JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of MON 87427, MON 87460, MON 89034, MIR162 and NK603 event-specific PCR-based Methods applied to DNA extracted from GM Stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize

European Union Reference Laboratory for Genetically Modified Food and Feed

2017

How to cite this report: European Union Reference Laboratory for GM Food and Feed, Joint Research Centre. "Report on the Verification of the Performance of MON 87427, MON 87460, MON 89034, MIR162 and NK603 event-specific PCR-based Methods applied to DNA extracted from GM Stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize", 2017. http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
Food & Feed Compliance

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European Union Reference Laboratory
for GM Food & Feed

Report on the Verification of the Performance of MON 87427, MON 87460, MON 89034, MIR162 and NK603 event-specific PCR-based Methods applied to DNA extracted from GM Stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize

20/10/2017

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Monsanto Company, represented by Monsanto Europe S.A. to request the authorisation of genetically modified stack (GM stack) MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize (glyphosate-tolerance from MON 87427 and NK603, insect-protection from MON 89034 and MIR162, reduced yield loss under water-limited conditions from MON 87460) and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, import and processing, in accordance with articles 5 and 17 of Regulation (EC) N° 1829/2003 GM Food and GM Feed. The unique identifier assigned to GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize is MON-87427-7 x MON 8746Ø-4 x MON-89Ø34-3 x SYN-IR162-4 x MON- ØØ6Ø3-6.

The GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize has been obtained by conventional crossing between the genetically modified maize events: MON 87427, MON 87460, MON 89034, MIR162 and NK603, without any new genetic modification.
The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events MON 87427, MON 87460, MON 89034, MIR162 and NK603 (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/MPR%20Report%20Application%2020_10_2015.pdf) the EURL GMFF has carried out an *in-house* verification of the performance of each validated method when applied to genomic DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 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 MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

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

Content

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: Belac 268 TEST (Flexible Scope for DNA extraction, DNA identification and real Time PCR) and ISO 17043:2010 accredited (certificate number: Belac 268 PT, proficiency test provider).

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

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1. Introduction

The EU legislative system [1, 2] for genetically modified food and feed foresees 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/MPR%20Report%20Application%2020_10_2015.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 MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 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 (EU) No 503/2013 (Annex III).

The results of the *in-house* verification study were evaluated with reference to ENGL method performance requirements [3] 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 when applied to genomic DNA extracted from the stack, and the corresponding control samples of DNA extracted from the GM stack maize MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 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 %) and precision (expressed as RSDr %) calculated by the applicant for the five methods applied to MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize genomic DNA. Means are the average of fifteen replicates obtained through five runs performed with ABI 7500 real-time PCR equipment. Percentages are expressed as GM DNA / total DNA x 100.

Note: Numerical values presented in the following tables were rounded keeping two digits for values ≤ 1, one digit for values between 1 and 10 and no digit for values ≥ 10, unless otherwise stated. The calculations in the MS Excel files however were done over not rounded data. This approach might create small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) provided by the applicant for the MON 87427, MON 87460, MON 89034, MIR162 and NK603 methods applied to GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

MON 87427*			
Sample GM %	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.076	0.83	9.94
RSD$_r$ (%)	23.71	6.42	6.39
Bias (%)	-10.9	-17.37	-0.62
MON 87460*			
Sample GM %	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.091	1.03	10.86
RSD$_r$ (%)	15.42	3.52	5.27
Bias (%)	7.6	3.36	8.6
MON 89034*			
Sample GM %	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.077	0.96	10.61
RSD$_r$ (%)	18.09	4.43	5.28
Bias (%)	-9.18	-4.02	6.12

Table 1 (continued)

MIR162*			
Sample GM %	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.102	1.01	9.80
RSD$_r$ (%)	20.01	21.25	9.13
Bias (%)	20.44	1.15	-2.04
NK603*			
Sample GM %	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.075	0.94	9.62
RSD$_r$ (%)	17.63	12.06	10.04
Bias (%)	-11.59	-6.13	-3.78

* Values are not rounded but are presented as reported by the applicant

The EURL GMFF verified the data and concluded that they were reliable and seemed to confirm that the methods meet the ENGL performance criteria[3].

One request of complementary information regarding methods, control samples and DNA sequences were 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 five methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA of GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize, hemizygous for the loci, as positive control sample.
- genomic DNA of conventional (non-GM) maize whose genetic background is LH244 x LH287, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize with the non-GM maize genomic DNA, in a constant amount of total maize genomic

DNA. The same GM concentrations as in the validation of the methods for the single lines were achieved, except for NK603 where the concentrations used in the single line are reported with a double asterisk in Table 2. Table 2 shows the five GM concentrations used in the verification of the MON 87427, MON 87460, MON 89034, MIR162 and NK603 methods when applying them to genomic DNA extracted from the GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

Table 2. Percentage (GM %) of MON 87427, MON 87460, MON 89034, MIR162 and NK603 in MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 stack genomic DNA contained in the verification samples.

MON 87427 GM %*	MON 87460 GM %*	MON 89034 GM %*	MIR162 GM %*	NK603 GM %**
8.00	8.00	8.00	5.00	5.00
3.00	3.00	3.00	2.00	2.00
0.90	0.90	0.90	0.90	0.90
0.20	0.40	0.40	0.40	0.50
0.06	0.09	0.09	0.10	0.10

* Percentage expressed in copy number ratio, [(GM DNA / total maize DNA x 100)]

** Validation of the single event was done at concentrations 0.1%, 0.49%, 0.98%, 1.96% and 4.91%.

The protocols described by the applicant for the individual MON 87427, MON 87460, MON 89034, MIR162 and NK603 maize events were implemented in the EURL GMFF laboratory with the deviations reported in § 4.4.1.

4.2 DNA extraction

A method for DNA extraction from maize 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.

Annex III to Reg. (EU) No 503/2013[2] requires the applicant to discuss the validity and limitations of the detection methods in the various types of foods and feeds (matrices) that are expected to be placed on the market. To this regard the applicant stated that the applicability of the quantitative real-time PCR methods developed for MON 87427, MON 87460, MON 89034, MIR162 and NK603 maize depends on the isolation of sufficient quality and quantity of purified DNA. Conceptually, the detection methods should work as far as good quality and intact DNA can be extracted from processed food and feed materials. The method is intended for extraction of genomic DNA from seeds, which results in high molecular weight DNA. The applicant also informed the EURL GMFF that during the processing of maize seeds into food and feed ingredients a number of steps are followed, which can influence the quality and intactness of the DNA contained in the final processed maize products [4,5,6]. DNA extraction from some of these processed matrices may require additional rounds of purification in order to achieve the quality standards needed for quantitative real-time PCR [7,8].

On a general note the EURL GMFF recommends that laboratories using this validated method for testing complex or difficult matrices always verify that the extracted genomic DNA is of sufficient quality.

The protocol for the DNA extraction method is available at http://gmo-crl.jrc.ec.europa.eu/summaries/MON88017_DNAExtr_report.pdf.

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

4.3   Experimental design

Eight PCR runs were carried out for each method. In each run, samples were analysed in parallel with both the GM-specific system and the reference systems high mobility group (*hmg*) and alcohol dehydrogenase 1 gene (*adh1*). Five GM levels were examined per run, each GM level in duplicate. PCR analysis was performed in triplicate for all samples. In total, for each method MON 87427, MON 87460, MON 89034, MIR162 and NK603, 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 the GM %.

4.4   PCR methods

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize using the single detection methods previously validated for the respective single GM events MON 87427, MON 87460, MON 89034, MIR162 and NK603 maize.

For detection of GM maize events MON 87427, MON 87460, MON 89034, MIR162 and NK603, DNA fragments of 95-bp, 82-bp, 77-bp, 92-bp and 108-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: 6-FAM (6-carboxyfluorescein) as reporter dye at their 5'-end and TAMRA (carboxytetramethylrhodamine) as a quencher dye at their 3'-end for four events and a non-fluorescent quencher MGBNFQ for MON 89034.

For quantification of GM maize events MON 87427, MON 87460, MON 89034 and NK603, a taxon-specific reference system amplifies a 79-bp fragment of high mobility group (hmg) gene a maize endogenous gene (GenBank AJ131373.1), using two *hmg* gene-specific primers and a gene-specific probe labelled with FAM and TAMRA.

For the relative quantification of GM maize events MON 87427, MON 87460, MON 89034 and NK603 maize standard curves are generated both for the GM and for the reference specific system by plotting Cq values of the calibration standards against the logarithm of the DNA

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amount and by fitting a linear regression into these data. Thereafter, the Cq values of the unknown samples are measured and, by means of the regression formula, the relative amount of MON 87427, MON 87460, MON 89034 and NK603 maize DNA is estimated.

For the relative quantification of GM event MIR162 DNA, a maize-specific reference system amplifies a 135 bp fragment of the maize endogenous alcohol dehydrogenase 1 gene (*adh1*), using two specific primers and a gene-specific probe labelled with VIC and TAMRA at the 5' and 3' end respectively. The fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Cq" value. For quantification of the amount of event MIR162 DNA in a test sample, the normalised Cq values of the calibration samples are used to calculate by linear regression a reference curve Cq-formula. The normalised Cq values of the unknown samples are measured and, by means of the regression formula, the relative amount of event MIR162 DNA is estimated.

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.

4.4.1 Deviations from the validated methods

The applicant applied a deviation to MON 87460 validated method in the context of MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 stack; the reaction volume of the MON 87460 validated method is 50 µl and the applicant reduced the volume to 25 µl.

TaqMan® buffer A (Life Technologies) in use with the *hmg* reference system validated for the relative quantification of event MON 89034 was phased out at the time of method verification. The applicant applied an *hmg* method where *hmg* is amplified with TaqMan Universal Master Mix. The EURL GMFF substituted the discontinued buffer with the method for the *hmg* reference system validated in the context of the relative quantification of maize event MON 87460 (EURL-VL-04/09VP, page 8/10 at http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27_MON87460_validated_Method.pdf). The latter method makes use of the TaqMan Universal Master Mix. Moreover, for MON 89034 the applicant performed an additional experiment to scale down the volume from 50 µl to 25 µl.

The quantification of GM event NK603 maize was verified following a method previously verified in the context of a maize stacked line (bridging study, EURL-VL-01/11VR, page 19/19 at http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-1507-59122-MON810-NK603%20.pdf; the final reaction volume was 25 µL for both the GM and the reference systems. The quantification of NK603 was performed relative to the validated maize reference system *hmg* in substitution of the suboptimal maize reference system *adh-70* bp (pages 10-11/14 of http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-03-10-VR.pdf).

Finally, the performance of GM event MIR162 method was verified by the applicant with a PCR assay containing a reference dye (sigma R4526) not present in the MIR162 validated method

(i.e. R4526 in replacement of sulforhodamine); moreover the final $MgCl_2$ concentration was different from the final concentration recommended in the validated method.

4.5 Results

Tables 3, 4, 5, 6 and 7 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 $[10^{(-1/slope)} - 1] \times 100$, and of the coefficient of determination (R^2) reported for all PCR systems in the eight runs, for GM maize events MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize. Slope values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 87427 method on GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

Run	MON 87427			hmg		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.25	103	0.99	-3.29	101	1.00
2	-3.16	107	1.00	-3.22	104	1.00
3	-3.23	104	0.99	-3.31	100	1.00
4	-3.37	98	1.00	-3.30	101	1.00
5	-3.33	100	1.00	-3.28	102	1.00
6	-3.25	103	1.00	-3.31	100	1.00
7	-3.20	105	1.00	-3.27	102	1.00
8	-3.20	105	1.00	-3.27	102	1.00
Mean	-3.25	103	1.00	-3.28	102	1.00

Table 4. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 87460 method on GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

Run	MON 87460			hmg		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.14	108	1.00	-3.29	101	1.00
2	-3.13	109	1.00	-3.31	100	1.00
3	-3.30	101	0.99	-3.30	101	1.00
4	-3.20	106	1.00	-3.34	99	1.00
5	-3.22	104	1.00	-3.30	101	1.00
6	-3.29	101	0.99	-3.32	100	1.00
7	-3.44	95	0.99	-3.34	99	1.00
8	-3.19	106	1.00	-3.36	99	1.00
Mean	**-3.24**	**104**	**1.00**	**-3.32**	**100**	**1.00**

Table 5. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 89034 method on GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

Run	MON 89034			hmg		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.34	99	1.00	-3.25	103	1.00
2	-3.39	97	1.00	-3.31	101	1.00
3	-3.41	96	1.00	-3.30	101	1.00
4	-3.40	97	1.00	-3.29	101	1.00
5	-3.41	97	1.00	-3.30	101	1.00
6	-3.41	96	1.00	-3.30	101	1.00
7	-3.36	98	1.00	-3.26	103	1.00
8	-3.35	99	1.00	-3.32	100	1.00
Mean	**-3.39**	**97**	**1.00**	**-3.29**	**101**	**1.00**

Table 6. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MIR162 method on GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

Run	MIR162		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.41	96	1.00
2	-3.47	94	1.00
3	-3.41	96	1.00
4	-3.21	105	0.99
5	-3.38	98	1.00
6	-3.37	98	1.00
7	-3.53	92	1.00
8	-3.50	93	1.00
Mean	-3.41	97	1.00

Table 7. Values of standard curve slope, PCR efficiency and R^2 coefficient for the NK603 method on GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

Run	NK603			hmg		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.65	88	0.99	-3.26	103	1.00
2	-3.64	88	0.99	-3.23	104	1.00
3	-3.52	92	0.99	-3.21	105	1.00
4	-3.62	89	0.99	-3.27	102	1.00
5	-3.58	90	0.99	-3.23	104	1.00
6	-3.51	93	0.99	-3.25	103	1.00
7	-3.72	86	0.99	-3.24	104	1.00
8	-3.59	90	0.99	-3.26	103	1.00
Mean	-3.60	89	0.99	-3.24	103	1.00

The mean PCR efficiencies of the GM and species-specific systems were above 97 % (103 % and 102 % for MON 87427 and *hmg* systems, 104 % and 100 % for MON 87460 and *hmg* systems, 97 % and 101 % for MON 89034 and *hmg* systems, 97 % for MIR162), with the exception of NK603 (89 % and 103 % for NK603 and *hmg* systems). The mean value obtained for the slope of NK603 in the context of MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 stacked line is 3.605 (rounded to 3.6), which is in line with ENGL performance requirements [3] and considered acceptable considering the mean slope value obtained in the validation of the single line (-3.66).

The mean R^2 coefficient of the methods was 1.00 for all, except for NK603 (0.99). The data presented in Tables 3, 4, 5, 6 and 7 confirm the appropriate performance characteristics of the

five methods when tested on GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize in terms of PCR efficiency and R^2 coefficient.

The EURL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) of the five methods applied to samples of DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize (see tables 8, 9, 10, 11 and 12).

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD$_r$ %) of the MON 87427 method applied to genomic DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

MON 87427					
Unknown sample GM %	**Expected value (GMO %)**				
	0.06	**0.20**	**0.9**	**3.0**	**8.0**
Mean	0.05	0.16	0.76	2.8	8.3
SD	0.01	0.02	0.05	0.30	0.61
RSD$_r$ (%)	14	13	6.1	11	7.3
Bias (%)	-18	-20	-15	-6.6	4.2

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD$_r$ %) of the MON 87460 method applied to genomic DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

MON 87460					
Unknown sample GM %	**Expected value (GMO %)**				
	0.09	**0.40**	**0.9**	**3.0**	**8.0**
Mean	0.08	0.37	0.78	2.8	8.1
SD	0.01	0.05	0.03	0.18	0.70
RSD$_r$ (%)	12	13	4.3	6.2	8.7
Bias (%)	-9.8	-7.0	-13	-5.3	1.3

Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD$_r$ %) of the MON 89034 method applied to genomic DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

MON 89034					
Unknown sample GM %	**Expected value (GMO %)**				
	0.09	**0.40**	**0.90**	**3.0**	**8.0**
Mean	0.09	0.37	0.77	2.6	7.3
SD	0.01	0.05	0.06	0.13	0.26
RSD$_r$ (%)	13	14	7.6	5.2	3.5
Bias (%)	-5.1	-7.2	-14	-15	-8.6

Table 11. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD$_r$ %) of the MIR162 method applied to genomic DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

MIR162					
Unknown sample GM %	**Expected value (GMO %)**				
	0.10	**0.40**	**0.90**	**2.0**	**5.0**
Mean	0.12	0.40	0.82	2.0	4.9
SD	0.01	0.04	0.09	0.26	0.33
RSD$_r$ (%)	11	9.8	10	13	6.6
Bias (%)	21	1.1	-8.8	2.0	-1.1

Table 12. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD$_r$ %) of the NK603 method applied to genomic DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

NK603					
Unknown sample GM %	**Expected value (GMO %)**				
	0.10	**0.50**	**0.90**	**2.0**	**5.0**
Mean	0.10	0.38	0.76	1.6	4.6
SD	0.01	0.04	0.04	0.21	0.28
RSD$_r$ (%)	13	12	5.5	13	6.1
Bias (%)	-3.8	-23	-15	-18	-8.3

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 less or equal to ± 25 % across the entire dynamic range. As shown in Tables 8, 9, 10, 11 and 12, the values range from -20 % to 4.2 % for MON 87427, from -13 % to 1.3 % for MON 87460, from -15 % to -5.1 % for MON 89034, from -8.8 % to 21 % for MIR162 and from -23 % to -3.8 % for NK603. Therefore, the five methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

Tables 8, 9, 10, 11 and 12 also show the relative repeatability standard deviation (RSD$_r$) estimated for each GM level. According to the ENGL acceptance criteria and method performance requirements, the RSD$_r$ values should be equal to or below 25%. As the values range between 6.1 % and 14 % for MON 87427, between 4.3 % and 13 % for MON 87460, between 3.5 % and 14 % for MON 89034, between 6.6 % and 13 % for MIR162 and between 5.5 % and 13 % for NK603, the five methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize.

5. Conclusions

The performance of the five event-specific methods for the detection and quantification of maize single line events MON 87427, MON 87460, MON 89034, MIR162 and NK603 maize, when applied to genomic DNA extracted from GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant.

Therefore these methods, developed and validated to detect and quantify the single maize events MON 87427, MON 87460, MON 89034, MIR162 and NK603, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack MON 87427 x MON 87460 x MON 89034 x MIR162 x NK603 maize or any of its sub-combinations, supposed that sufficient genomic DNA of appropriate quality is available.

6. References

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