

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of FG72 and A5547-127 event-specific PCR- based Methods applied to DNA extracted from GM Stack FG72 x A5547-127 Soybean

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 Bayer CropScience AG to request the authorisation of genetically modified stack (GM stack) FG72 x A5547-127 soybean (containing genes for tolerance to glyphosate, isoxaflutole and glufosinate ammonium) 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 GM Feed. The unique identifier assigned to GM stack FG72 x A5547-127 is MST-FGØ72-2 x ACS-GMØØ6-4.

The GM stack FG72 x A5547-127 soybean has been obtained by conventional crossing between the genetically modified soybean events: FG72 and A5547-127, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events FG72 and A5547-127 (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](http://gmo-crl.jrc.ec.europa.eu/doc/Min%20Perf%20Requirements%20Analytical%20methods.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 FG72 x A5547-127 soybean.

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 FG72 x A5547-127 soybean.

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: 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 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 method 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 FG72 x A5547-127 soybean.

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)

Bayer CropScience AG submitted the detection methods, the 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 soybean FG72 x A5547-127 and from non-GM soybean.

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 two methods applied to FG72 x A5547-127 soybean genomic DNA. Means are the average of eighteen replicates obtained through three runs performed with 7900HT 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 FG72 and A5547-127 methods applied to GM stack FG72 x A5547-127 soybean.

FG72*			
Sample* GM%	Expected value (GMO %)		
	0.08	0.9	4.5
Mean	0.077	0.93	4.43
RSD _r (%)	7.79	6.12	5.92
Bias (%)	7.45	3.00	2.59
A5547-127*			
Sample GM%	Expected value (GMO %)		
	0.08	0.9	4.5
Mean	0.075	0.86	4.40
RSD _r (%)	9.84	7.47	3.99
Bias (%)	5.87	5.70	5.27

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

The applicant expressed the relative method repeatability as a RSD (relative standard deviation): every set of replicates (triplicates) yielded a variance estimate (standard deviation) and their mean was used to calculate the RSD_r. The applicant expressed the bias % as the absolute value of the mean deviation from the reference value.

The EURL GMFF verified the data and concluded that they seemed to confirm that the methods meet the ENGL performance criteria ⁽³⁾.

Two requests of complementary information regarding 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 two methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack FG72 x A5547-127.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from leaves of GM stack FG72 x A5547-127 soybean, homozygous for the loci FG72 and A5547-127, as positive control sample.
- genomic DNA extracted from leaves of conventional (non-GM) soybean, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack FG72 x A5547-127 soybean with the non-GM soybean genomic DNA, in a constant amount of total soybean 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 FG72 and A5547-127 methods when applying them to genomic DNA extracted from the GM stack FG72 x A5547-127 soybean.

Table 2. Percentage (GM %) of FG72 and A5547-127 in FG72 x A5547-127 stack genomic DNA contained in the verification samples.

FG72 GM%* [[GM DNA / total soybean DNA x 100]]	A5547-127 GM%* [[GM DNA / total soybean DNA x 100]]
0.10	0.08
0.40	0.40
0.90	0.90
3.0	4.0
8.0	8.0

* 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

FG72 and A5547-127 GM events (available at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>).

4.2 DNA extraction

A method for DNA extraction from soybean was previously evaluated by the EURL GMFF with regard to its performance characteristics and was considered valid, i.e. fit the purpose of providing soybean DNA of appropriate quality and amount for being used in subsequent PCR experiments.

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/A2704-12_soybean_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 system *le1*, amplifying a fragment of the lectin gene. 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 FG72 and A5547-127, 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 the 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 FG72 x A5547-127 soybean using the single detection methods previously validated for the respective single GM events FG72 and A5547-127.

For detection of GM soybean events FG72 and A5547-127, DNA fragments of 70-bp and 75-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 FAM (6-carboxyfluorescein) as reporter dye at the 5'-end for FG72 and A5547-127 probes and MGB-NFQ (minor groove binding non-fluorescent quencher) and TAMRA (6-carboxytetramethylrhodamine) as quencher dyes at the 3'-end of FG72 and A5547-127, respectively.

For quantification of GM soybean events FG72 and A5547-127, a taxon-specific reference system amplifies a 102-bp fragment of *lectin (le1)* a soybean endogenous gene (GenBank K00821), using two *le1* gene-specific primers and a gene-specific probe labelled with VIC as reporter and TAMRA as quencher dye.

For the relative quantification of GM soybean events FG72 and A5547-127 standard curves are generated both for the FG72/A5547-127 and for the *le1* specific systems by plotting the 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 FG72 and A5547-127 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

No deviations from the original validated methods were introduced

4.5 Results

Tables 3 and 4 present the values of the slope 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 soybean events FG72 and A5547-127. Slope and R^2 values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and R² coefficient for the FG72 method on GM stack FG72 x A5547-127 soybean.

Run	FG72			<i>le1</i>		
	Slope	PCR Efficiency (%)	R ² coefficient	Slope	PCR Efficiency (%)	R ² coefficient
1	-3.40	97	1.00	-3.36	98	1.00
2	-3.42	96	1.00	-3.50	93	0.99
3	-3.39	97	1.00	-3.53	92	0.99
4	-3.44	95	1.00	-3.43	96	0.99
5	-3.32	100	1.00	-3.36	98	0.99
6	-3.44	95	1.00	-3.44	95	0.99
7	-3.46	94	1.00	-3.46	95	1.00
8	-3.37	98	1.00	-3.45	95	1.00
Mean	-3.40	97	1.00	-3.44	95	1.00

Table 4. Values of standard curve slope, PCR efficiency and R² coefficient for the A5547-127 method on GM stack FG72 x A5547-127 soybean.

Run	A5547-127			<i>le1</i>		
	Slope	PCR Efficiency (%)	R ² coefficient	Slope	PCR Efficiency (%)	R ² coefficient
1	-3.23	104	1.00	-3.24	104	1.00
2	-3.26	102	1.00	-3.29	101	0.99
3	-3.36	98	1.00	-3.27	102	1.00
4	-3.28	102	1.00	-3.36	98	1.00
5	-3.24	103	1.00	-3.26	102	1.00
6	-3.27	102	1.00	-3.32	100	1.00
7	-3.28	102	1.00	-3.29	101	1.00
8	-3.25	103	1.00	-3.33	100	1.00
Mean	-3.27	102	1.00	-3.30	101	1.00

The mean PCR efficiencies were 97% for FG72 and 102% for the A5547-127 system, respectively; the mean PCR efficiencies for the *le1* system were 95% and 101%. The mean R² coefficient of the methods was 1.00 for all systems in all cases. The data presented in Tables 3 and 4 confirm the appropriate performance characteristics of the FG72 and A5547-127 methods when tested on GM stack FG72 x A5547-127 soybean in terms of PCR efficiency and R² 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 methods applied to

samples of DNA extracted from GM stack FG72 x A5547-127 soybean; see tables 5 and 6. Values of standard deviation were rounded to two digits.

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r%) of the FG72 method applied to genomic DNA extracted from GM stack FG72 x A5547-127 soybean.

FG72					
Unknown sample GM%	Expected value (GMO%)				
	0.10	0.40	0.90	3.0	8.0
Mean	0.11	0.44	0.90	3.0	7.7
SD	0.01	0.04	0.07	0.27	0.53
RSD _r (%)	13	9.4	7.5	8.8	7.0
Bias (%)	9.2	9.3	0.17	0.66	-3.9

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r%) of the A5547-127 method applied to genomic DNA extracted from GM stack FG72 x A5547-127 soybean

A5547-127					
Unknown sample GM%	Expected value (GMO%)				
	0.08	0.40	0.90	4.0	8.0
Mean	0.07	0.38	0.87	3.9	8.1
SD	0.01	0.05	0.06	0.26	0.56
RSD _r (%)	7.2	13	7.4	6.7	6.9
Bias (%)	-12	-5.5	-3.1	-3.3	1.5

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 less or equal to $\pm 25\%$ across the entire dynamic range. As shown in Tables 5 and 6, the values range from -3.9% to 9.3% for FG72 and from -12% to 1.5% for A5547-127. Therefore, the two methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack FG72 x A5547-127 soybean.

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 7.0% and 13% for FG72 and between 6.7% and 13% for A5547-127, the two methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack FG72 x A5547-127 soybean.

5. Conclusions

The performance of the two event-specific methods for the detection and quantification of soybean single line events FG72 and A5547-127, when applied to genomic DNA extracted from GM stack FG72 x A5547-127, 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 soybean events FG72 and A5547-127, can be equally applied for the detection and quantification of the respective events in genomic DNA extracted from the GM stack FG72 x A5547-127 soybean, 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 FG72 x A5547-127 soybean.

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.
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