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Community Reference Laboratory
CRL
GM Food and Feed

Verification of Performance of a Bt11 Event-specific Method on Bt11 "Field Maize" Using Real-Time PCR

Validation Report

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Directorate General-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 the Bt11 event-specific method to detect and quantify the Bt11 transformation event in "field maize" DNA (unique identifier SYN-BTØ11-1). The method has been previously validated to detect and quantify the Bt11 event in "sweet maize" DNA. The 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 provided the detection method and the control samples (genomic DNA extracted from Bt11 field maize leaf tissue and genomic DNA from non-GM field maize leaf tissue). 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.it/doc/Method%20requirements.pdf) and to the validation results obtained for Bt11 sweet maize (http://gmo-crl.jrc.it/statusofdoss.htm).

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

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Report on Steps 1-3 of the Validation Process

Syngenta Crop Protection AG submitted the detection method and control samples for the Bt11 maize event (unique identifier SYN-BTØ11-1) under Article 8 and 20 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 to 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 requests of complementary information were addressed to the applicant. Upon reception of the last complementary information in January 2007, the scientific assessment of the detection method for Bt11 field maize was positively concluded.

The event-specific detection method for Bt11 sweet maize was validated by the CRL-GMFF following the conclusion of an international collaborative study and the publication of the validation report (http://gmo-crl.jrc.it/statusofdoss.htm). Hence, the detection method for Bt11 field maize did not undergo a full validation process. The CRL-GMFF performed an in-house verification of the detection method to verify that it exhibits a comparable performance on samples of Bt11 field maize (as provided in accordance to Annex I-2.C.2 of Commission Regulation (EC) No 641/2004).

In February 2007, the CRL-GMFF concluded the experimental verification of the method characteristics (step 3, experimental testing of the samples and methods) by quantifying five blind GM-levels within the range 0.1%-2%, on a DNA copy number basis. The experiments were performed in repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision of the quantifications were 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 Crop Protection AG submitted the detection method and control samples for the Bt11 field maize event (unique identifier SYN-BTØ11-1) under Article 8 and 20 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Directorate General-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) No 1829/2003, carried out the in-house verification of the event-specific method for the detection and quantification of Bt11 sweet maize, on samples of Bt11 field maize. The method had been previously validated by international collaborative study on sweet maize event (http://gmo-crl.jrc.it/statusofdoss.htm).

Upon reception of the method, 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 method (step 3) according to the requirements of Regulation (EC) 641/2004 and following CRL-GMFF operational procedures. The in-house method verification was performed in January-February 2007.

A method for DNA extraction from maize seeds, submitted by the applicant, was evaluated by the CRL-GMFF. Laboratory testing of the method was carried out in order to confirm its performance characteristics. The protocol for DNA extraction and a report on method testing is available at http://gmo-crl.jrc.it/.

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

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology consists of an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of Bt11 DNA to total maize DNA in field maize. The procedure is a simplex system, in which the Bt11 event is quantified in reference to a maize *adh1* (Alcohol dehydrogenase-1) endogenous system [(3)].

The study was carried out in accordance with 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 detection method, two vials of DNA extracted from leaf tissue were provided by the applicant as control samples. The Bt11 genomic DNA was extracted from hybrid "NX3707", while the near isogenic conventional counterpart was extracted from hybrid "Pelican".

Samples containing mixtures of 100% Bt11 and non-GM maize genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the control samples provided, in a constant amount of total maize DNA.

The protocol (reagents, concentrations, primer/probe sequences) followed in the in-house verification is the one already published as validated method for the detection and quantification of the Bt11 event (http://gmo-crl.jrc.it/statusofdoss.htm).

Table 1 shows the five GM levels used in the verification of the Bt11 detection method.

Table 1. Bt11 GM contents

Bt11 GM % (GM copy number/maize genome copy number *100)
0.1
0.3
0.7
1.0
2.0

3. Experimental design

Eight PCR runs using the Bt11 event-specific method were carried out. In each run, samples were analysed in parallel with both the GM-specific and the reference systems (*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, 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 maize event Bt11, a 70-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers (3′ event-specific junction). PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe 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 the relative quantification of maize event Bt11, a maize-specific reference system amplifies a 135-bp fragment of maize endogenous gene *adh1* (*alcohol dehydrogenase 1*), using a pair of specific primers and an *adh1* gene-specific probe labelled with FAM and TAMRA [(3)].

Standard curves are generated for both the GM specific system (Bt11) and the reference gene (*adh1*), by plotting the Ct-values measured for the calibration samples against the logarithm of the DNA copy number, and by fitting a linear regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For the determination of the amount of Bt11 DNA in an unknown sample, the Bt11 copy number is divided by the copy number of the maize reference gene (*adh1*) and multiplied by 100 to obtain the percentage value (GM% = GM-specific system/maize reference system*100).

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

5. Deviations reported

No deviations from the protocol of the previously validated method were introduced.

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), are presented in Table 2.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the Bt11 detection method (Bt11 assay and endogenous *Adh1* assay) on Bt11 field maize DNA

Run	Bt11			*Adh1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.300	99.1	0.997	-3.340	99.2	0.994
2	-3.209	95.1	0.986	-3.303	99.2	0.993
3	-3.314	99.7	0.995	-3.313	99.7	0.996
4	-3.310	99.5	0.984	-3.381	97.6	0.992
5	-3.432	95.6	0.995	-3.339	99.3	0.989
6	-3.294	98.8	0.995	-3.245	96.7	0.986
7	-3.424	95.9	0.995	-3.305	99.3	0.995
8	-3.195	94.4	0.996	-3.361	98.4	0.994
Mean	-3.310	97.3	0.993	-3.324	98.7	0.993

The mean PCR efficiencies were higher than 97% and the linearity of the method for both assays (R^2 value) was above 0.99. Data reported in Table 2 confirm the appropriate performance characteristics of the method tested on Bt11 field maize material in terms of PCR efficiency and linearity.

7. Method performance requirements

The results of the in-house verification study for the Bt11 detection method on Bt11 field maize DNA are reported in Table 3. All results were evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by CRL-GMFF (http://gmo-crl.jrc.it/guidancedocs.htm, see also Annex 1). Further, Table 3 details estimates of accuracy and precision for each GM level and for all methods.

Table 3. Estimates of accuracy (expressed as bias %) and precision of the Bt11 detection method on Bt11 field maize DNA

Bt11 Field Maize					
Unknown sample GM%	**Expected value (GMO %)**				
	0.1	**0.3**	**0.7**	**1.0**	**2.0**
Mean	0.09	0.30	0.66	0.88	2.11
SD	0.01	0.03	0.05	0.16	0.15
RSDr (%)	14	9.7	7.3	18	7.3
Bias (%)	-13	-0.1	-5.2	-12	5.4

The *trueness* of the method is estimated using the measure of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, trueness, measured as bias from the accepted value, should be ± 25% across the entire

dynamic range. As shown in Table 3, the method satisfies the above requirement across the entire dynamic range tested.

Table 3 further documents 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 be 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 3 the method satisfies this requirement throughout the dynamic range tested.

8. Comparison of method performance between Bt11 sweet maize and Bt11 field maize

A synoptic comparison of the method performance on sweet maize DNA and field maize DNA is shown in Table 4.

Table 4. Comparison of accuracy and precision of the Bt11 detection method on sweet maize DNA and field maize DNA

Accuracy and precision of Bt11 quantification on Bt11 field maize			**Accuracy and precision of Bt11 quantification on Bt11 sweet maize***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.1	-13	14	**0.1**	0	33
0.3	-0.1	9.7	**0.3**	0	19
0.7	-5.2	7.3	**0.7**	0	24
1.0	-12	18	**1.0**	0	10
2.0	5.4	7.3	**2.0**	-10	15

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

The Bt11 event-specific method shows comparable performance in terms of precision of quantification and accuracy when applied to sweet maize DNA and to field maize DNA. All values are within the limits of the ENGL acceptance criteria and method performance requirements.

Therefore, the in-house method verification has demonstrated that the Bt11 method developed to detect and quantify Bt11 sweet maize DNA can be equally applied for the quantification of Bt11 field maize DNA.

9. Conclusions

The overall method performance of the Bt11 event-specific method for the quantitative detection of Bt11 field maize DNA has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (as detailed under http://gmo-crl.jrc.it/guidancedocs.htm), and to the validation results obtained for Bt11 sweet maize DNA (http://gmo-crl.jrc.it/statusofdoss.htm).

The results obtained during the present verification study indicate that the analytical module of the method submitted by the applicant complies with ENGL performance criteria. The method is 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.

3. Hernandez, M., Duplan, M.N., Berthier, G., Vaitilingom, M., Hauser, W., Freyer, R., Pla, M., Bertheau, Y. (2004). Development and Comparison of Four Real-Time Polymerase Chain Reaction Systems for Specific Detection and Quantification of Zea mays L. J.Agric. Food Chem. 52, 4632-4637.

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.