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Report on the Verification of the Performance of NK603, T25 and DAS-40278-9 event-specific PCR-based Methods applied to DNA extracted from GM Stack NK603 x T25 x DAS-40278-9 maize

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European Union Reference Laboratory for Genetically Modified Food and Feed

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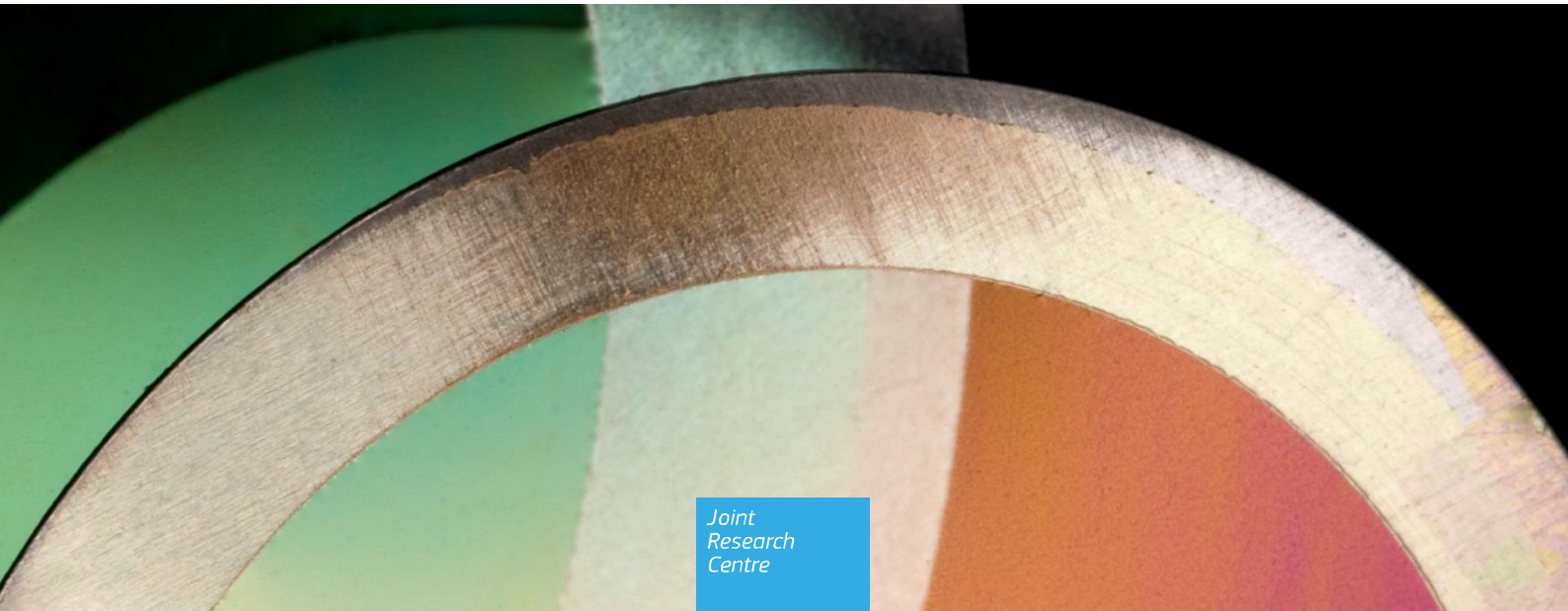

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Report on the Verification of the Performance of NK603, T25 and DAS-40278-9 event-specific PCR-based Methods applied to DNA extracted from GM Stack NK603 x T25 x DAS-40278-9 maize

21 August 2021

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Pioneer Overseas Corporation to request the authorisation of the genetically modified stack (GM stack) NK603 x T25 x DAS-40278-9 maize (tolerance to glyphosate, glufosinate, 2,4-dichlorophenoxyacetic acid (2,4-D) and aryloxyphenoxypropionate (AOPP) herbicides), and all sub-combinations of the individual events as present in the segregating progeny; the application was for food and feed uses, 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 x DAS-40278-9 maize is MON-ØØ6Ø3-6xACS-ZMØØ3-2xDAS-4Ø278-9.

The GM stack NK603 x T25 x DAS-40278-9 maize was obtained by conventional crossing between the genetically modified maize events: NK603, T25 and DAS-40278-9, 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, T25 and DAS-40278-9 (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 x DAS-40278-9 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 x DAS-40278-9 maize.

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

Content

Quality assurance

The EURL GMFF is ISO 17025:2017 accredited [certificate number: Belac 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) in food and feed by DNA extraction, DNA identification and Real-time PCR and for determination of Genetically Modified content in % (cp/cp) in food and feed by DNA extraction and digital PCR)].

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/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 x T25 x DAS-40278-9 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. Dossier reception and acceptance (step 1)

Pioneer Overseas Corporation, 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 NK603 x T25 x DAS-40278-9 and from non GM maize.

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

3. Dossier scientific assessment (step 2)

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.

3.1 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 quantitative real-time PCR methods for NK603, T25 and DAS-40278-9 were pre-validated on maize seed and tissues. These methods can, in principle, be applied to any sample from which sufficient quantities of maize DNA, free of PCR inhibitors, can be purified.

However, the processing of maize grain involves varying degrees of mechanical, enzymatic, solvent, heat, acid, pressure treatment, or combinations of these steps […]. These steps influence the quality and intactness of DNA contained in the final processed maize products [4-7] which may result in significant degradation of high molecular weight DNA and failure to PCR amplify products greater than a few 100 base pairs [4,5]. Random DNA fragmentation is known to lead to variability in quantifying DNA by qPCR [8], thus affecting the ability to accurately quantify the presence of a GM event and taxon-specific target in processed fractions. Moreover, the DNA extraction procedure necessary for some of these processed matrices may need additional rounds of processing to clean-up the DNA, to eliminate PCR inhibitors in order to achieve quality genomic DNA suitable for PCR testing [9-10]. These extraction methods are widely used for plant-based materials, are economical and can be easily scaled [11].

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 https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-02-14-XP.pdf.

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

3.2 PCR methods

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSD_r %) calculated by the applicant for the three methods applied to NK603 x T25 x DAS-40278-9 maize genomic DNA. Means are the average of thirty replicates obtained through five runs performed with the Applied Biosystems™ 7500 Fast 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 indicated. 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, RSD_r %) provided by the applicant for the NK603, T25 and DAS-40278-9 methods applied to GM stack NK603 x T25 x DAS-40278-9 maize.

NK603 *				
Sample GM %	**Expected value (GMO %)**			
	0.1	**0.9**	**2.0**	**8.0**
Mean	0.099	0.834	1.737	7.842
RSD_r (%)	18.18	7.91	9.50	6.04
Bias (%)	-1.00	-7.33	-13.15	-1.98
T25 *				
Sample GM %	**Expected value (GMO %)**			
	0.09	**0.9**	**2.0**	**8.0**
Mean	0.077	0.818	1.791	7.999
RSD_r (%)	22.08	8.92	7.48	10.03
Bias (%)	-14.44	-9.11	-10.45	-0.01
DAS-40278-9 *				
Sample GM %	**Expected value (GMO %)**			
	0.08	**0.9**	**2.0**	**8.0**
Mean	0.07	0.890	2.088	7.891
RSD_r (%)	21.43	15.51	11.21	7.45
Bias (%)	-12.5	-1.11	4.40	-1.35

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

3.2.1 Deviations from the validated methods introduced by the applicant

The event-specific detection method for NK603 maize was applied by the applicant as published and modified in EURL-VL-01/11, Annex 1, Tables 3 and 4[1].The event-specific detection methods for T25 maize[2] and DAS-40278-9 maize[3] were followed without deviations.

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).

The dossier was therefore moved to step 3.

4. EURL GMFF experimental testing (step 3)

In step 3 the EURL GMFF used the three methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack NK603 x T25 x DAS-40278-9 maize.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenised ground seeds of GM stack NK603 x T25 x DAS-40278-9 maize, hemizygous for the locus, as positive control sample.
- genomic DNA extracted from homogenized seeds of conventional (non-GM) near isogenic line maize, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack NK603 x T25 x DAS-40278-9 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 obtained. Table 2 shows the five GM concentrations used in the verification of the NK603, T25 and DAS-40278-9 methods when applying them to genomic DNA extracted from the GM stack NK603 x T25 x DAS-40278-9 maize.

[1] https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-1507-59122-MON810-NK603%20.pdf
[2] https://gmo-crl.jrc.ec.europa.eu/summaries/CRLVL0804VP%20corrected%20version%201%20-%20EURL%20Web.pdf
[3] https://gmo-crl.jrc.ec.europa.eu/summaries/2012-08-15_EURL-VL-10-10%20VM_JRC76621.pdf

Table 2. Percentage (GM %) of NK603, T25 and DAS-40278-9 in NK603 x T25 x DAS-40278-9 stack genomic DNA contained in the verification samples.

NK603 GM %* (GM DNA / total maize DNA x 100)	**T25 GM %*** (GM DNA / total maize DNA x 100)	**DAS-40278-9 GM %*** (GM DNA / total maize DNA x 100)
0.10	0.15	0.10
0.50	0.40	0.40
0.90	0.90	0.90
2.0	2.0	2.0
5.0	3.3	5.0

* Percentage expressed in copy number ratio.

The protocols described by the applicant were implemented precisely in the EURL GMFF laboratory and were in accordance with the protocols already published for the individual NK603, T25 and DAS-40278-9 GM events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx), as indicated in §3.2.1.

4.2 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 system *hmg*, high mobility group, for the NK603 and DAS-40278 method and *Adh*1 alcohol dehydrogenase 1 gene for the T25 method. 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 NK603, T25 and DAS-40278-9, 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). All runs were performed with the Applied Biosystems™ 7500 real-time PCR equipment An Excel spreadsheet was used for determination of the GM %.

4.3 PCR methods

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack NK603 x T25 x DAS-40278-9 maize using the single detection methods previously validated for the respective single GM events NK603, T25 and DAS-40278-9.

For detection of GM maize events NK603, T25 and DAS-40278-9, DNA fragments of 108-bp, 102-bp and 98-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 (carboxytetramethylrhodamine) as a quencher dye at their 3′-end for all three events.

For quantification of GM maize events NK603 and DAS-40278-9, a taxon-specific reference method amplifies a 79-bp fragment of *high mobility group* (*hmg*) a maize endogenous gene

(GenBank accession number AJ131373.1), using two *hmg* gene-specific primers and a gene-specific probe labelled with FAM and TAMRA.

For quantification of GM maize event T25, a taxon-specific reference method amplifies a 135-bp fragment of *Alcohol delydrogenase-1,* (*Adh-1*), a maize endogenous gene (GenBank X04050), using two *Adh-1* gene-specific primers and a gene-specific probe labelled with FAM and TAMRA.

For the relative quantification of GM maize events NK603, T25 and DAS-40278-9 standard curves are generated both for the NK603 and DAS-40278-9 and for the maize-specific reference method *hmg* 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 NK603 and DAS-40278-9 DNA is estimated.

For relative quantification of GM maize event T25 DNA in a test sample, the ΔCq values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCq values against the logarithm of the relative amount of T25 event DNA). The ΔCq values of the unknown samples are measured and, by means of the regression formula, the relative amount of T25 event 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.

4.3.1 Deviations from the validated methods introduced by the EURL

The event-specific detection method for NK603 maize was applied as published and modified in EURL-VL-01/11, Annex 1, Tables 3 and 4[4].The event-specific detection methods for T25 maize and DAS-40278-9 maize were followed without deviations.

4.4 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 coefficient of determination (R^2) reported for all PCR systems in the eight runs, for GM maize events NK603, T25 and DAS-40278-9. Slope values were rounded to two digits.

[4] https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-1507-59122-MON810-NK603%20.pdf

Table 3. Values of standard curve slope, PCR efficiency and R^2 coefficient for the NK603 method on GM stack NK603 x T25 x DAS-40278-9 maize.

Run	NK603			*hmg*		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.63	89	1.00	-3.41	96	1.00
2	-3.57	91	1.00	-3.37	98	1.00
3	-3.54	92	1.00	-3.42	96	1.00
4	-3.60	89	1.00	-3.38	98	1.00
5	-3.71	86	1.00	-3.44	95	1.00
6	-3.55	91	1.00	-3.43	96	1.00
7	-3.65	88	1.00	-3.45	95	1.00
8	-3.54	92	1.00	-3.41	96	1.00
Mean	-3.60	90	1.00	-3.41	96	1.00

Table 4. Values of standard curve slope, PCR efficiency and R^2 coefficient for the T25 method on GM stack NK603 x T25 x DAS-40278-9 maize.

Run	T25		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.35	99	1.00
2	-3.34	99	1.00
3	-3.34	99	1.00
4	-3.31	101	1.00
5	-3.37	98	1.00
6	-3.45	95	1.00
7	-3.41	96	1.00
8	-3.39	97	1.00
Mean	-3.37	98	1.00

Table 5. Values of standard curve slope, PCR efficiency and R2 coefficient for the DAS-40278-9 method on GM stack NK603 x T25 x DAS-40278-9 maize

Run	DAS-40278-9			*hmg*		
	Slope	**PCR Efficiency (%)**	**R^2 Coefficient**	**Slope**	**PCR Efficiency (%)**	**R^2 coefficient**
1	-3.34	99	1.00	-3.42	96	1.00
2	-3.33	100	1.00	-3.38	98	1.00
3	-3.35	99	1.00	-3.46	94	1.00
4	-3.33	100	1.00	-3.31	100	1.00
5	-3.33	100	1.00	-3.43	96	1.00
6	-3.29	101	1.00	-3.45	95	1.00
7	-3.27	102	1.00	-3.42	96	1.00
8	-3.33	100	1.00	-3.37	98	1.00
Mean	-3.32	100	1.00	-3.40	97	1.00

The mean PCR efficiencies of the GM and species-specific methods were equal or above 90 % (90 % for NK603, 98 % for T25 and 100 % for the DAS-40278-9 systems, respectively). The mean PCR efficiencies were 96-97 % for the *hmg* maize-specific reference method. The mean R^2 coefficient of the methods was 1.00 for all systems in all cases. The data presented in Tables 3, 4 and 5 confirm the appropriate performance characteristics of the three methods when tested on GM stack NK603 x T25 x DAS-40278-9 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, RSD_r %) of the three methods applied to samples of DNA extracted from GM stack NK603 x T25 x DAS-40278-9 maize see Tables 6, 7 and 8.

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSDr %) of the NK603 method applied to genomic DNA extracted from GM stack NK603 x T25 x DAS-40278-9 maize.

NK603					
Unknown sample GM %	**Expected value (GMO %)**				
	0.10	**0.50**	**0.90**	**2.0**	**5.0**
Mean	0.10	0.48	0.85	1.8	4.6
SD	0.01	0.03	0.03	0.11	0.14
RSD_r (%)	10	5.4	3.6	6.2	3.1
Bias (%)	2.5	-3.5	-5.6	-10	-7.4

Table 7. 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 x T25 x DAS-40278-9 maize.

T25					
Unknown sample GM %	**Expected value (GMO %)**				
	0.15	**0.40**	**0.90***	**2.0**	**3.3**
Mean	0.13	0.38	0.79	1.9	3.0
SD	0.01	0.02	0.03	0.09	0.08
RSD_r (%)	8.9	4.3	3.7	4.7	2.7
Bias (%)	-16	-4.0	-12	-3.7	-8.1

*based on 15 replicates

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSDr %) of the DAS-40278-9 method applied to genomic DNA extracted from GM stack NK603 x T25 x DAS-40278-9 maize.

DAS-40278-9					
Unknown sample GM %	**Expected value (GMO %)**				
	0.10	**0.50**	**0.90**	**2.0**	**5.0**
Mean	0.08	0.40	0.85	2.1	4.8
SD	0.01	0.02	0.03	0.10	0.12
RSD_r (%)	6.9	5.9	3.2	4.9	2.5
Bias (%)	-16	-21	-5.7	6.1	-3.4

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 6-8, the values range from -10 % to 2.5 % for NK603, from -16 % to -3.7 % for T25 and from -21 % to 6.1 % for DAS-40278-9. Therefore, the three methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack NK603 x T25 x DAS-40278-9 maize.

Tables 6-8 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 3.1 % and 10 % for NK603, between 2.7 % and 8.9 % for T25 and between 2.5 % and 6.9 % for DAS-40278-9, the three methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack NK603 x T25 x DAS-40278-9 maize.

5. Conclusions

The performance of the three event-specific methods for the detection and quantification of maize single line events NK603, T25 and DAS-40278-9, when applied to genomic DNA

extracted from GM stack NK603 x T25 x DAS-40278-9 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 NK603, T25 and DAS-40278-9, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack NK603 x T25 x DAS-40278-9 maize or any of its sub-combinations, provided that sufficient genomic DNA of appropriate quality is available.

6. References

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