fitting a linear regression into these data. Thereafter, the normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of event Bt11 or GA21 DNA is estimated respectively.

For detailed information on the preparation of the standard curve calibration samples please refer to the protocols of the validated methods at http://gmo-crl.jrc.it/statusofdoss.htm.

5. Deviations reported

The Sigma JumpStart Taq Ready Mix was supplemented with 600 nM sulforhodamine for all PCR reactions (Bt11, GA21 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 linearity

The values of the slopes of the standard curves, from which the PCR efficiency is calculated using the formula [10^(-1/slope)-1]*100, and of the R^2 (expressing the linearity of the regression) reported for all PCR systems in the eight runs, are presented in Table 2 and 3 for Bt11 and GA21 methods, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the Bt11 method on event Bt11 x GA21.

Run	Bt11		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.57	90.7	1.00
2	-3.46	94.5	1.00
3	-3.34	99.2	1.00
4	-3.33	99.5	0.99
5	-3.46	94.5	1.00
6	-3.37	97.9	1.00
7	-3.37	98.2	1.00
8	-3.36	98.4	1.00
Mean	-3.41	96.6	1.00

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the GA21 method on event Bt11 x GA21.

Run	GA21		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.43	95.8	1.00
2	-3.59	89.9	0.99
3	-3.35	98.9	1.00
4	-3.41	96.3	1.00
5	-3.43	95.6	0.99
6	-3.47	94.4	1.00
7	-3.39	97.3	1.00
8	-3.52	92.3	1.00
Mean	-3.45	95.1	1.00

The mean PCR efficiencies of the GM specific systems were 96.6% and 95.1% respectively for the Bt11 and GA21 specific systems. The linearity of the methods was 1.00 for both systems.

Overall, data reported in Table 2 and 3 confirm the appropriate performance characteristics of the two methods tested on Bt11 x GA21 maize samples in terms of PCR efficiency and linearity.

7. Method performance requirements

The results of the in-house verification study for the Bt11 and GA21 detection methods applied to event Bt11 x GA21 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 CRL-GMFF (http://gmo-crl.jrc.it/guidancedocs.htm, see also Annex 1). In addition, Tables 4 and 5 report estimates the trueness and repeatability standard deviation for each GM level and 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 GA21 maize DNA.

Bt11					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.4**	**0.9**	**5.0**	**8.0**
Mean	0.10	0.43	0.95	5.02	7.99
SD	0.02	0.03	0.12	0.60	0.73
RSDr (%)	16.0	7.0	12.3	11.9	9.1
Bias (%)	16.1	7.4	6.0	0.4	-0.1

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the GA21 method on event Bt11 x GA21 maize DNA

GA21					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.5**	**0.9**	**5.0**	**8.0**
Mean	0.11	0.53	0.98	4.67	7.77
SD	0.02	0.05	0.11	0.77	1.05
RSDr (%)	17.6	9.2	11.6	16.4	13.5
Bias (%)	22.3	6.6	9.3	-6.6	-2.9

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 satisfy the above requirement throughout their respective dynamic ranges.

Tables 4 and 5 further document the relative repeatability standard deviation (RSD_r) as estimated for each GM level. In order to accept methods for collaborative trial evaluation, the CRL-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.it/guidancedocs.htm]). As it can be observed from the values reported in Tables 4 and 5, the two methods satisfy this requirement throughout their respective dynamic ranges.

8. Comparison of method performance between event Bt11 x GA21 and the single trait events

A synoptic comparison of the two method performances on the maize event Bt11 x GA21 and 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 event Bt11 x GA21 and on event Bt11.

Trueness and repeatability of Bt11 quantification on Bt11 x GA21			**Trueness and repeatability of Bt11 quantification on single event Bt11***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.09	16.1	16	**0.09**	2.2	17
0.4	7.4	7	**0.4**	-1.9	13
0.9	6.0	12	**0.9**	1.8	11
5.0	0.4	12	**5.0**	-5.2	13
8.0	-0.1	9	**8.0**	-1.2	9

*method validated (http://gmo-crl.jrc.it/statusofdoss.htm)

Table 7. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the GA21 detection method on event Bt11 x GA21 and on event GA21.

Trueness and repeatability of GA21 quantification on Bt11 x GA21			**Trueness and repeatability of GA21 quantification on single event GA21***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.09	22.3	18	**0.09**	-8.7	23
0.5	6.6	9	**0.5**	0.8	17
0.9	9.3	12	**0.9**	1.6	20
5.0	-6.6	16	**5.0**	-5.6	20
8.0	-2.9	14	**8.0**	-8.5	17

*method validated (http://gmo-crl.jrc.it/statusofdoss.htm)

For trueness, the Bt11 even-specific method (Table 6), when applied to event Bt11 x GA21 and compared to the single line, shows higher bias (%) at low levels of GM (0.09, 0.4 and 0.9%), and lower bias at high GM levels (5 and 8%). The GA21 event-specific method (Table 7), when applied to event Bt11 x GA21, shows higher bias (%) at low levels of GM (0.09, 0.5

and 0.9%). In all cases, however, the trueness is within the acceptance range set by ENGL (± 25%).

For relative repeatability standard deviation (RSDr %), the Bt11 and GA21 event-specific methods (Table 6 and 7) show similar values when applied to the single events or Bt11 x GA21. In all cases, the results are below the ENGL acceptance level established at maximum 25%.

Therefore, the in-house method verification has demonstrated that the Bt11 and GA21 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 GA21.

9. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events Bt11 and GA21 combined in maize event Bt11 x GA21 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.it/guidancedocs.htm), and to the validation results obtained for the single trait events (http://gmo-crl.jrc.it/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 CRL-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.