EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Event-specific method for the quantitation of maize line NK603 using real-time PCR

Validation Report

Biotechnology & GMOs Unit
Institute for Health and Consumer Protection
DG JRC

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Executive Summary

The JRC as Community Reference Laboratory (CRL) for the GM Food and Feed (see Regulation EC 1829/2003), in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the NK603 transformation event in maize flour. The collaborative trial was conducted according to internationally accepted guidelines.

Monsanto Company provided the method-specific reagents (primers, probes, reaction master mix), whereas the IRMM/JRC prepared the test samples (GM and non-GM maize flour). The trial involved thirteen laboratories from eleven European Countries. Since one laboratory reported of not having been able to extract DNA from the maize flour samples, data sets from twelve participants were received.

The results of the collaborative trial have met ENGL's performance requirements and the scientific understanding about satisfactory method performance. Therefore, the JRC as Community Reference Laboratory considers the method validated as fit for the purpose of regulatory compliance.

The results of the collaborative study are publicly available under http://gmo-crl.jrc.it/. The method will also be submitted to CEN, the European Standardisation body, to be considered as international standard.

Contents

Document Approval		
Name / *Function*	**Date**	**Signature**
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1. Introduction

The Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for the GM Food and Feed (see Regulation EC 1829/2003) organised the collaborative trial of the event-specific method for the detection and quantification of NK603 maize. The study involved thirteen European laboratories, members of the European Network of GMO Laboratories (ENGL).

A pre-validation study involving three laboratories was carried out between December 2003 and January 2004. Following the evaluation of the pre-validation results, the ring trial was organized and took place between March and April 2004.

The operational procedure of the collaborative study comprised the following modules:

- ✓ DNA extraction: an enhanced CTAB DNA extraction and purification protocol adopted from the prEN ISO 21571:2002[1].
- ✓ Spectrophotometric quantification of the amount of total DNA extracted, adopted from prEN ISO 21571:2002.
- ✓ Real-time PCR (Polymerase Chain Reaction) monitor run (inhibition test).
- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event NK603 DNA to total maize DNA. The procedure is a simplex system, in which a maize *Adh*1 endogenous assay (reference gene) and the target assay (NK603) are performed in separate wells. The PCR assay has been optimised for use in an ABI Prism® 7700 sequence detection system. Although other systems may be used, thermal cycling conditions must be verified.

The ring-trial was carried out in accordance with the following internationally accepted guidelines:

- ✓ ISO 5725, especially considered in relation to the measure of precision (i.e. repeatability and reproducibility) and trueness.
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995).

2. List of Participants

The method was tested in thirteen ENGL laboratories to determine its performance. Each laboratory was requested to carefully follow the protocol provided. The participating laboratories are listed in Table 1.

[1] Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Nucleic Acid Extraction. CEN/TC 275/WG11N0031. Draft November 2002.

Table 1. ENGL laboratories participating in the validation study of NK603.

Laboratory	Country
Ministère des Classes Moyennes et de l'Agriculture - Centre de Recherches Agronomiques (CRA)	Belgium
Department of Plantgenetics and Breeding	Belgium
Landesamt für Verbraucherschutrz, Gesundheit und Arbeitsschutz (LVGA)	Germany
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Lebensmitteluntersuchung und Forschung Wien (AGES-LUVIE)	Austria
Laboratoire Interrégional de la Direction Générale de la Concurrence, Consommation et Répression des Fraudes de Strasbourg	France
Research Institute of Crop Production	Czech Republic
National Institute of Biology	Slovenia
National Veterinary Institute	Norway
National Food Administration	Sweden
Danish Institute for Food and Veterinary Research	Denmark
The Food and Consumer Product	Netherlands
Institute of Public Health	Belgium
Istituto Zooprofilattico Sperimentale Lazio e Toscana	Italy

Legend: one of the 13 laboratories did not provide the results for validation on NK603.

3. Materials

Samples of maize flour containing wild type maize and NK603 maize at different concentrations were used. The samples used in the study (GM and non-GM maize flour) were prepared by the Institute for Reference Materials and Measurements (IRMM) of the European Commission Joint Research Centre. Monsanto Company developed and optimised the method and provided the specific reagents.

The participants received the following materials:

- ✓ Calibration maize flour sample 1g (4.91% NK603 maize, CRM 415-5). From this sample, DNA was extracted, quantified and diluted to generate the standard curve.
- ✓ Unknown samples, represented by 10 unknown maize flour samples (CRM samples) labelled from U1 to U10, 1g each.
- ✓ Negative DNA target control (labelled C1): Bt176 maize DNA (200 µl @20ng/µl).
- ✓ Negative DNA target control (labelled C2): one maize flour sample, (CRM 415-0), nominal 0% NK603 maize, 1g. In addition to the negative DNA target controls, amplification reagent control (nucleic acid free water provided by each trial participant) was used on each PCR plate.
- ✓ Reaction reagents, primers and probes for the *Adh*1 reference gene and for the NK603 specific systems as follows:
  - ❑ 5X TaqMan Universal PCR Master Mix (2X) (ABI, Cat No. 4304437): total amount 25 ml.

- ✓ Primers and probes (1 tube each) as follows:
  - ❑ NK603 primer F 300 µl 5`-ATGAATGACCTCGAGTAAGCTTGTTAA -3`
  - ❑ NK603 primer R 300 µl 5`-AAGAGATAACAGGATCCACTCAAACACT-3`
  - ❑ NK603 probe PR 200 µl 6-FAM-TGGTACCACGCGACACACTTCCACTC-TAMRA
  - ❑ *Adh*1 primer F 500 µl 5-CCAGCCTCATGGCCAAAG-3`
  - ❑ *Adh*1 primer R 500 µl 5-CCTTCTTGGCGGCTTATCTG-3`
  - ❑ *Adh*1 probe PR 330 µl 6-FAM-CTTAGGGGCAGACTCCCGTGTTCCCT- TAMRA

Table 2 shows the certified value over the dynamic range for the unknown samples.

Table 2. NK603 certified values.

NK603 Certified GM concentration (w/w)
0.10
0.49
0.98
1.96
4.91

4. Experimental design

Ten unknown samples, representing five GM levels, were used to extract DNA. The two replicates for each GM level were analyzed on two PCR plates. The PCR analysis was triplicated for standard curve and control samples, but quadruplicated for unknown samples. Thus, each unknown sample was quantified based on four repetitions. Each participating laboratory carried out the determination of the GM% according to the instructions provided in the protocol.

5. Methods

Description of the operational steps

DNA was extracted from the flour samples by using an enhanced CTAB DNA extraction and purification protocol adopted from the prEN (ISO 21571:2002)[2]. Subsequently, purified DNA was quantified by means of spectrophotometry in order to determine the amount of DNA to be analysed in real-time PCR. The procedure "Basic ultraviolet spectrometric method" was adopted from the Annex B "Methods for the quantification of the extracted DNA" of the prEN ISO 21571:2002. The method has been widely used and ring-tested in the past (Anon, 2002). After the DNA quantification, a real-time PCR monitor run was carried out to provide data about possible PCR inhibition.

For specific detection of event NK603 genomic DNA, a 108-bp fragment of the region that spans the 3′ insert-to-plant junction in maize event NK603 is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For relative quantification of event NK603 DNA, a maize-specific reference system amplifies a 70-bp fragment of *Adh*1, a maize endogenous gene, using a pair of *Adh*1 gene-specific primers and an *Adh*1 gene-specific probe labelled with FAM and TAMRA.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event NK603 DNA in a test sample, the Ct-values of a certain sample for maize event NK603 and *Adh*1 are determined. Standard curves are then used to calculate relative content of event NK603 DNA to total maize DNA.

The first standard curve point S1 was derived from the 4.91% NK603 CRM (IRMM-415-5). This corresponds to 5,405 GM copies in 300 ng of DNA when 4 µl per reaction/well are used (75 ng/µl). Standard curve points S2 – S5 were obtained by serial dilution of the 4.91% standard S1. The absolute copy numbers in the calibration curve samples are determined by dividing the sample DNA weight (nanograms) by the published average 1C value for maize genomes (2.725 pg). The copy number values, which were used in the quantification, are provided in Table 3.

[2] Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Nucleic Acid Extraction. CEN/TC 275/WG11N0031. Draft Novermber 2002.

Table 3. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Maize genome copies	110,092	36,697	12,232	3,058	765
NK603 GM copies	5,405	1,802	601	150	38

6. Summary of results

Table 4 shows the mean values of both replicates for each GM level as computed by the JRC on raw data provided by all laboratories. When raw data were not available, the JRC has taken into account the mean value of the replicates provided by the laboratories. Each mean value is the average of four PCR repetitions.

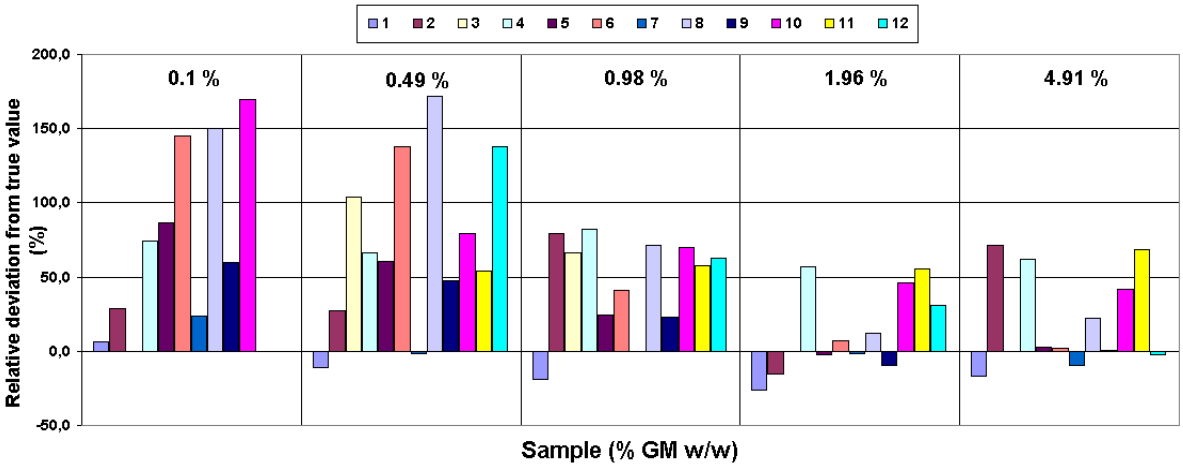
Table 4. Replicates' mean value by laboratories and by all unknown samples.

Laboratories	**Certified value (GM content w/w)**									
	0.10%		**0.49**		**0.98**		**1.96**		**4.91**	
	Rep 1	*Rep 2*	*Rep 1*	*Rep 2*	*Rep 1*	*Rep 2*	*Rep 1*	*Rep 2*	*Rep 1*	*Rep 2*
Lab 1	0.102	0.111	0.410	0.460	0.667	0.924	1.280	1.613	3.117	5.079
Lab 2	0.066	0.191	0.707	0.541	2.089	1.424	1.654	1.674	10.210	6.600
Lab 3	0.700	0.144	1.120	0.880	1.940	1.319	10.270	2.938	21.640	11.421
Lab 4	0.160	0.189	0.894	0.737	1.640	1.935	2.928	3.212	7.937	7.950
Lab 5	0.180	0.193	0.793	0.778	1.257	1.181	1.934	1.898	4.996	5.114
Lab 6*	0.290	0.200	0.970	1.360	1.170	1.600	2.100	2.100	4.190	5.840
Lab 7	0.140	0.107	0.478	0.484	0.919	1.042	1.879	1.966	3.982	4.890
Lab 8	0.211	0.289	1.302	1.363	1.923	1.432	2.369	2.036	5.883	6.146
Lab 9	0.184	0.135	0.646	0.800	1.237	1.168	1.775	1.777	4.648	5.237
Lab 10	0.285	0.254	0.886	0.872	1.709	1.619	3.114	2.627	6.610	7.309
Lab 11	0.138	13.874	0.860	0.648	1.559	1.535	2.885	3.199	6.335	10.227
Lab 12	0.637	0.58	1.041	1.29	1.524	1.660	2.602	2.540	4.434	5.130

* Raw data were not available for this laboratory.

In Figure 1 the deviation from the true value for each GM level tested (expressed as the average of the two replicates) is shown for each laboratory. As it can be observed, most of the laboratories overestimated the true value of NK603 content over the range of concentrations tested; it can be noted that the magnitude of overestimation at each GM level and the number of laboratories overestimating tend to decrease towards the upper end of the dynamic range.

Figure 1. Relative deviation (%) from the true value for all laboratories and NK603 levels



7. Method performance requirements

The results of the collaborative trial are reported in table 5. These are evaluated with respect to the method acceptance criteria and to the method performance requirements, as established by ENGL and adopted by CRL. In table 5 estimates of both repeatability and reproducibility for each GM level are reported, after identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

Table 5. NK603 validation data.

	Certified value (GM content w/w)				
	0.10	**0.49**	**0.98**	**1.96**	**4.91**
Laboratories having returned results	12	12	12	12	12
Samples per laboratory	2	2	2	2	2
Laboratories excluded	3	0	0	1	1
Reason	2 C. test & 1 G. test	0	0	1 C.test	1 C. test
Mean value	0.183	0.847	1.436	2.235	5.994
Repeatability relative standard deviation (%)	24.249	15.239	17.163	7.694	21.629
Repeatability standard deviation	0.044	0.129	0.247	0.172	1.296
Reproducibility relative standard deviation (%)	37.075	34.450	25.425	26.055	31.041
Reproducibility standard deviation	0.068	0.292	0.365	0.582	1.861
Bias (absolute value)	0.083	0.357	0.456	0.275	1.084
Bias (%)	83.00	72.86	46.50	14.03	22.08

C. test = Cochran's test; G. test = Grubbs' test

The *relative reproducibility standard deviation (RSD_R),* that describes the inter-laboratory variation, should be below 33% at the target concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range. As it can be observed in table 5, the method satisfies this requirement at all GM level, with the exception of level 0.49 where the RSD_R is slightly above the limit (34.450). However, this small deviation is not seen as sufficient to consider the method unsatisfactory.

In the same table are also reported the relative repeatability standard deviation (RSD_r) values estimated from ring trial results for each GM level. In order to accept methods for collaborative trial evaluation, the CRL requires that RSD_r is below 30%, as indicated by ENGL. As it can be observed from the values reported in table 5, the method satisfies this requirement throughout the whole dynamic range tested.

In table 5 measures of method bias, which allow estimating trueness, are also shown for each GM level. Bias is estimated according to ISO 5725 data analysis protocol. According to ENGL method performance requirements, trueness should be ±25% throughout the whole dynamic range. In this case the method satisfies such requirement only for GM values above 0.98%; the values of bias are high at lower GM values, and tend to decrease towards the upper end of the dynamic range tested.

8. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (available under http://gmo-crl.jrc.it). The method acceptance criteria were reported by the applicant and used to evaluate the method prior the collaborative study.

The results obtained during the collaborative trial indicate that the method can be considered as fit for enforcement purposes with respect to its intra and inter-laboratory variability. The method bias, although satisfying performance requirements at higher GM levels, is high at lower GM concentrations. Therefore, it is recommended to take into consideration this factor when the method is routinely applied.

In conclusion, the method is considered complying with the current labeling requirements in Europe.

9. References

Anon, (2002). Swiss food manual, Chapter 52B, Section 1 to 5. Eidgenössische Drucksachen und Materialzentrale, CH-5005 Bern.

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