European Commission

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of MON 89034, TC1507, NK603 and DAS-40278-9 event-specific PCR-based Methods applied to DNA extracted from GM Stack MON 89034 x TC1507 x NK603 x DAS-40278-9 Maize

European Union Reference Laboratory for Genetically Modified Food and Feed

2016

Joint Research Centre

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This publication is a Validated Methods, Reference Methods and Measurements Report
by the Joint Research Centre of the European Commission.

JRC101373

Abstract

An application was submitted by Dow AgroSciences LLC to request the authorisation of genetically modified stack (GM stack) MON 89034 x TC1507 x NK603 x DAS-40278-9 maize 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) No 1829/2003 on GM Food and Feed. The unique identifier assigned to GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize is MON-89Ø34-3 x DAS-Ø15Ø7 x MONØØ6Ø3-6 x DAS4Ø278-9.

The GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize has been obtained from traditional breeding methods between progeny of the four genetically modified maize single events MON 89034, TC1507, NK603 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 MON 89034, TC1507, NK603 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 the in-house verification of the performance of each validated method when applied to genomic DNA extracted from GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize. Deviations from the original methods were observed and are reported in the dedicated paragraphs of this document.

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 89034 x TC1507 x NK603 x DAS-40278-9 maize.

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10 June 2016

Executive Summary

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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) - 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.

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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 method, if the method has previously been validated by the EURL GMFF for the parental single-line event and the events have been stacked by conventional crossing. These criteria are met for the GM stack MON 89034 x TC1507 x NK603 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 (EC) No 641/2004 (Annex I).

The results of the *in-house* verification study were evaluated with reference to the ENGL method performance requirements[3] and to validation results on the individual events.

2. Step 1 (dossier reception and acceptance)

Dow AgroSciences LLC submitted the detection methods, data demonstrating their adequate performance when applied to genomic DNA extracted from stack, and the corresponding control sample of DNA extracted from GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize and from conventional 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 RSD_r %) calculated by the applicant for the four methods applied to MON 89034 x TC1507 x NK603 x DAS-40278-9 maize genomic DNA. Means are the average of fifteen replicates obtained through one run performed with ABI Prism® 7900HT for TC1507 and DAS-40278-9, with ABI7500 Fast real-time PCR system standard mode for NK603, and with Agilent Mx3005P™ QPCR system for MON 89034. Percentages are expressed as GM DNA/ total DNA x 100.

Note: Numerical values presented in the following tables (except performance data reported by the applicant) 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, RSD_r %) reported by the applicant for the MON 89034, TC1507, NK603 and DAS-40278-9 methods applied to GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize.

MON 89034 (w/w)						
Unknown sample GM%	**Expected value (GMO %)**					
	0.085	**0.90**	**5.0**	**10**		
Mean	0.081	0.805	4.10	9.47		
RSD_r (%)	10.9	12.5	13.4	7.8		
Bias (%)	-4.7	-10.6	-18	-5.3		
TC1507 (HGE)*						
Unknown sample GM%	**Expected value (GMO %)**					
	0.085	**0.90**	**2.0**	**5.0**		
Mean	0.088	0.957	2.27	5.03		
RSD_r (%)	14.3	11.0	9.7	5.2		
Bias (%)	3.5	6.3	13.5	0.60		
NK603 (w/w)						
Unknown sample GM%	**Expected value (GMO %)**					
	0.085	**0.10**	**0.49**	**0.98**	**1.96**	**4.91**
Mean	0.075	0.090	0.400	0.905	1.79	4.70
RSD_r (%)	15.3	13.3	5.3	5.6	7.3	4.5
Bias (%)	-11.8	-10.0	-18.4	-7.7	-8.7	-4.3
DAS-40278-9 (HGE)*						
Unknown sample GM%	**Expected value (GMO %)**					
	0.080	**0.90**	**2.0**	**5.0**		
Mean	0.070	0.881	2.01	4.93		
RSD_r (%)	11.0	3.9	4.0	4.3		
Bias (%)	-12.5	-2.1	0.5	-1.4		

*hge: haploid genome equivalents

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].

Several requests for complementary information regarding purity of control samples, reagents quantity and expiration dates, naming of documentation files, DNA quality, and DNA sequences were addressed to and responded by the applicant. The EURL GMFF verified the data and the complementary information received and accepted the 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 four methods in its own laboratory and performed a verification of their performance when applied to DNA extracted from GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize.

4.1 Materials

The following control samples were provided by the applicant:

- Genomic DNA from homogenized seeds of GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize;
- Genomic DNA from homogenized seeds of conventional (non-GM) maize

The EURL GMFF prepared test samples of different GMO concentrations by mixing DNA extracted from GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize with the non-GM maize DNA, in a constant amount of total maize DNA.
Table 2 shows the five GM concentrations used in the verification of the MON 89034, TC1507, NK603 and DAS-40278-9 methods when applying them to genomic DNA extracted from the GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize. These are the same concentrations used in the validation of these methods for the parental single line GMOs, except for NK603 where the concentrations used in the single line are reported with an asterisk (*).

Table 2. Percentage of MON 89034, TC1507, NK603 and DAS-40278-9 in MON 89034 x TC1507 x NK603 x DAS-40278-9 stack genomic DNA contained in the verification samples.

MON 89034 GM% (GM DNA/ total maize DNA x 100)	**TC1507 GM%** (GM DNA/ total maize DNA x 100)	**NK603* GM%** (GM DNA/ total maize DNA x 100)	**DAS-40278-9 GM%** (GM DNA/ total maize DNA x 100)
0.09	0.10	0.10	0.10
0.40	0.50	0.50	0.40
0.90	0.90	0.90	0.90
3.00	2.00	2.00	2.00
8.00	5.00	5.00	5.00

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

The protocols validated for the individual GM events MON 89034, TC1507, NK603 and DAS-40278-9 (available athttp://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx), were followed in the *in-house* verification with the deviation 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 for the purpose of providing genomic 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/TCTC1507-DNAextrc.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 maize reference system *hmg*, maize *high mobility group*. 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 89034, TC1507, NK603 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). An Excel spreadsheet was used for determination of the GM %.

4.4 PCR methods

During the verification study, the EURL GMFF carried out tests on DNA extracted from GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize using the single detection methods previously validated for the respective single GM events MON 89034, TC1507, NK603 and DAS-40278-9, unless otherwise stated (refer to paragraph 4.5 deviations from validated methods).

For detection of GM maize events MON 89034, TC1507, NK603 and DAS-40278-9, DNA fragments of 77-bp, 58-bp, 108-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 (6-carboxytetramethylrhodamine) or in the case of MON 89034, MGBNFQ (minor groove binder non-fluorescent quencher), as a quencher dye at their 3'-end.

For quantification of GM maize events MON 89034, TC1507, NK603 and DAS-40278-9, a taxon-specific reference system amplifies a 79-bp fragment of high mobility group *hmg* maize endogenous gene (GenBank AJ131373.1), using two *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM and TAMRA.

For quantification of GM maize events MON 89034, TC1507, NK603 and DAS-40278-9, standard curves are generated both for the MON 89034, TC1507, NK603 and DAS-40278-9 and for the *hmg* specific system by plotting Cq values of the calibration standards against the logarithm of the DNA copy numbers and by fitting a linear regression into these data. Thereafter, the normalised Cq values of the unknown samples are measured and, by means of the regression formula, the relative amounts of MON 89034, TC1507, NK603 and DAS-40278-9 DNA are 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 verification of the GM event NK603 was done by the EURL GMFF using *hmg* as the reference system in a final reaction volume of 25 µl for both the GM and the reference system in line with the NK603 protocol already modified and verified in the context of the maize stacked event verification (bridging study) EURL-VL-01/11VR, (page 19/19 of http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-TC1507-59122-MON810-NK603%20.pdf).
The NK603 method was modified in EURL-VL-01/11VR following the modularity principle: the NK603 quantification was performed relative to the validated maize reference system *hmg* in substitution of the maize reference system *adh*1 that was originally validated but lately demonstrated to be suboptimal for quantitative purposes (pages 10-11/14 of http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-03-10-VR.pdf). The use of *hmg* was suggested by the applicant as well, in substitution of the previously validated *adh*1 reference system.

For the MON 89034-method, the buffer suggested by the applicant to be used with the *hmg* reference system is discontinued (TaqMan® buffer A, Life Technologies). Therefore, the EURL GMFF decided to maintain all conditions in accordance with the initial validation, i.e. primers and probes, volume and cycling conditions, but to use TaqMan® Universal Master Mix. In fact,

the EURL GMFF implemented the *hmg* reference system as validated in the context of the dossier EURL-VL-04/09VP (page 8/10 of http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27_MON87460_validated_Method.pdf).

4.5 Results

Tables 3, 4, 5 and 6 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] x 100, and of the the coefficient of determination (R2) reported for all PCR systems in the eight runs, for GM maize events MON 89034, TC1507, NK603 and DAS-40278-9.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 89034 method on GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize.

Run	MON 89034			*hmg**		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.51	93	1.00	-3.33	100	1.00
2	-3.49	93	1.00	-3.36	98	1.00
3	-3.45	95	1.00	-3.38	98	1.00
4	-3.53	92	1.00	-3.40	97	1.00
5	-3.45	95	1.00	-3.32	100	1.00
6	-3.43	96	1.00	-3.34	99	1.00
7	-3.47	94	1.00	-3.38	98	1.00
8	-3.48	94	1.00	-3.37	98	1.00
Mean	-3.48	94	1.00	-3.36	98	1.00

* method validated in the context of EURL-VL-04/09VP http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27_MON87460_validated_Method.pdf

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the TC1507 method on GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize.

Run	TC1507			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.14	108	0.99	-3.22	104	1.00
2	-3.24	104	0.99	-3.32	100	1.00
3	-3.16	107	0.99	-3.21	105	1.00
4	-3.07	111	1.00	-3.26	103	1.00
5	-3.21	105	0.99	-3.27	102	1.00
6	-3.23	104	1.00	-3.25	103	1.00
7	-3.11	110	0.99	-3.24	104	1.00
8	-3.22	104	0.99	-3.27	102	1.00
Mean	-3.17	107	0.99	-3.26	103	1.00

Table 5. Values of standard curve slope, PCR efficiency and linearity (R^2) for the NK603 method on GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize.

Run	NK603			*hmg***		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.68	87	1.00	-3.43	96	1.00
2	-3.75	85	1.00	-3.45	95	1.00
3	-3.71	86	1.00	-3.43	96	1.00
4	-3.81	83	1.00	-3.43	96	1.00
5	-3.61	89	1.00	-3.41	96	1.00
6	-3.71	86	1.00	-3.43	96	1.00
7	-3.72	86	1.00	-3.40	97	1.00
8	-3.70	86	1.00	-3.45	95	1.00
Mean	-3.71	86	1.00	-3.43	96	1.00

**method validated in the context of EURL-VL-01/11VR (http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-TC1507-59122-MON810-NK603%20.pdf).

Table 6. Values of standard curve slope, PCR efficiency and linearity (R^2) for the DAS-40278-9 method on GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize.

Run	DAS-40278-9			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.24	103	0.99	-3.26	103	1.00
2	-3.26	103	1.00	-3.28	102	1.00
3	-3.31	101	1.00	-3.23	104	1.00
4	-3.23	104	1.00	-3.36	99	1.00
5	-3.28	102	0.99	-3.34	99	1.00
6	-3.35	99	1.00	-3.29	101	1.00
7	-3.26	102	1.00	-3.27	102	1.00
8	-3.27	102	1.00	-3.32	100	1.00
Mean	-3.28	102	1.00	-3.29	101	1.00

The mean PCR efficiencies of the four GM systems were 94 % for MON 89034, 107 % for TC1507, and 102 % for the DAS-40278-9 and 86 % for NK603. The value for NK603, being slightly below 90 %, was deemed acceptable as it was in-line with previous verifications performed by the EURL-GMFF (e.g. http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-01-11-VR-TC1507-59122-MON810-NK603%20.pdf). The efficiency of the maize reference system (*hmg*) ranged between 96 % and 101 %. The mean coefficient of determination of all methods (R^2) was 1.00, apart from the TC1507 GM system which had a (R^2) of 0.99. The data presented in Tables 3 to 6 confirm the appropriate performance of the four methods when tested on GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 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 four methods applied to samples of DNA extracted from GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize. Tables 7 to 10 report the trueness and precision for each GM level for each of the four methods.

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 89034 x TC1507 x NK603 x DAS-40278-9 maize.

MON 89034					
Unknown sample GM%	**Expected value (GMO %)**				
	0.09	**0.40**	**0.90**	**3.0**	**8.0**
Mean	0.09	0.39	0.90	2.96	7.78
SD	0.01	0.03	0.04	0.12	0.33
RSD_r (%)	12	6.6	5.0	4.2	4.2
Bias (%)	-3.8	-3.5	-0.25	-1.5	-2.8

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the TC1507 method applied to genomic DNA extracted from GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize.

TC1507					
Unknown sample GM%	**Expected value (GMO %)**				
	0.10	**0.50**	**0.90**	**2.0**	**5.0**
Mean	0.08	0.43	0.78	1.81	4.82
SD	0.01	0.04	0.05	0.19	0.36
RSD_r (%)	13	10	6.3	10	7.5
Bias (%)	-20	-15	-13	-10	-3.6

Table 9. 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 89034 x TC1507 x NK603 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	0.46	0.88	1.79	4.73
SD	0.01	0.02	0.03	0.05	0.21
RSD_r (%)	8.5	4.0	3.3	3.0	4.5
Bias (%)	3.4	-7.4	-2.2	-11	-5.3

Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the DAS-40278-9 method applied to genomic DNA extracted from GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize.

DAS-40278-9					
Unknown sample GM%	**Expected value (GMO %)**				
	0.10	**0.40**	**0.90**	**2.0**	**5.0**
Mean	0.09	0.39	0.84	1.98	5.04
SD	0.01	0.02	0.03	0.08	0.22
RSD_r (%)	12	6.0	3.3	4.3	4.4
Bias (%)	-14	-2.6	-6.3	-0.86	-0.82

The trueness of the method is estimated using the measurements of the method bias for each GM level. According to the ENGL, the trueness of the method should be ±25 % across the entire dynamic range. As shown in Tables 7 to 10, the values range from -3.8 % to -0.25 % for MON 89034, from -20 % to -3.6 % for TC1507, from -11 % to 3.4 % for NK603 and from -14 % to -0.82 % for DAS-40278-9. Therefore, all four methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to genomic DNA extracted from GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize.

Tables 7 to 10 also show the relative repeatability standard deviation (RSD_r) as estimated for each GM level. According to the ENGL, the RSD_r values should be below 25 %. As the values range between 4.2 % and 12 % for MON 89034, between 6.3 % and 13 % for TC1507, between 3.0 % and 8.5 % for NK603, and between 3.3 % and 12 % for DAS-40278-9, the four methods satisfy this requirement throughout their respective dynamic ranges when applied to genomic DNA extracted from GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 maize.

5. Conclusions

The performance of the four event-specific methods for the detection and quantification of maize events MON 89034, TC1507, NK603 and DAS-40278-9, when applied to the control samples provided by the applicant, i.e. genomic DNA extracted from GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9, meets ENGL performance requirements.

Therefore these methods, developed and validated to detect and quantify the single maize events MON 89034, TC1507, NK603 and DAS-40278-9, can be equally applied for the detection and quantification of the respective events combined in GM stack MON 89034 x TC1507 x NK603 x DAS-40278-9 or any of its sub-combinations, provide sufficient genomic DNA at appropriate quality is available.

References

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European Commission
Joint Research Centre – Institute for Health and Consumer Protection

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Author(s): European Union Reference Laboratory for GM Food and Feed

2016 – 18 pp. – 21.0 x 29.7 cm

JRC101373

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