In-house Validation of an Event-specific Method for the Quantification of Maize Bt176 Using Real-time PCR

Validation Report

13 July 2011

Joint Research Centre
Institute for Health and Consumer Protection
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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 an in-house validation study to assess the performance of a quantitative event-specific method to detect maize event Bt176 (unique identifier SYN-EV176-9) 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 Crop Protection provided the detection methods and the control samples [genomic DNA extracted from leaves of maize line Bt176 (inbred NP1760) and from leaves of near-isogenic maize counterpart (NP2131)]. The EURL-GMFF prepared the samples (calibration samples and blind samples at different GM percentages [DNA/DNA]).

On April 25th 2007, further to a communication from the applicant indicating no intention to submit an application for renewal of the authorisation of SYN-EV176-9 maize under Regulation (EC) No 1829/2003, the European Commission issued Decision 2007/304/EC on withdrawal from the market of the maize line Bt176 and its derived products.

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

The results of this EURL-GMFF in-house validation study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

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Report on Steps 1-3 of the Validation Process

Syngenta Crop Protection submitted the detection method and control samples of the maize event Bt176 (unique identifier SYN-EV176-9) according to Articles 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 procedures ("Description of the EURL-GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/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 (ENGL) and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/doc/Method%20requirements.pdf) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, three scientific assessments were performed and two requests of complementary information were addressed to the applicant. Upon reception of the complementary information of the detection method, bioinformatics analysis carried out by the EURL-GMFF on 10/08/2007 for maize Bt176 highlighted that major parts of the insert(s) sequence were missing.

On 25th April 2007, further to a communication from the notifier indicating no intention to submit an application for renewal of the authorisation of SYN-EV176-9 maize under Regulation (EC) No 1829/2003, the European Commission issued Decision 2007/304/EC on withdrawal from the market of the maize line Bt176 and its derived products. However, in order to enforce Article 2 of the above Commission Decision, the EURL-GMFF performed an in-house validation of the detection method to verify that is fit for purpose.

In June 2008 the EURL-GMFF concluded the in-house validation of the method characteristics (step 3, experimental testing of the samples and methods) by quantifying five blind GM levels within the validated the range 0.1%-4.5%. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were 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 Crop Protection provided the detection method and control samples of the maize event Bt176 (unique identifier SYN-EV176-9) according to Articles 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), established by Regulation (EC) No 1829/2003, carried out an in-house validation of the event-specific method for the detection and quantification of maize Bt176.

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

The in-house validation aimed at assessing the performance of a quantitative real-time polymerase chain reaction (qPCR) method. The methodology consists of an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event Bt176 DNA to total maize DNA. The procedure is a simplex system, in which the event Bt176 is quantified in reference to the maize *adh1* (alcohol dehydrogenase) endogenous gene.

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

- ISO 5725: 1994[(1)]. Accuracy (trueness and precision) of measurement methods and results.
- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies"[(2)].

2. Materials

The following samples were provided by the applicant:

- DNA extracted from leaves of maize line Bt176 (inbred NP1760)
- DNA extracted from leaves of a near-isogenic maize counterpart (NP2131)

Samples 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 Bt176 and non-GM maize genomic DNA at different GMO percentages were prepared by the EURL-GMFF in a constant amount of total maize DNA.

The five GM percentages used in the in-house validation of the Bt176 method are reported in Table 1.

Table 1. Bt176 GM contents (%) in total maize DNA

Bt176 (GM DNA / Non-GM DNA x 100)
0.10
0.45
0.90
2.00
4.50

3. Experimental design

Eight runs 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. In total, the quantification of the five GM levels was performed as an average of sixteen replicates per GM level. PCR analysis was performed in triplicate for all samples.

4. Method

For detection of event Bt176, a specific fragment of 82 bp spanning the 3' insert-to-plant junction is amplified using specific primers.
PCR products are measured at each cycle (real-time) by means of a specific oligonucleotide probe labelled with FAM dye (6-carboxyfluorescein) and TAMRA (carboxytetramethylrhodamine) as quencher dye.

For the relative quantification of GM event Bt176, a maize specific reference system amplifies a 135-bp fragment of the maize endogenous gene *adh1* (alcohol dehydrogenase 1, GenBank Accession No. AY691949), using *adh1* gene-specific primers and an *adh1* probe labelled with VIC and TAMRA.

For relative quantification of event Bt176 DNA in an unknown sample, the normalised ΔCt values of the calibration samples are used to calculate, by linear regression, the parameters of a standard curve (plotting ΔCt values against the logarithm of the relative amount of event Bt176). The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of event Bt176 is estimated.

Calibration samples (S1-S5) are prepared by mixing the appropriate amount of Bt176 DNA with non-GM maize DNA to obtain the following five relative contents of Bt176: 5.0%, 2.5%, 0.9%, 0.4% and 0.09%. The total DNA amount per reaction is 250 ng, with 5 µL of a DNA solution at the concentration of 50 ng/µL for reaction.

The Bt176 contents of the calibration samples and the total DNA quantity used in the PCR are indicated in Table 2.

The in-house validation was conducted on an ABI 7900HT platform.

Table 2. GMO content (%) in the standard curve samples.

Sample	S1	S2	S3	S4	S5
Total amount (ng) of DNA in reaction	250	250	250	250	250
GM% (GM copy number/maize genome copy number x 100)	5.0	2.5	0.9	0.4	0.09

5. Results

PCR efficiency and linearity

The values of the slope of the ΔCt-curve [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) in the eight runs for the GM event are summarised in Table 3.

Table 3. Values of slope, PCR efficiency and R^2

Run	Slope	PCR Efficiency (%)	R^2
1	-3.20	105	0.99
2	-3.13	109	1.00
3	-3.27	102	1.00
4	-3.39	97	1.00
5	-3.15	108	1.00
6	-3.38	98	1.00
7	-3.41	96	1.00
8	-3.31	100	1.00
Mean	**-3.28**	**102**	**1.00**

The mean PCR efficiency was 102%. The R^2 of the method was 1.00. These results confirm the appropriate performance characteristics of the Bt176 method in terms of PCR efficiency and linearity.

6. Method performance requirements

The results of the in-house validation of the Bt176 detection method are reported in Table 4. 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). Table 4 reports the trueness and precision for each GM level.

Table 4. Trueness (expressed as bias %) and relative repeatability standard deviation (RSDr %) of the Bt176 method.

Bt176					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.45**	**0.9**	**2.0**	**4.5**
Mean	0.10	0.43	0.83	1.96	4.11
SD	0.010	0.037	0.063	0.169	0.364
RSDr (%)	11	8.7	7.5	8.6	8.9
Bias (%)	-4.5	-4.9	-7.5	-2.2	-8.7

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 in Table 4, the values ranged from -2.2% to -8.7%. Therefore, the method satisfied the above requirement throughout the respective range of applicability.

Table 4 reports on the relative repeatability standard deviation (RSD_r %) estimated for each GM level. As indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), the EURL-GMFF requires that the RSD_r % is below 25%. As it can be observed in Table 4, the values ranged between 7.5% and 11%. Therefore, the method satisfied this requirement throughout the range tested.

7. Conclusions

The method performance of the event-specific method for the quantitative detection of event Bt176 has 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).

The results obtained during the in-house validation indicate that the method comply 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.

8. Quality assurance

The EURL-GMFF operates according to ISO 9001:2008 (certificate number: CH-32232) and technical activities under ISO 17025:2005 [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative /quantitative PCR) - Accredited tests available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

9. References

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

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

10. 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 Quantification (LOQ)

Definition: The limit of quantification 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.