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Report on the Verification of the Performance of NK603 and T25 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack NK603 x T25 Maize

European Union Reference Laboratory for Genetically Modified Food and Feed

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Joint Research Centre
Institute for Health and Consumer Protection

Contact information

Molecular Biology and Genomics Unit
Address: Joint Research Centre, Via Enrico Fermi 2749, TP 201, 21027 Ispra (VA), Italy
E-mail: eurl-gmff@jrc.ec.europa.eu
Tel.: +39 0332 78 5165
Fax: +39 0332 78 9333

<https://ec.europa.eu/jrc>

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Report on the Verification of the Performance of NK603 and T25 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack NK603 x T25 Maize

28 April 2015

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Monsanto Company to request the authorisation of genetically modified stack (GM stack) NK603 x T25 maize and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, and import and processing, in accordance with articles 5 and 17 of Regulation (EC) No 1829/2003 GM Food and GM Feed. The unique identifier assigned to GM stack NK603 x T25 maize is MON-ØØ6Ø3-6 x ACS-ZMØØ3-2.

The GM stack NK603 x T25 maize has been obtained by conventional crossing between two genetically modified maize events: NK603 and T25, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events NK603 and T25 (see <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EURL GMFF has carried out only an *in-house* verification of the performance of each validated method when applied to genomic DNA extracted from GM stack NK603 x T25 maize.

The results of the *in-house* verification led to the conclusion that the individual methods meet the ENGL performance criteria also when applied to genomic DNA extracted from the GM stack NK603 x T25 maize.

This report is published at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

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Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172 (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

The EURL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EURL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection (IHCP) provided by SGS.

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit (MBG)
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, 21027 Ispra (VA) – Italy
Functional mailbox: eurl-gmff@jrc.ec.europa.eu

1. Introduction

The EU legislative system ^(1, 2) for genetically modified food and feed provides that any GMO for food and feed use shall undergo the authorisation process before it can be placed on the market. This holds true also for a GMO containing more than one single GM event obtained by conventional crossing, co-transformation or re-transformation (genetically modified stack).

Consequently, the application for authorisation of a GM stack shall be accompanied, among others, by an event-specific method for detection, identification and quantification for each GM event composing the stack, and by samples of the stack and food and feed derived from it. The EURL GMFF shall validate the event specific methods of detection proposed by the applicant with regard to their performance when applied to DNA extracted from the stack, and shall report to the European Food Safety Authority, who will include the EURL GMFF report in the overall opinion concerning the risk assessment and potential authorisation of the assessed stack. In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EURL GMFF carries out an *in-house* verification of the performance of each event-specific methods if this method has previously been validated by the EURL GMFF for the parental single-line event and these events have been stacked by conventional crossing. These criteria are met for the GM stack NK603 × T25 maize.

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* verification of the methods (step 3) according to the requirements of Regulation (EC) No 641/2004 (Annex I).

The results of the *in-house* verification study were evaluated with reference to ENGL method performance requirements and to the validation results on the individual events.

2. Step 1 (dossier reception and acceptance)

Monsanto Company submitted the detection methods, data demonstrating their adequate performance, and the corresponding control samples as DNA extracted from GM stack NK603 × T25 maize and from non GM maize.

The dossier was found to be complete and thus was moved to step 2.

3. Step 2 (dossier scientific assessment)

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL ⁽³⁾ and with regard to their documentation and reliability.

Table 1 shows values of trueness (expressed as bias %) based on the data provided by the applicant, and precision (expressed as RSDr %), calculated by the applicant for each method applied on the stack DNA. Means are the average of fifteen replicates obtained through one run performed. Percentages are expressed as GM DNA / total DNA x 100.

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) for the NK603 and T25 methods applied to GM stack NK603 x T25 maize.

NK603			
Unknown sample GM%	Expected value (GMO %)		
	0.085	1.0	10
Mean	0.098	1.08	10.49
RSD _r (%)	24.10	14.06	10.11
Bias (%)	15.29	8.00	4.90
T25			
Unknown sample GM%	Expected value (GMO %)		
	0.085	1.0	10
Mean	0.088	0.94	9.63
RSD _r (%)	11.31	11.36	4.84
Bias (%)	3.53	-6.00	-3.70

The EURL GMFF calculated the RSDr (%) and the bias (%), verified the data and concluded that they were reliable and seemed to confirm that the methods meet the ENGL performance criteria ⁽³⁾.

One request for complementary information regarding the sequences of the events and the flanking region of NK603 x T25 was addressed to the applicant. The EURL GMFF verified the data and the complementary information received and accepted the received clarifications as satisfactory.

The dossier was therefore moved to step 3.

4. Step 3 (EURL GMFF experimental testing)

In step 3 the EURL GMFF implemented the two methods in its own laboratory and performed a verification of their performance when applied to DNA extracted from GM stack NK603 x T25 maize.

The procedures are simplex systems, in which the event T25 was quantified in reference to a maize taxon-specific sequence belonging to the alcohol dehydrogenase 1 (*adh1*) maize gene (Accession No M32984) for which the method was validated in the context of the respective single line event (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>). The event NK603 was quantified in reference to two maize taxon-specific sequences: *adh1* (Accession No M32984) and *hmg* (Accession No AJ131373).

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenised seeds of GM stack NK603 × T25 maize
- genomic DNA extracted from homogenized seeds of non GM maize.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack NK603 × T25 maize and genomic DNA extracted from non GM maize in a constant amount of total maize DNA. The same concentrations as in the validation of the methods for the single lines were achieved, with small adaptations for NK603. Table 2 shows the five GM concentrations used in the verification of the NK603 and T25 methods when applying them to genomic DNA extracted from the GM stack NK603 × T25 maize.

Table 2. Percentage of NK603 and T25 in NK603 × T25 verification samples.

NK603 GM% (GM DNA / Non-GM DNA x 100)	T25 GM% (GM DNA / Non-GM DNA x 100)
0.10	0.15
0.50	0.40
0.90	0.90
2.0	2.0
5.0	3.3

The protocols (reagents, concentrations, primer/probe sequences) described by the applicant were implemented precisely in the EURL GMFF laboratory. The *in-house* verification followed the protocols already published as validated methods for the individual NK603 and T25 events (available at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>).

4.2 DNA extraction

A method for DNA extraction from maize seeds was previously evaluated by the EURL GMFF with regard to its performance characteristics and was considered valid, i.e. fit the purpose of providing maize DNA of appropriate quality and amount for being used in subsequent PCR experiments. The protocol for the DNA extraction method is available at http://gmo-crl.jrc.ec.europa.eu/summaries/MON89788_Soya_DNAExtrSampl_report.pdf.

Consequently, the EURL GMFF did not verify the DNA extraction method proposed by the applicant.

4.3 Experimental design

Eight PCR runs for each method were carried out. In each run, samples were analysed in parallel with both the GM-specific module and the maize reference module. Five GM levels

were examined per run, for each GM level in duplicate. PCR analysis was performed in triplicate for all samples. In total, for each method, the quantification of the five GM levels was performed as an average of sixteen replicates per GM level (8 runs x 2 replicated levels per run). An Excel spreadsheet was used for determination of GM%.

4.4 PCR methods

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack NK603 × T25 maize using the single detection methods previously validated for the respective single GM events NK603 and T25.

For detection of GM maize events NK603 and T25, DNA fragments of 108-bp and 102-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 (6-carboxyfluorescein) as reporter dye at their 5'-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at their 3'-end.

The *adh1* primers/probe sets used as maize reference modules and validated for the relative quantification of events NK603 and T25 differ in their oligonucleotide sequences, in the position of the annealing sites along the target gene and in the corresponding amplicon lengths. The *adh1* modules validated for NK603 and T25 generate amplicons of 70 bp and 135 bp length, respectively. To distinguish between the two reference modules, they will be called *adh1-70* and *adh1-135* hereinafter. The *adh1-70* was shown to target a DNA region harbouring a SNP (single nucleotide polymorphism) in the middle of the annealing site of the *adh1 primer F* (validation report for the single line event NK603). The presence of the SNP in *adh1-70* was extensively investigated and shown to be in relation with the bias of quantification of NK603 depending on the *adh1* genotype of the maize varieties originating the sample and the calibrant ⁽⁴⁾.

In the context of the verification of the performance of the validated NK603 method applied to NK603 × T25 product, the NK603 event was also quantified in reference to the maize taxon-specific sequence belonging to the high mobility group (*hmg*) maize gene for which the analytical module is described for instance in the validation of the single line event MON89034 maize (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>). The *hmg* module was confirmed to determine good accuracy of quantification (trueness and precision) in a number of maize events validated by the EURL GMFF. The *hmg* module was also shown to be endowed with good performance characteristics in investigations conducted by the scientific community ^(5,6).

For detailed information on the preparation of the respective standard curve calibration samples please refer to the protocols of the validated methods at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

4.5 Deviations from the validated methods

No deviations from the original validated methods were introduced but *hmg* was evaluated in addition to *adh1-70* for quantification purposes of NK603 DNA in GM stack NK603 × T25 maize.

4.6 Results

Tables 3 and 4 present the values of the slopes of the different standard curves generated by the EURL GMFF when using DNA extracted from the GM stack, from which the PCR efficiency is calculated using the formula $[10^{(-1/\text{slope})} - 1] \times 100$, and of the R^2 (expressing the linearity of the regression) reported for all PCR modules in the eight runs, for GM maize events NK603 and T25.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the NK603 and *adh1* (70 bp) modules on GM stack NK603 × T25 maize.

Run	NK603			<i>adh1-70</i>		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.72	86	0.98	-2.91	121	1.00
2	-3.59	90	0.99	-3.04	113	0.99
3	-3.62	89	0.99	-3.08	111	1.00
4	-3.29	102	0.99	-3.08	111	1.00
5	-3.40	97	0.97	-3.05	113	1.00
6	-3.61	89	1.00	-3.07	112	0.99
7	-3.44	95	1.00	-3.12	109	1.00
8	-3.90	81	0.98	-3.08	111	1.00
Mean	-3.57	91	0.99	-3.05	113	1.00

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the NK603 and *hmg* modules on GM stack NK603 × T25 maize.

Run	NK603			<i>hmg</i>		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.62	89	0.99	-3.29	101	1.00
2	-3.56	91	0.99	-3.20	105	0.99
3	-3.48	94	1.00	-3.23	104	0.99
4	-3.58	90	0.99	-3.17	107	0.99
5	-3.50	93	0.99	-3.27	102	0.99
6	-3.58	90	0.99	-3.20	105	0.99
7	-3.56	91	0.98	-3.24	104	0.99
8	-3.44	95	0.99	-3.19	106	0.99
Mean	-3.54	92	0.99	-3.22	104	0.99

Table 5. Values of standard curve slope, PCR efficiency and linearity (R^2) for the T25 method on GM stack NK603 × T25 maize.

Run	T25		
	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.31	100	1.00
2	-3.30	101	1.00
3	-3.15	108	1.00
4	-3.23	104	1.00
5	-3.19	106	1.00
6	-3.28	102	1.00
7	-3.30	101	1.00
8	-3.34	99	1.00
Mean	-3.26	103	1.00

The mean PCR efficiencies of the GM and taxon-specific modules were above 90% (Tables 3 and 4). The NK603 module displays acceptable PCR efficiency (between 91% and 92%) and correlation coefficient (R^2 equal to 0.99). The same holds true for *hmg* (efficiency of 104% and R^2 equal to 0.99), but not for *adh1-70*, whose amplification efficiency (113%) is above the limit set by the ENGL, though the R^2 is close to 1.00. The mean PCR efficiency of the Δ CT calibration curve T25 was 103% and the R^2 was 1.00, well within the ENGL acceptance criteria (Table 5).

With the exception of *adh1-70*, the data presented in Tables 3, 4 and 5 confirm the appropriate performance characteristics of the methods when tested on GM stack NK603 × T25 maize in terms of PCR efficiency and linearity.

The EURL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSD_r %) of the two methods applied to samples of DNA extracted from GM stack NK603 × T25 maize (Tables 6, 7 and 8).

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the NK603-*adh1-70* method applied to genomic DNA extracted from GM stack NK603 × T25 maize.

NK603-<i>adh1-70</i>					
Unknown sample GM%	Expected value (GMO%)				
	0.10	0.50	0.90	2.0	5.0
Mean	0.16	0.56	1.2	2.1	6.2
SD	0.02	0.08	0.09	0.31	0.81
RSD _r (%)	11	14	7.5	15	13
Bias (%)	58	11	31	3.91	24

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the NK603-*hmg* method applied to genomic DNA extracted from GM stack NK603 × T25 maize.

NK603-<i>hmg</i>					
Unknown sample GM%	Expected value (GMO%)				
	0.10	0.50	0.90	2.0	5.0
Mean	0.11	0.44	0.90	1.8	4.8
SD	0.01	0.03	0.05	0.16	0.32
RSD _r (%)	9.9	6.8	5.9	8.8	6.6
Bias (%)	5.3	-13	-0.48	-9.4	-3.4

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the T25 method applied to genomic DNA extracted from GM stack NK603 × T25 maize.

T25-<i>adh1-135</i>					
Unknown sample GM%	Expected value (GMO%)				
	0.15	0.4	0.9	2.0	3.3
Mean	0.14	0.36	0.85	1.8	3.3
SD	0.02	0.05	0.06	0.23	0.32
RSD _r (%)	16	15	7.3	13	9.6
Bias (%)	-9.4	-11	-5.0	-7.8	-0.11

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 $\pm 25\%$ across the entire dynamic range. As shown in Table 6, the trueness of the method, measured as bias, showed important deviations at the 0.1% (+58%) and at the 0.9% (+31%) GM-levels when *adh1-70* is used, in both cases exceeding the limits set by the ENGL method performance criteria. The trueness of the NK603 method when the samples were quantified using *hmg* was well below the ENGL limits over the whole dynamic

range (Table 7), the greatest deviation being represented by the bias at the 0.5% GM-level (-13%).

The underperformance of the *adh1-70* module was expected as it targets a region in the maize genome that shows a sequence polymorphism, which may affect the efficiency of amplification. The EURL GMFF had already demonstrated through bridging studies that it is possible to replace *adh1-70* with *hmg*; this holds true also considering the data presented in this report.

Table 8 documents the trueness for the T25 method in combination with the *adh1-135* module, and illustrates that it is well within the ENGL acceptance criteria, with the highest bias of -11% at the 0.4% GM-level. Therefore, the NK603 and T25 methods used in combination with *hmg* and *adh1-135* respectively satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack NK603 × T25.

Tables 6, 7 and 8 also show the relative repeatability standard deviation (RSD_r) as estimated for each GM level. According to the ENGL acceptance criteria and method performance requirements, the EURL GMFF requires RSD_r values to be below 25%. As it can be observed in Tables 6, 7 and 8 the values range between 7.5% and 15% for the NK603 quantified relatively to *adh1-70*, between 5.9% and 9.9% for NK603 quantified relatively to *hmg*, and between 7.3% and 16% for T25.

Hence, the methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack NK603 × T25 maize.

5. Comparison of method performance on NK603 × T25 and on the single events

An indicative comparison of the performance (bias, RSD_r %) of the two methods applied to GM stack NK603 × T25 maize and on the single-line events is shown in Tables 9 and 10. The performance of the methods on the single lines was previously validated through international collaborative trials (<http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>).

Note: the comparison of data generated in different testing conditions and different times is intended to be only of qualitative nature; differences in the figures reported are not necessarily statistically significant.

Table 9. Qualitative comparison of the performance of the NK603-*adh1-70* method applied to genomic DNA extracted from GM stack NK603 × T25 maize and to genomic DNA extracted from the single line event NK603.

Trueness and repeatability of NK603 quantification on NK603 × T25			Trueness and repeatability of NK603 quantification on single event NK603*		
GM%	Bias (%)	RSD _r (%)	GM%	Bias (%)	RSD _r (%)
0.10	58	11	0.10	83	24
0.50	11	14	0.49	73	15
0.90	31	7.5	0.98	47	17
2.0	3.9	15	1.96	14	7.7
5.0	24	13	4.91	22	22

*method validated in inter-laboratory study (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

Table 10. Qualitative comparison of the performance of the T25 detection method applied to genomic DNA extracted from GM stack NK603 × T25 maize and to genomic DNA extracted from the single line event T25.

Trueness and repeatability of T25 quantification on NK603 × T25			Trueness and repeatability of T25 quantification on single event T25*		
GM%	Bias (%)	RSD _r (%)	GM%	Bias (%)	RSD _r (%)
0.15	-9.4	16	0.15	-27	26
0.40	-11	15	0.40	-6	22
0.90	-5.0	7.3	0.90	-9	10
2.0	-7.8	13	2.0	-12	22
3.3	-0.11	9.6	3.3	6	11

*method validated in inter-laboratory study (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

When NK603 was quantified in relation to *adh1-70* the bias was lower than the single trait, but still at 0.1% and 0.9% it was outside the ENGL method performance requirements (Table 9). On the other hand, Table 7 above shows that when the NK603 event was quantified in relation to *hmg* the bias was within the ENGL acceptance criteria.

Throughout the whole range of applicability, the T25 event-specific method (Table 10) showed a comparable or lower bias when applied to the combined event compared to the single trait. In all cases, the relative repeatability standard deviation (RSD_r %) of the event-specific methods when applied to NK603 x T25, were below the ENGL acceptance level established at maximum 25%.

6. Conclusions

This method verification demonstrated that the substitution of the *hmg* module in place of the *adh1-70* module in the quantification of event NK603 allows to minimise the bias of quantification to acceptable levels according to the limits set by the ENGL method performance requirements.

The performance of the two event-specific methods (NK603/*hmg* and T25/*adh1-135*) for the detection and quantification of maize events NK603 and T25, when applied to genomic DNA extracted from GM stack NK603 × T25 maize, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant. The method verification has demonstrated that the PCR efficiency, linearity, trueness and repeatability of the methods were within the limits established by the ENGL.

In conclusion, the verification study confirmed that the methods are capable to detect, identify and quantify each of the GM events when applied to genomic DNA of suitable quality, extracted from GM stack NK603 × T25 maize.

Therefore these methods, developed and validated to detect and quantify the single events, can be equally applied for the detection and quantification of the respective events combined in GM stack NK603 × T25 maize.

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The results of the in-house verification led to the conclusion that the individual methods meet the ENGL performance criteria also when applied to genomic DNA extracted from the GM stack NK603 x T25 maize.

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