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Report on the Verification of the Performance of MON 87427, MON 89034, NK603 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack MON 87427 x MON 89034 x NK603 maize

European Union Reference Laboratory for Genetically Modified Food and Feed

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24 June 2016

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Monsanto Company, as represented by Monsanto Europe S.A. to request the authorisation of genetically modified stack (GM stack) MON 87427 x MON 89034 x NK603 and all sub-combinations of the individual events as present in the segregating progeny, (herbicide tolerance and insect resistance) 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 89034 x NK603 maize is MON-87427-7 x MON-89Ø34-3 x MONØØ6Ø3-6.

The GM stack MON 87427 x MON 89034 x NK603 maize has been obtained by conventional crossing between the genetically modified maize events: MON 87427, MON 89034, 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 89034 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/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 MON 87427 x MON 89034 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 89034 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: ACCREDIA 1172 (Flexible Scope for DNA extraction and qualitative/quantitative PCR)].

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.

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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/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 MON 87427 x MON 89034 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 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 of DNA extracted from the GM stack MON 87427 x MON 89034 x NK603 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 %) and precision (expressed as RSDr %) calculated by the applicant for the three methods verified on the stack DNA. Means are the average of fifteen replicates 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 89034, and NK603 methods applied to GM stack MON 87427 x MON 89034 x NK603 maize.

MON 87427 **			
Unknown sample* GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.087	0.84	8.83
RSD_r (%)	15.18	7.92	5.90
Bias (%)	2.21	-16.28	-11.66
MON 89034 **			
Unknown sample* GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.079	0.92	8.93
RSD_r (%)	17.07	8.70	6.88
Bias (%)	-6.61	-7.74	-10.74
NK603 **			
Unknown sample* GM%	**Expected value (GMO %)**		
	0.085	**1.0**	**10**
Mean	0.076	0.93	8.98
RSD_r (%)	21.08	8.09	7.97
Bias (%)	-11.15	-6.90	-10.24

*Unknown samples are DNA samples containing different levels of GM DNA from stack material and non-GM DNA from conventional material.

**Numbers 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)]. Two requests for complementary information regarding the zygosity of the positive control sample and the 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 three methods in its own laboratory and performed a verification of their performance when applied to DNA extracted from GM stack MON 87427 x MON 89034 x NK603 maize.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA of MON 87427 x MON 89034 x NK603 extracted from homogenized maize seeds, hemizygous for MON 87427, MON 89034 and NK603, as positive control sample.
- genomic DNA extracted from non GM maize seeds 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 89034 x NK603 maize and 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. Table 2 shows the five GM concentrations used in the verification of the MON 87427, MON 89034 and NK603 methods when applying them to genomic DNA extracted from the GM stack MON 87427 x MON 89034 x NK603 maize. These are the same concentrations used in the validation of these methods for the parental single line GMOs.

Table 2. Percentage (GM %) of MON 87427, MON 89034, and NK603 in MON 87427 x MON 89034 x NK603 DNA of verification samples.

MON 87427 GM%* [(GM DNA / total maize DNA x 100)]	**MON 89034 GM%*** [(GM DNA / total maize DNA x 100)]	**NK603 GM%*** [(GM DNA / total maize DNA x 100)]
0.06	0.09	0.10
0.20	0.40	0.50
0.90	0.90	0.90
3.0	3.0	2.0
8.0	8.0	5.0

*percentage expressed in copy number ratio.

The protocols described by the applicant for the individual MON 87427, MON 89034 and NK603 GM events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx) 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.

Note: the EURL GMFF recommends that laboratories using a validated method always verify that the extracted genomic DNA is of sufficient quality and quantity, particularly when testing complex or difficult matrices

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

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 system and the maize reference system high mobility group *(hmg).* 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 MON 87427, MON 89034 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 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 89034 x NK603 maize using the single detection methods previously validated for the respective single GM events MON 87427, MON 89034 and NK603.

For detection of GM maize events MON 87427, MON 89034 and NK603, DNA fragments of 95 bp, 77 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: FAM (6-carboxyfluorescein) as reporter dye at the 5′-end for all three events and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at their 3′-end for MON 87427 and NK603, and MGBNFQ (minor groove binding non-fluorescent quencher) for MON 89034, respectively.

For quantification of GM maize events MON 87427, 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 Accession No AJ131373.1), using two *hmg* specific primers and a gene-specific probe labelled with FAM and TAMRA.

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

TaqMan® buffer A (Life Technologies) in use with the *hmg* reference system validated for the relative quantification of event MON 89034 is discontinued by the manufacturer. Therefore, the EURL GMFF substituted it 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 (same manufacturer).

The performance of the quantification of GM event NK603 maize was verified following a method previously verified in the context of a maize stacked event verification (bridging study, EURL-VL-01/11VR, page 19/19 at http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-TC1507-59122-MON810-NK603%20.pdf); the final reaction volume is 25 µL for both the GM and the reference system. The quantification of NK603 is 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).

4.5 Results

Tables 3, 4, and 5 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 R^2 (expressing the R^2 coefficient of the regression) reported for all PCR systems in the eight runs, for GM maize events MON 87427, MON 89034 and NK603. Slope and R^2 coefficient 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 89034 x NK603 maize.

Run	MON 87427			*hmg*		
	Slope	**PCR Efficiency (%)**	**R^2 coefficient**	**Slope**	**PCR Efficiency (%)**	**R^2 coefficient**
1	-3.39	97	1.00	-3.37	98	1.00
2	-3.34	99	1.00	-3.42	96	1.00
3	-3.33	100	0.99	-3.39	97	1.00
4	-3.45	95	1.00	-3.36	98	1.00
5	-3.41	96	1.00	-3.36	99	1.00
6	-3.29	101	0.99	-3.33	100	1.00
7	-3.30	101	0.99	-3.39	97	1.00
8	-3.41	96	1.00	-3.36	99	1.00
Mean	-3.36	98	1.00	-3.37	98	1.00

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

Run	MON 89034			*hmg*		
	Slope	**PCR Efficiency (%)**	**R^2 coefficient**	**Slope**	**PCR Efficiency (%)**	**R^2 coefficient**
1	-3.39	97	1.00	-3.29	101	1.00
2	-3.26	102	1.00	-3.32	100	1.00
3	-3.43	96	1.00	-3.21	105	1.00
4	-3.36	98	1.00	-3.26	103	1.00
5	-3.38	98	1.00	-3.25	103	1.00
6	-3.35	99	1.00	-3.21	105	1.00
7	-3.40	97	1.00	-3.30	101	1.00
8	-3.32	100	1.00	-3.24	103	1.00
Mean	-3.36	98	1.00	-3.26	103	1.00

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

Run	NK603			*hmg*		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.55	91	1.00	-3.37	98	1.00
2	-3.77	84	0.99	-3.40	97	1.00
3	-3.83	82	0.94	-3.37	98	1.00
4	-3.55	91	0.99	-3.32	100	1.00
5	-3.73	85	1.00	-3.38	97	1.00
6	-3.62	89	0.99	-3.36	98	1.00
7	-3.55	91	0.99	-3.33	100	1.00
8	-3.78	84	0.99	-3.33	100	1.00
Mean	-3.67	87	0.99	-3.36	99	1.00

The mean PCR efficiencies of the GM and species-specific system were around 98% for both MON 87427 and MON 89034 methods, and between 98% and 103% for *hmg* system. The mean PCR efficiency of the NK603 method was 87%, slightly below 90 %, which is in line with the historical recordings of efficiency of the NK603 amplification system (see: http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).
The R^2 coefficient of the methods was between 0.99 and 1.00 for all systems. The data presented in Tables 3, 4 and 5 confirm the appropriate performance characteristics of the three methods when tested on genomic DNA extracted from the GM stack MON 87427 x MON 89034 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 three methods applied to samples of genomic DNA extracted from GM stack MON 87427 x MON 89034 x NK603 maize, see tables 6, 7, and 8.

Table 6. 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 89034 x NK603 maize.

MON 87427					
Unknown sample GM%	**Expected value (GMO%)**				
	0.06	**0.20**	**0.90**	**3.0**	**8.0**
Mean	0.06	0.19	0.86	3.2	8.0
SD	0.01	0.02	0.06	0.26	0.51
RSD_r (%)	10	8.6	6.6	8.2	6.3
Bias (%)	-7.0	-2.8	-4.1	6.5	-0.40

Table 7. 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 89034 x NK603 maize.

MON 89034					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.40**	**0.90**	**3.0**	**8.0**
Mean	0.08	0.38	0.82	2.8	7.7
SD	0.01	0.03	0.06	0.17	0.65
RSD_r (%)	13	7.2	7.2	6.2	8.4
Bias (%)	-13	-5.5	-8.8	-6.0	-3.3

Table 8. 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 89034 x NK603 maize.

NK603					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.50**	**0.90**	**2.0**	**5.0**
Mean	0.09	0.46	0.86	1.8	4.9
SD	0.01	0.04	0.07	0.09	0.30
RSD_r (%)	12	9.3	8.7	5.0	6.2
Bias (%)	-9.9	-7.3	-4.8	-12	-2.5

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 Tables 6, 7 and 8, the values range from -7.0% to 6.5% for MON 87427, from -13% to -3.3% for MON 89034, and from -12% to -2.5% for NK603. Therefore, the three methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack MON 87427 x MON 89034 x NK603 maize.

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 the values range between 6.3% and 10% for MON 87427, between 6.2% and 13% for MON 89034, and between 5.0% and 12% for NK603 the three methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack MON 87427 x MON 89034 x NK603 maize.

5. Conclusions

The performance of the three event-specific methods for the detection and quantification of maize single line events MON 87427, MON87701 and NK603 when applied to genomic DNA extracted from GM stack MON 87427 x MON 89034 x NK603 maize, 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, MON87701, and NK603, can be equally applied, with the modification described above, for the detection and quantification of the respective events in DNA extracted from the GM stack MON 87427 x MON 89034 x NK603 maize or any of its sub-combinations, supposed that sufficient genomic DNA of appropriate quality is available.

This statement is valid for all types of food and feed products that could contain the GM stack MON 87427 x MON 89034 x NK603 maize or any of its sub-combinations.

6. References

1. Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed (Text with EEA relevance). OJ L 268, 18.10.2003, p. 1–23.

2. Regulation (EU) No 503/2013 of 3 April 2013 "on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006".

3. European Network of GMO Laboratories (ENGL), 'Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing', 2015. http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf.

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