Validation of the GMO specific detection method developed by NVI/INRA for Bt11 in sweet maize

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Executive Summary

The JRC in collaboration with the European Network of GMO Laboratories (ENGL) has carried out a full validation study (ring-trial) by following internationally accepted guidelines to test the performance of a quantitative event-specific method to detect and quantify the Bt11 transformation event in sweet maize.

The method validated has been developed by the National Veterinary Institute of Norway and INRA, France, within the EU shared cost action project QPCRGMOFOOD. The 14 participants of the validation study were members of the ENGL from nine different European countries. The materials needed in the study (GM and non-GM DNA as well as the method-specific reagents) have been provided by Syngenta.

The JRC considers the method performance to be appropriate for its aimed purpose, taken into account the performance criteria proposed by the ENGL for methods submitted for regulatory compliance as well as the current scientific understanding about satisfactory method performance. The method will also be proposed to CEN, the European Standardisation body, for the consideration to become European and/or international standard.

In conclusion, the JRC confirms that the method validated is fit for the purpose of regulatory compliance.

Contents

1. Introduction

The validation process of the event-specific method for the detection and quantification of Bt11 maize was organised by the JRC (Biotechnology and GMOs Unit of the Institute of Health and Consumer Protection) and participating laboratories were members of the European Network of GMO Laboratories (ENGL).

The method is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event Bt11 DNA to total maize DNA. The procedure is a simplex system, in which a maize adh endogenous assay (reference gene, Hernández M. *et. al.,* 2004) and the target assay (Bt11) are amplified in separate wells. The PCR assay has been optimized for use in an ABI Prism® 7700 sequence detection system. Other systems may be used, but thermal cycling conditions must be verified. The method has been developed by the National Veterinary Institute of Norway and INRA, France. The method development and pre-validation took place within the EU share cost action project QPCRGMOFOOD (contract no. QLK1–1999–01301).

The ring-trial was carried out according to internationally accepted guidelines and took in particular into account the following:

- ISO 5725: Accuracy (trueness and precision) of measurement methods and results.
- IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz 1995).
- General requirements and definitions: Draft European standard prEN ISO 24276:2002
- Quantitative nucleic acid based methods: Draft European standard prEN ISO 21570:2002
- ENGL: Definition of minimum performance requirements for analytical methods of GMO testing. Available from http://gmo-crl.jrc.it.

The validation process concerning this particular method was initiated in May 2003. The experimental work of the ring-trial took place during the period 1.8. – 15.9.2003, and the results were firstly reported to the Competent Authorities of the Member States in October 2003.

2. Ring-trial participants

The method was tested in the 14 ENGL laboratories to determine the performance parameters. The purpose of the ring-trial was to test the performance of the method and not that of a laboratory and so each laboratory was requested to carefully follow the provided protocol. The participating laboratories are listed in Table 1[1].

Table 1. The ENGL laboratories, which participated in the validation study.

Laboratory	Country
Ministère des Classes Moyennes et de l'Agriculture - Centre de Recherches Agronomiques (CRA) - Département Qualité des Productions Agricoles	BE
CLO – Departement van Plantengenetica en-veredeling	BE
Ministerio de Sanidad y Consumo - Instituto de Salud Carlos III - Centro Nacional de Alimentación	ES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH - Lebensmitteluntersuchung und Forschung Wien (AGES-LUVIE)	AT
Laboratoire Interrégional de la Direction Générale de la Concurrence - Consommation et Répression des Fraudes de Strasbourg	FR
Bio-GEVES	FR
Laboratory of the Government Chemist (LGC) - BioAnalytical Innovation Team	GB
DEFRA - Central Science Laboratory (CSL)	GB
Swedish National Food Administration - Food Control - Division 2	SE
Danish Veterinary and Food Administration - Institute of Food Research and Nutrition	DK
Inspectie Waren en Veterinaire Zaken Regio Noord-West - Signaleringsafdeling Primaire Land- en Tuinbouwproducten	NL
NAK - Nederlandse Algemene Keuringsdienst	NL
Istituto Superiore di Sanità (ISS) - Laboratorio di chimica dei cereali	IT
Istituto Zooprofilattico Sperimentale Lazio e Toscana - Dipartimento di Virologia e Biotecnologie	IT

[1] The laboratory numbering does not follow the order in which the laboratories have been listed in the table.

3. Materials

Samples containing wild type sweet maize and Bt11 sweet maize at different DNA concentrations were used. The stocks of non-GM maize and 100% Bt11 sweet maize DNA were obtained from Syngenta. The DNA extraction method used was based on a Magnesil Lysis solution and magnetic separation of DNA. The details of the DNA are as follows:

- Wild type leaf genomic DNA from hybrid field maize (Brasco);
- Bt11 sweet maize grain genomic DNA (GH0937).

The standard curve, control and unknown samples were produced by the JRC. All participants of the ring-trial received the following materials:

- Five DNA samples for standard curves (labelled with S1 – S5). The details are provided in Table 1.
- Twelve unknown DNA samples (labelled from U1 to U12). The unknown samples consisted of six pairs of blind duplicates with the following Bt11 sweet maize percentages: 0.1, 0.3, 0.7, 1.0, 1.3, and 2%.
- Negative DNA target control (labeled with C1): Bt176 maize DNA.
- Negative DNA target control (labeled with C2): 0% Bt11 maize DNA.
- In addition to the negative DNA target controls, amplification reagent control (nucleic acid free water provided by each trial participant) was used.
- Primers and probes for the adh reference gene and for the Bt11 specific systems as follows:
  - ***ADH method*** ***Label***
  - ADH forward (20µM) ADH F
  - ADH reverse (20µM) ADH R
  - ADH probe (10µM) pr ADH

  - ***Bt11 maize method*** ***Label***
  - Bt113JFor (20µM) Bt11 F
  - Bt113JRev (20µM) Bt11 R
  - Bt113JFT probe (10µM) pr Bt11

Table 2. Concentration levels of the standard curve samples.

Sample code	***S1***	***S2***	***S3***	***S4***	***S5***
Total quantity (ng/6µl)	***258***	***129***	***65***	***16***	***5***
Concentration (ng/µl)	***43.0***	***21.5***	***10.8***	***2.7***	***0.9***
Final *Zea mays* copy number	***94,679***	***47,340***	***23,670***	***5,917***	***1,972***
Final Bt11 GMO copy number	***2,367***	***1,183***	***592***	***148***	***49***

4. Methods

The method has been optimised for DNA extracted from pure ground Bt11 maize kernels, Bt11 maize leaves and certified reference material of Bt11 maize dried powder from ground kernels containing mixtures of genetically modified Bt11 and conventional maize. The method was originally described for LightCycler and TaqMan™ chemistry, and adapted and optimised for ABI Prism 7700 and 7900 Sequence Detection System® prior to conducting the collaborative trial validation. One of the laboratories also used ABI Prism 7000 Sequence Detection System® in the ring-trial.

In the reference gene system, a fragment of the *adh*1 gene is amplified using two maize *adh*1-specific primers. In the Bt11 specific system, a fragment of the Bt11 maize specific 3' integration border region is amplified using two specific primers; the forward primer target a pUC18 derived sequence (internal to the inserted DNA sequence) and the reverse primer target the 3' integration border region (11 nucleotides from pUC18 derived sequence, 9 nucleotides from the maize genome). Accumulation of PCR products is measured at the end of each PCR cycle (real-time) by means of a system-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as reporter dye and TAMRA as quencher. For that purpose TaqMan® chemistry is employed.

The measured fluorescence signal trespasses a user defined threshold value after a certain number of cycles. This number is called the Ct-value. For quantitation of the amount of maize *adh*1-DNA in an unknown sample the Ct-value is converted into a corresponding copy number value by comparison with a calibration curve whose Ct-values are directly linked with known copy numbers by the means of linear regression analysis.

For the determination of the amount of Bt11 DNA in the test sample, the Bt11 copy number is divided by the copy number of the maize reference gene (adh) and multiplied by 100 to obtain the percentage value.

5. Results

The samples provided were analysed on two different PCR plates. The real-time PCR was triplicated for standard curve and control samples and quadruplicated for unknown samples. Thus, each unknown sample was quantified based on four replicates. Each participating laboratory carried out the quantitation and determination of the GM% according to the instructions provided in the protocol.

The results of one laboratory were excluded from the data analysis due to the fact that the master-mix as described in the protocol was not used in the analysis. Thereafter, the outliers were treated by using Cochran test and Grubb's tests according to the IUPAC harmonized protocol (Horwitz 1995).

The main results are summarized in Table 3. The unknown samples covered the dynamic range of 0.1 – 2.0% of Bt11 in maize. The average measured GM% is equal to or very close to the true one (Figure 1). The variability in the quantitation results is highest at the 0.1% GM level, where the relative reproducibility standard deviation equals to 33.5%. For the higher GM percentages, the reproducibility relative standard deviation is between 18.4% and 27.0% (Table 3).

Table 3. The main results from the analysis of unknown samples.

Unknown sample GM%	**0.1**	**0.3**	**0.7**	**1.0**	**1.3**	**2.0**
Number of laboratories having returned results	13	13	13	13	13	13
Number of samples per laboratory	2	2	2	2	2	2
Number of excluded laboratories	2	2	1	3	1	3
Number of laboratories retained after exclusion	11	11	12	10	12	10
Number of accepted samples	22	22	24	20	24	20
Mean value	0.1	0.3	0.7	1.0	1.2	1.8
Median value	0.1	0.3	0.7	1.0	1.2	1.9
Coefficient of variation (CV%)	33.4	17.7	21.5	12.1	27.0	18.5
Repeatability relative standard deviation RSD_r (in %)	33.5	19.0	24.4	10.4	25.0	14.9
Repeatability standard deviation s_r	0.04	0.06	0.17	0.11	0.31	0.28
Reproducibility relative standard deviation RSD_R (in %)	33.5	19.0	24.4	12.7	27.0	18.4
Reproducibility standard deviation s_R	0.04	0.06	0.17	0.13	0.33	0.34
Reproducibility limit R ($R=2,8 \times s_R$)	0.11	0.17	0.48	0.36	0.92	0.95

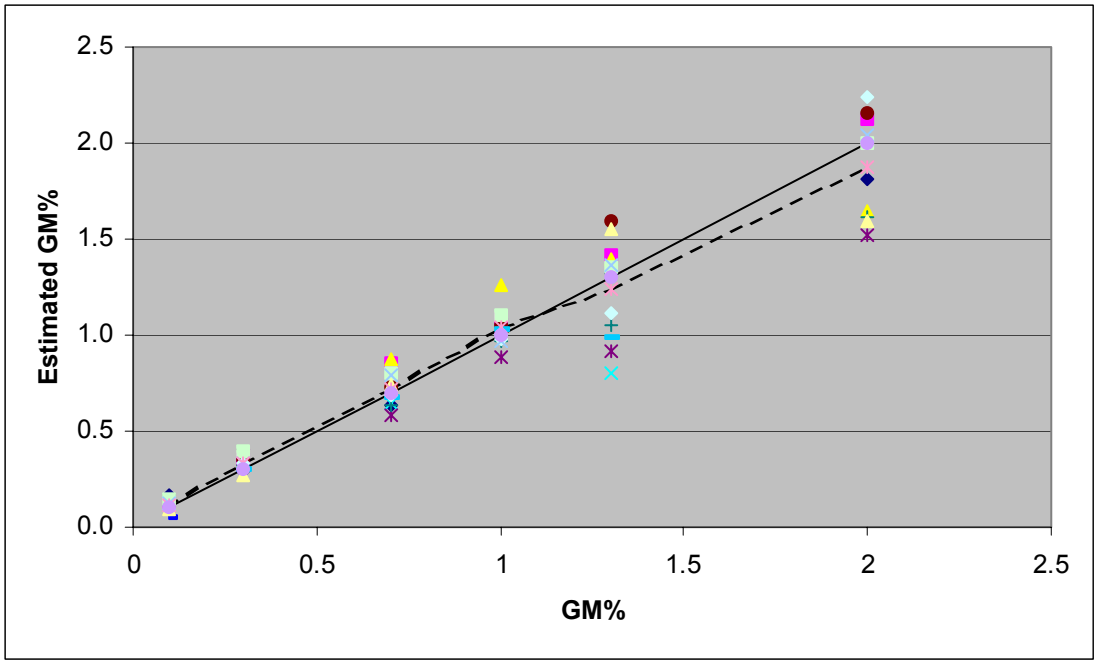

Figure 1. The estimated GM% of each laboratory (points) as well as the average measured GM% (dotted line) and true GM% (continued line) of the unknown samples.

An analysis of variance was carried out to test the differences between the results obtained by different laboratories and to test if the measurements of different GM percentages were significantly different from one another (e.g. to test if the estimated GM% at 1.0% GM level and 0.7% GM level could be differentiated). Several tests were used in the analysis (t-, Tukey- and Student-Newman-Keuls tests). There was no clear laboratory effect in any of the concentrations, i.e. the results of the different laboratories did not differ from one another in a statistically significant way. It is also possible to distinguish all the tested GM levels one from another, i.e. there was a statistically significant difference between all the concentrations.

6. Evaluation

The overall method performance has been evaluated with respect to the ENGL method performance requirements (available from http://gmo-crl.jrc.it. The "method performance requirements" define the minimum performance criteria, which the method should demonstrate upon completion of a validation study according to internationally accepted guidelines in order to certify that the method validated is fit for the purpose of compliance with regulation (EC) No 1829/2003.

The method is quantitative and event-specific, i.e. it detects only the Bt11 GM event and no other currently available GMOs (Rønning et al. 2003). The method is applicable for the enforcement purposes and it has shown robustness in that it has been tested by 14 different laboratories in nine EU countries. The results from different laboratories did not vary significantly one from another. The method performance over the tested dynamic range (from 0.1% to 2% GM level) confirms that the method is capable for complying with the current labeling requirements in Europe. Within this range, the method performs in dynamic manner.

The accuracy and trueness of the method are fit for the purpose: The estimated GM concentrations were very close to or equal to the true ones over the dynamic range tested. The method is sensitive in that it is able to differentiate between rather small GM concentration differences (i.e. between 0.1; 0.3; 0.7; 1.0; 1.3 and 2%). The reproducibility and repeatability (i.e. precision) at all the GM levels is considered small enough to comply with the performance requirements. With the current technologies, it is very difficult to obtain a variability significantly lower than the one obtained in this study. The method is quantitative down to the 0.1% GM level. GM concentrations lower than that were not tested in this study. Detection limit as such was not tested in the ring-trial. However, the previous method testing indicates that this criteria should be fulfilled with no problems also for instance at 0.05% GM level (Rønning et al. 2003).

7. Conclusions

The JRC considers the method performance to be appropriate for its aimed purpose, taken into account the performance criteria proposed by the ENGL for methods submitted for regulatory compliance as well as the current scientific understanding about satisfactory method performance. In conclusion, the JRC confirms that the method validated is fir for the purpose of regulatory compliance.

8. References

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