

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of GA21 and T25 event- specific PCR-based Methods applied to DNA extracted from GM Stack GA21 x T25 maize

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Report on the Verification of the Performance of GA21 and T25 event-specific PCR-based Methods applied to DNA extracted from GM Stack GA21 x T25 maize

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European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Syngenta Crop Protection NV/SA, to request the authorisation of genetically modified stack (GM stack) GA21 x T25 maize (the intended function of the genetic modification of GA21 x T25 maize is to facilitate the control of weeds by conferring tolerance to glyphosate and glufosinate-ammonium in herbicide products) 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 GA21 x T25 maize is MON-ØØØ21-9 X ACS-ZMØØ3-2.

The GM stack GA21 x T25 maize has been obtained by conventional crossing between *the* genetically modified maize events: GA21 and T25, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events GA21 and T25 (see <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EURL GMFF has carried out only an *in-house* verification of the performance of each validated method when applied to genomic DNA extracted from GM stack GA21 x T25 maize.

The results of the *in-house* verification led to the conclusion that the individual methods meet the ENGL performance criteria also when applied to genomic DNA extracted from the GM stack GA21 x T25 maize.

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

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Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: 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/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 GA21 x T25 maize.

Upon reception of methods, samples and related data (step 1), the EURL GMFF carried out the assessment of the documentation (step 2) and the *in-house* verification of the methods (step 3) according to the requirements of Regulation (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)

Syngenta Crop Protection NV/SA 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 GA21 x T25 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. The applicant supplemented the JumpStart™ Taq Ready Mix 2x with sulforhodamine at 600 nM and 11 mM MgCl₂ (final concentration) for the GA21 event-specific method in order to get passive reference fluorescence values clearly above the background.

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSD_r %) calculated by the applicant for the two methods applied to GA21 x T25 maize genomic DNA. Means are the average of sixteen replicates obtained through eight runs performed with 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 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 %) provided by the applicant for the GA21 and T25 methods applied to GM stack GA21 x T25 maize.

GA21 *					
Sample GM %	Expected value (GMO %)				
	0.08	0.5	0.9	2.0	5.0
Mean	0.08	0.50	0.95	2.21	5.67
RSD _r (%)	14.5	15.2	10.9	15.4	8.8
Bias (%)	0.0	0.4	5.4	10.5	13.4
T25 *					
Sample GM %	Expected value (GMO %)				
	0.08	0.5	0.9	2.0	5.0
Mean	0.08	0.55	0.96	2.25	5.27
RSD _r (%)	14.1	7.5	9.2	7.6	11.7
Bias (%)	3.8	9.4	7.0	12.5	5.4

* 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⁾.

One request of complementary information regarding the method and control samples was addressed to the applicant. The EURL GMFF verified the data and the complementary information received and accepted the received clarifications as satisfactory.

The dossier was therefore moved to step 3.

4. Step 3 (EURL GMFF experimental testing)

In step 3 the EURL GMFF implemented the two methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack GA21 x T25 maize.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA from seeds of GM stack GA21 x T25 maize, hemizygous for the loci, as positive control sample.
- genomic DNA from seeds of conventional (non-GM) near-isogenic maize, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack GA21 x T25 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. Table 2 shows the five GM concentrations used in the verification of the GA21 and T25 methods when applying them to genomic DNA extracted from the GM stack GA21 x T25 maize.

Table 2. Percentage (GM %) of GA21 and T25 in GA21 x T25 stack genomic DNA contained in the verification samples.

GA21 GM %* [(GM DNA / total maize DNA x 100)]	T25 GM %* [(GM DNA / total maize DNA x 100)]
0.05	0.15
0.50	0.40
0.90	0.90
5.0	2.0
8.0	3.3

* percentage expressed in copy number ratio.

The protocols described by the applicant were implemented in the EURL GMFF laboratory and were in accordance with the protocols already published for the individual GA21 and T25 GM events (available at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). The concentration of sulforhodamine was increased in the GA21 method (§4.5).

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 GA21 and T25 in the combined trait product GA21 × T25 maize have been validated in-house using DNA derived from homogenized maize seeds; however, in principle, the method can be applied to any sample from which sufficient quantities of inhibitor-free genomic maize DNA can be purified.

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/MIR604_DNAExtr.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 system *adh1* (alcohol dehydrogenase 1). 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 GA21 and T25, 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 GA21 x T25 maize using the single detection methods previously validated for the respective single GM events GA21 and T25.

For detection of GM maize events GA21 and T25, DNA fragments of 101-bp and 102-bp respectively are amplified using specific primers. PCR products are measured during each cycle (real-time) by means of target-specific oligonucleotide probes labelled with two fluorescent dyes: FAM™ (6-carboxyfluorescein) as reporter dye at their 5'-end and TAMRA™ (6-carboxytetramethylrhodamine) as a quencher dye at their 3'-end for the both events.

For quantification of GM maize event GA21 and T25, a taxon-specific reference system amplifies a 135-bp fragment of alcohol dehydrogenase 1 (*adh1*) a maize endogenous gene (NCBI accession number, AY691949.1), using two alcohol dehydrogenase 1 gene-specific primers and a gene-specific probe labelled with VIC® and TAMRA™ for the relative quantification of event GA21 and labelled with FAM™ and TAMRA™ for the relative quantification of event T25.

For relative quantification of GM maize event GA21 and T25 DNA in a test sample, the ΔC_q values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔC_q values against the logarithm of the relative amount of GA21 and T25 event DNA, respectively). The ΔC_q values of the unknown samples are measured and, by means of the regression formula, the relative amount of GA21 and T25 events 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 Sulforhodamine concentration specified in the protocol for the GA21 event-specific PCR method previously validated (http://gmo-crl.jrc.ec.europa.eu/summaries/GA21Syngenta_validated_Method_correctedVersion1.pdf) was doubled following the modification introduced by the applicant (§ 3).

The sulforhodamine concentration used in the verification of the performance of the maize GA21 event-specific method in the stacked GA21 x T25 corresponds to the one validated in the context of maize Bt11 (http://gmo-crl.jrc.ec.europa.eu/summaries/Bt11_CRLVL1007_Validated_Method%20doc.pdf) and MIR162 (http://gmo-crl.jrc.ec.europa.eu/summaries/MIR162_validated_Method.pdf) event-specific methods; the Sigma Jumpstart Ready Mix (2X) contained 20 µl instead of 10 µl of 10000x Sulforhodamine 101.

4.5 Results

Tables 3 and 4 present the values of the slopes of the different standard curves generated by the EURL GMFF when using DNA extracted from the GM stack, from which the PCR efficiency is calculated using the formula $[10^{(-1/\text{slope})} - 1] \times 100$, and of the coefficient of determination (R^2) reported for all PCR systems in the eight runs, for GM maize events GA21 and T25. Slope values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and R^2 coefficient for the GA21 method on GM stack GA21 x T25 maize.

Run	GA21		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.23	104	1.00
2	-3.23	104	1.00
3	-3.21	105	1.00
4	-3.21	105	1.00
5	-3.17	107	1.00
6	-3.26	103	1.00
7	-3.17	107	1.00
8	-3.26	103	1.00
Mean	-3.22	105	1.00

Table 4. Values of standard curve slope, PCR efficiency and R^2 coefficient for the T25 method on GM stack GA21 x T25 maize.

Run	T25		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.47	94	1.00
2	-3.58	90	1.00
3	-3.47	94	1.00
4	-3.21	105	0.99
5	-3.42	96	1.00
6	-3.42	96	1.00
7	-3.39	97	1.00
8	-3.33	100	1.00
Mean	-3.41	97	1.00

The mean PCR efficiencies of the GM and species-specific systems were above 90 % (105 % for GA21 and 97 % for T25, respectively). The mean R^2 coefficient of the methods was 1.00

for both systems. The data presented in Tables 3 and 4 confirm the appropriate performance characteristics of the two methods when tested on GM stack GA21 x T25 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 two methods applied to samples of DNA extracted from GM stack GA21 x T25 maize see Tables 5 and 6.

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the GA21 method applied to genomic DNA extracted from GM stack GA21 x T25 maize.

GA21					
Unknown sample GM %	Expected value (GMO %)				
	0.09	0.50	0.90	5.0	8.0
Mean	0.09	0.49	0.87	5.2	8.0
SD	0.01	0.06	0.07	0.38	0.62
RSD_r (%)	10	12	8.2	7.2	7.8
Bias (%)	-1.8	-1.2	-3.0	4.1	-0.46

Table 6. 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 GA21 x T25 maize.

T25					
Unknown sample GM %	Expected value (GMO %)				
	0.15	0.40	0.90	2.0	3.3
Mean	0.14	0.37	0.78	1.8	2.9
SD	0.02	0.03	0.05	0.15	0.21
RSD_r (%)	14	7.4	6.8	8.3	7.3
Bias (%)	-8.4	-7.9	-13	-8.5	-11

The trueness of the method is estimated using the measurements of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method should be less or equal to ± 25 % across the entire dynamic range. As shown in Tables 5 and 6, the values range from -3.0 % to 4.1 % for GA21 and from -13 % to -7.9 % for T25. Therefore, the two methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack GA21 x T25 maize.

Tables 5 and 6 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 7.2 % and 12 % for GA21 and between 6.8 % and 14 % for T25, the two methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack GA21 x T25 maize.

5. Conclusions

The performance of the two event-specific methods for the detection and quantification of maize single line events GA21 and T25, when applied to genomic DNA extracted from GM stack GA21 x T25 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 GA21 and T25, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack GA21 x T25 maize, supposed that sufficient genomic DNA of appropriate quality is available.

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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