



EUROPEAN COMMISSION  
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection  
Molecular Biology and Genomics Unit



# Event-specific Method for the Quantification of Soybean FG72 Validation Report

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European Union Reference Laboratory for GM Food and Feed  
Joint Research Centre  
Institute for Health and Consumer Protection  
Molecular Biology and Genomics Unit

## Executive Summary

In line with its mandate<sup>1</sup> the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific PCR method for detecting and quantifying soybean event FG72 (unique identifier MST-FGØ72-2). The validation study was conducted according to the EU-RL GMFF validation procedure [<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>] and internationally accepted guidelines<sup>2</sup>, involving 12 laboratories.

In accordance with current EU legislation<sup>1</sup>, Bayer CropScience has provided the detection method and the samples (genomic DNA from soybean seeds harbouring the FG72 event as positive control DNA, genomic DNA from conventional soybean seeds as negative control DNA). The EU-RL GMFF prepared the validation samples (calibration samples and blind samples at test GM percentage [DNA/DNA]), organised an international collaborative study and analysed the results.

The study confirms that the method meets the method performance requirements as established by the EU-RL and the ENGL detailing the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004 and it fulfils the analytical requirements of Regulation (EU) No 619/2011.

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

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<sup>1</sup> Regulation (EC) No 1829/2003 of 22 September 2003 “on genetically modified food and feed” and with Regulation (EC) No 641/2004 of 6 April 2004 “on detailed rules for the implementation of Regulation (EC) No 1829/2003”,

<sup>2</sup> The IUPAC “Protocol for the design, conduct and interpretation of method-performance studies” (Horwitz, 1995); and ISO 5725 (1994).

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## 1. Summary

In line with Regulation (EC) No 1829/2003, Bayer CropScience provided the EU-RL GMFF with an event specific method for the detection and quantification of soybean event FG72 (unique identifier MST-FGØ72-2) together with control samples.

In response to this early submission of a draft dossier (step 1), the EU-RL GMFF started the step-wise validation procedure in advance respect to the declaration of valid application by EFSA (October 2011).

The scientific assessment (step 2) focused on the method performance characteristics assessed against the ENGL method acceptance criteria<sup>3</sup> ([http://gmo-crl.jrc.ec.europa.eu/doc/Min\\_Perf\\_Requirements\\_Analytical\\_methods.pdf](http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) for a summary of method acceptance criteria and method performance requirements). The scientific assessment performed for soybean event FG72 was positively concluded in July 2010.

In step 3 of the validation procedure (Experimental testing), the EU-RL GMFF verified the purity of the control samples provided, conducted the in-house testing of samples and methods. The positive control DNA - submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Reg.(EC) No 1829/2003 – was found contaminated with DNA not related to the specific application. Further to request for replacement of positive control sample, its reception and testing, and following a scientific assessment the sample was considered acceptable (July 2011).

The method characteristics were verified by quantifying five blind GM levels within the range 0.1%-8% on a copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision were within the limits established by the ENGL. In addition, and in line with the requirements of Reg. (EU) No 619/2011, the EU-RL GMFF verified *i)* the zygosity ratio of the positive control sample submitted, by investigating the GM- to reference- target ratio by means of digital PCR, in order to determine the conversion factor between copy numbers and mass fractions; *ii)* the method's precision (relative repeatability standard deviation, RSDr %) at the 0.1% related to mass fraction of GM-material on fifteen replicates. Step 3 was concluded in May 2012.

The collaborative trial (step 4) was organised and took place in August/September 2011. It demonstrated that the method is well suited to analyse DNA, appropriately extracted from food or feed, with regard to identifying the presence of GM event FG72 and is therefore applicable for this purpose.

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<sup>3</sup> EURL/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>)

## 2. Summary of the EU-RL GMFF scientific assessment on applicants' data

Documentation and data provided by the applicant were evaluated by the EU-RL GMFF for compliance with the ENGL method acceptance criteria for the parameters of the calibration curves (slope,  $R^2$  coefficient) used to quantify in three runs three test samples at different GM-levels in haploid genome copy number.

Table 1. Values of slope and  $R^2$  obtained by the applicant

	FG72		<i>le1</i>	
	$R^2$	Slope	$R^2$	Slope
Run 1	1.00	-3.4	1.00	-3.3
Run 2	1.00	-3.4	0.99	-3.4
Run 3	1.00	-3.4	1.00	-3.3

Table 1 indicates that the slope and  $R^2$  coefficient of the standard curves for the GM-system (FG72) and the soybean-specific reference system (*le1*) were within the ENGL acceptance criteria (slope between -3.1 to -3.6 and  $R^2$  coefficient  $\geq 0.98$ ).

Table 2. Mean %, precision and trueness measured at three GM-levels by the applicant

	Test Sample Expected GMO %		
	0.08	0.9	4.5
Mean %	0.07	0.91	4.53
Precision (RSDr %)	15	5	7
Trueness (bias %)	-14	2	7

Table 2 reports precision and trueness for the three GM-levels tested by the applicant. Eighteen values for each GM-level were provided. Both parameters were within the ENGL acceptance criteria (trueness  $\pm 25\%$ , RSDr  $\leq 25\%$  across the entire dynamic range).

### 3. Materials and methods

#### 3.1 DNA extraction

A "Dellaporta-derived" protocol for DNA extraction suitable for the isolation of genomic DNA from ground soybean seeds and grains, submitted by the applicant in support of the validation of the method for detection of event soy A2704-12, was evaluated and tested by the EU-RL GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on testing were published in 2007 at [http://gmo-crl.jrc.ec.europa.eu/summaries/A2704-12\\_soybean\\_DNAExtr\\_report.pdf](http://gmo-crl.jrc.ec.europa.eu/summaries/A2704-12_soybean_DNAExtr_report.pdf). In agreement with the ENGL position, which endorses the modularity principle (see also Annex I to Reg. (EC) No 641/2004), the EU-RL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for analysis of event FG72 soybean given the similarity of the soybean matrix.

#### 3.2 Method protocol for the PCR analysis

The PCR analysis method that was provided by the applicant (see the corresponding Validated Method at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>) and subsequently validated by the EU-RL GMFF is an event-specific, quantitative, real-time TaqMan<sup>®</sup> PCR (Polymerase Chain Reaction) procedure for the determination of the relative content of GM event FG72 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean *le1* (*lectin 1*) specific assay, and the target assay (FG72) are performed in separate wells.

For the detection of GM event FG72, a 70-bp fragment of the region spanning the 3' insert-to-plant junction in soybean FG72 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and MGB-NFQ at its 3' end as quencher dye.

For the relative quantification of GM event FG72, a soybean specific reference system amplifies a 102-bp fragment of *lectin1* (*le1*), a soybean endogenous gene, using *le1* gene-specific primers and a *le1* gene-specific probe labelled with VIC as reporter dye at its 5' end, and TAMRA (carboxytetranethylrhodamine) as quencher at its 3' end.

Standard curves are generated for both the FG72 and the *le1* systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves.

For relative quantification of event FG72 DNA in a test sample, the FG72 copy number is divided by the copy number of the soybean reference gene (*le1*) and multiplied by 100 to obtain the percentage value ( $GM\% = FG72/le1 \times 100$ ).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (nanograms) by the published average 1C value for the soybean genome (1.13 pg) (Arumuganathan & Earle, 1991). The copy number values used in the quantification, the GMO contents of the calibration samples and total DNA quantity used in PCR are listed in Table 3.

Table 3. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	150	37.5	9.4	2.35	0.6
Target taxon <i>le1</i> copies	132743	33186	8296	2074	519
FG72 Soybean GM copies	13274	3319	830	207	52

### ***3.3 EU-RL GMFF experimental testing***

#### **3.3.1 Determination of the zygosity ratio in the positive control sample**

Standard and test samples were prepared at the EU-RL GMFF in terms of GM DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes. Annex II of Reg. (EU) No 619/2011 requires that "when results are primarily expressed as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EU-RL GMFF." In order to satisfy this requirement, the EU-RL GMFF conducted an experimental assessment on the zygosity (GM-target to reference target ratio) in the positive control sample that was submitted by the applicant for the preparation of standard and test items at various GM-concentrations.

The copy number of the FG72 and of the *Le1* targets in the positive control sample submitted by the applicant were determined by digital PCR (dPCR).

Digital PCR experiments were performed on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Genomic DNA was provided by the applicant as positive control sample for FG72 soy event.

Three micrograms of genomic DNA were digested at 37 °C overnight with 20 units of the four-base cutter restriction enzyme Taq I. The latter does not cleave within the annealing sites for the primers of the FG72 or *le1* amplification systems. Taq I restriction sites are located outside the respective targeted sequences. Further to digestion, the DNA was precipitated with ammonium acetate 2.5 M final and two volumes of absolute ethanol. The outcome of enzymatic digestion was

controlled by running approximately 100 nanograms of Taq I digested and 100 nanograms of undigested DNA in comparison with DNA molecular marker in 1% agarose-gel electrophoresis.

Digested template DNA was used in digital PCR experiments. Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix with UNG (Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probe, at the reaction concentrations indicated in the corresponding Validated Method (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>), 1 µL of DNA at a concentration of 0.5 ng/µL, suitable to avoid panel saturation after analysis (optimal between 200<positive partitions<500).

Loading of the digital chip was performed according to the manufacturer's instructions by using the IFC controller (Fluidigm). A volume of 9 µL of reaction mix was loaded into each well of which only approximately 4.6 µL were distributed into the 765 chambers (or partitions) constituting one panel. The analysis was repeated in three days; five replicates in five panels were run each day for both the GM- and reference assay, with a total number of fifteen data sets for both targets. No template controls were included. Amplification conditions were as reported in the Validated Method. Data analysis and copy number calculation was performed using the BioMark digital PCR Analysis software applying the default settings.

Calculations of mean and variances are implemented according to the procedure outlined for random variables in the Annex 4 of the ENGL guidance document "Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods"<sup>4</sup>.

### **3.3.2 In-house verification of the method performance against ENGL method acceptance criteria**

The method performance characteristics were verified (EU-RL GMFF step 3) by quantifying on a copy number basis five blinded test samples with known GM levels, within the range 0.1%-8%. The experiments were performed on an ABI 7900 real-time platform under repeatability conditions. Test samples with GM-levels 8.0%, 3.0%, 0.9% and 0.4% were tested in two real-time PCR runs with two replicates for each GM-level on each plate (total of four replicates per GM-level). The test sample with GM-level 0.1% was tested in 15 replicates in an additional run. Average values of the slope and of the R<sup>2</sup> coefficient of the standard curves and method trueness and precision of quantification over the dynamic range were evaluated for compliance against the ENGL method acceptance criteria.

In order to assess the method compliance to Reg. (EU) No 619/2011, the EU-RL GMFF also implemented tests to determine the zygosity of the GM-insert in the positive control sample and estimated the method precision (RSDr) at 0.1% GM level in mass fraction on 15 replicates.

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<sup>4</sup> Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. European Network of GMO laboratories (ENGL), 2011.  
<http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf>



### ***3.4 International collaborative study***

The international collaborative study (EU-RL GMFF step 4) involved twelve laboratories, all being “National reference laboratories, assisting the CRL for testing and validation of methods for detection”, as listed in annex to Regulation (EC) No 1981/2006. The study was carried out in accordance with the following internationally accepted guidelines:

The IUPAC “Protocol for the design, conduct and interpretation of method-performance studies” (Horwitz, 1995)  
ISO 5725 (1994)

The objective of the international collaborative study was to verify in experienced laboratories the trueness and reproducibility of the PCR analytical method described under 3.2, above, that was provided by the applicant.

#### **3.4.1 List of participating laboratories**

The participants in the FG72 validation study were randomly selected, using a validated software, from within the 35 NRL that offered to participate.

Clear guidance was given to the selected laboratories with regards to the standard operational procedures to follow for the execution of the protocol (the report of the Validated Method is available at, <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>). The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the validation of the detection method for soybean FG72

Laboratory	Country
Bavarian Health and Food Safety Authority	DE
Environment Agency Austria	AT
Food and Environment Research Agency	UK
Genetically Modified Organism Controlling Laboratory	PL
Institute for Consumer Protection, Department 3 - Food Safety - Halle	DE
LGC Limited	UK
National Centre for Food, Spanish Food Safety Agency and Nutrition	ES
National Centre of Public Health Protection - Bulgarian National Laboratory for Genetically Modified Food	BG
Plant Health Laboratory	FR
Science and Advice for Scottish Agriculture (SASA)	UK
State Office for Agriculture, Food Safety and Fisheries	DE
Walloon Agricultural Research Centre (CRA-W)	BE

#### 3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: six laboratories used the ABI 7500, four used the ABI 7900, one used the ABI 7300 and one used the Stratagene Mx 3005.

#### 3.4.3 Materials used in the international collaborative study

For the validation of the quantitative event-specific method, control samples were provided by the EU-RL GMFF to the participating laboratories. They consisted of:

- i)* genomic DNA extracted from homozygous soybean seeds harbouring the event FG72, and
- ii)* genomic DNA extracted from conventional soybean seeds genetically similar to those harbouring the FG72 event.

The control samples were prepared by the EU-RL GMFF from the samples provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11<sup>5</sup>.

<sup>5</sup> Control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample). Reg. (EC) No 1829/2003, Art. 2 (11)

These positive and negative control samples were used by the EU-RL GMFF to prepare standard and test samples (of unknown GM-content), containing mixtures of FG72 soybean and non-GM soybean, as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes. Given the ratio of 1 between GM and non-GM targets in the positive control sample, the same concentration levels of standards and test samples are equally expressed in terms of DNA copy number ratios or DNA mass fractions.

The calibration sample S1 was prepared by mixing the appropriate amount of FG72 DNA in control non-GM soybean DNA to obtain a 10% GM FG72. Calibration samples S2-S5 were prepared by 4-fold dilution from the S1 sample.

The 12 NRLs participating in the validation study received the following materials:

- ✓ Five calibration samples with known concentrations of GM-events (175 µL of DNA solution each) labelled from S1 to S5 (Table 1).
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution each) labelled from U1 to U20, representing 5 GM levels (Table 5).

Table 5. FG72 GM contents in genome copy number and DNA mass

FG72 GM%
GM copy number/soybean genome copy number x 100
8.00
3.00
0.90
0.40
0.10

- ✓ Reaction reagents:
  - Universal PCR Master Mix (2x), one vial: 15 mL
  - distilled sterile water, one vial: 10 mL
- ✓ Primers and probes (1 tube each) as follows:
  - le1* taxon-specific assay
    - KVM164 (10 µM): 160 µL
    - KVM165 (10 µM): 160 µL
    - TM021 (10 µM): 160 µL
  - FG72 assay provided by the applicant
    - MAE071 (10 µM): 320 µL
    - SHA097 (10 µM): 320 µL
    - TM325 (10 µM): 160 µL

#### 3.4.4 Design of the collaborative study

Twenty test samples (labelled from U1 to U20), representing five GM levels, each in 4 replicates, were included in the validation study (Table 3). The detailed design of the PCR plates and other details were communicated to the participating laboratory in a validation protocol. On each PCR plate, the samples were analysed for the FG72 specific system and for the *le1* taxon-specific system. In total, two plates were run per participating laboratory. Participants determined the GM% according to the instructions provided) and using the excel sheet provided.

Each participating laboratory received the above described materials and prepared the master-mixes for the FG72 and lection assay in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per recommended plate lay-out. The amplification reactions followed the cycling program provided. Raw data were reported according to instructions provided on an excel sheet designed, validated and distributed to participating laboratories by the EU-RL GMFF. The original data recorded in the excel sheets were provided to the EU-RL GMFF and back-up copies on CDs were subsequently delivered via mail. All the data received are stored by the EU-RL GMFF on a dedicated and protected server.

The EU-RL GMFF then analysed the data against parameters and limits set by the ENGL: trueness, precision, amplification efficiency and linearity

#### 3.4.5 Deviations reported from the protocol

Nine laboratories reported no deviations from the method protocol

One laboratory sealed the plate with optical cap strips (Applied Biosystems, part number 4323032) instead of adhesive covers.

One laboratory found one of the test tubes empty (U14), corresponding to one replicate for GM-level 8.0%.

Another laboratory, due to pipetting error, could not return the value for U19, corresponding to one replicate for GM-level 3.0%.

## 4. Results

### 4.1 *EU-RL GMFF experimental testing*

Results of the EU-RL GMFF experimental testing were already reported, in chapter 2.

#### 4.1.1 Determination of the zygoty ratio in the positive control sample

The results of the tests to determine the zygoty ratio in the positive control sample are shown in Table 6.

Table 6. Summary of dPCR analysis conducted on the FG72 and *Le1* targets in the positive control sample.

<b>Mean ratio (FG72/<i>Le1</i>)</b>	<b>0.976</b>
Standard deviation	0.064
<b>RSD<sub>r</sub> (%)</b>	<b>6.6</b>
Standard error of the mean	0.017
<b>Upper 95% CI of the mean</b>	<b>1.011</b>
<b>Lower 95% CI of the mean</b>	<b>0.941</b>

In conclusions, the 95% confidence interval (CI) spans around 1 and therefore the mean ratio is not significantly different from an expected ratio of 1, assuming a GM homozygous and a single-copy reference target, for an alpha = 0.05.

Hence, given the above experimental data, in the positive control sample provided by the applicant in support to the validation of the method for detection of FG72 soybean the GM% expressed in GM- DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes equals the GM% expressed in DNA mass fraction:

$$\text{GM \% in DNA copy number ratio} = \text{GM \% in mass fraction}$$

#### 4.1.2 In-house verification of method performance against ENGL method acceptance criteria

Test samples with GM-levels 8.0%, 3.0%, 0.9% and 0.4% were tested in two real-time PCR runs (run A and B) with two replicates for each GM-level on each plate (total of four replicates per GM-level). The test sample with GM-level 0.1% was tested in 15 replicates in one run (run C). Results are shown in Table 7 and 8.

Table 7. Standard curve parameters

	FG72-system			Lec reference system		
	Slope	PCR efficiency*	R <sup>2</sup>	Slope	PCR efficiency*	R <sup>2</sup>
Run A	-3.377	97.8	0.999	-3.321	100.1	0.997
Run B	-3.250	103.1	0.998	-3.412	96.4	0.999
Run C	-3.404	96.7	0.998	-3.292	101.3	0.995

\* PCR efficiency (%) is calculated using the formula  $\text{Efficiency} = (10^{(-1/\text{slope})} - 1) \times 100$

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R<sup>2</sup> coefficient shall be  $\geq 0.98$ .

Table 7 documents that that the slope of the standard curve, and the R<sup>2</sup> coefficient were within the limits established by the ENGL.

Table 8. Outcome of the in-house verification, with regards to the quantification of the five test samples

GM-levels %	Measured GM %	Bias %	Precision (RSDr %)
8.0	8.56	6.9	1.2
3.0	2.96	-1.3	11.1
0.9	0.85	-5.5	11.2
0.4	0.38	-5.3	4.6
0.1	0.10	-0.5%	18%

According to the ENGL method acceptance criteria, the method trueness (measured as bias %) should be within  $\pm 25\%$  of the accepted reference value over the dynamic range. The method's precision as RSDr % should be  $\leq 25\%$  over the dynamic range. Table 8 documents that trueness (estimated through bias %) and precision (estimated through RSDr, relative standard deviation of repeatability) of quantification were within the limits established by the ENGL.

## 4.2 Results of the international collaborative study

### 4.2.1 PCR efficiency and linearity

Standard curve slopes [from which the PCR efficiency (%) is calculated using the formula  $\text{Efficiency} = (10^{(-1/\text{slope})} - 1) \times 100$ , and R<sup>2</sup> values (expressing the linearity of the regression) reported by participating laboratories for the FG72 and the *le1* assays are reported in Table 9.

Table 9. Values of slope, PCR efficiency and R<sup>2</sup> obtained during the validation study

Lab	Plate	FG72			<i>le1</i>		
		Slope	PCR Efficiency (%)	R <sup>2</sup>	Slope	PCR Efficiency (%)	R <sup>2</sup>
1	A	-3.41	97	1.00	-3.44	95	1.00
	B	-3.35	99	1.00	-3.46	94	1.00
2	A	-3.42	96	1.00	-3.65	88	1.00
	B	-3.48	94	1.00	-3.54	91	1.00
3	A	-3.60	90	1.00	-3.77	84	1.00
	B	-3.44	95	1.00	-3.66	88	1.00
4	A	-3.53	92	0.99	-3.60	90	1.00
	B	-3.45	95	1.00	-3.64	88	1.00
5	A	-3.45	95	1.00	-3.47	94	1.00
	B	-3.37	98	1.00	-3.50	93	1.00
6	A	-3.44	95	1.00	-3.75	85	0.99
	B	-3.40	97	1.00	-3.85	82	1.00
7	A	-3.45	95	1.00	-3.57	91	1.00
	B	-3.47	94	1.00	-3.55	91	1.00
8	A	-3.42	96	1.00	-3.68	87	1.00
	B	-3.40	97	1.00	-3.58	90	1.00
9	A	-3.49	94	1.00	-3.55	91	1.00
	B	-3.45	95	1.00	-3.48	94	1.00
10	A	-3.56	91	1.00	-3.56	91	1.00
	B	-3.43	96	1.00	-3.52	92	1.00
11	A	-3.58	90	1.00	-3.73	85	1.00
	B	-3.50	93	1.00	-3.59	90	1.00
12	A	-3.60	90	1.00	-3.65	88	1.00
	B	-3.50	93	1.00	-3.60	90	1.00
<b>Mean</b>		<b>-3.47</b>	<b>94</b>	<b>1.00</b>	<b>-3.60</b>	<b>90</b>	<b>1.00</b>

Table 9 indicates that the efficiency of amplification for the FG72 system ranges from 90 to 99% with a linearity of 1.00 and the amplification efficiency for the soy-specific reference system ranges from 82% to 95% with a linearity of 1.00. The mean PCR efficiency was 94% for the FG72 assay and 90% for the *le1* assay, with both values within the ENGL acceptance criteria. The R<sup>2</sup> of the methods was 1.00 both for FG72 and *le1* assays.

The results confirm the appropriate performance characteristics of the method tested in terms of efficiency and linearity.

#### 4.2.2 GMO quantification

Table 10 reports the mean values of the four replicates for each GM level as provided by all laboratories, before application of the Cochran and Grubbs tests for identification of outlying values, as per ISO 5725.

Table 10. GM% mean values determined by laboratories for test samples

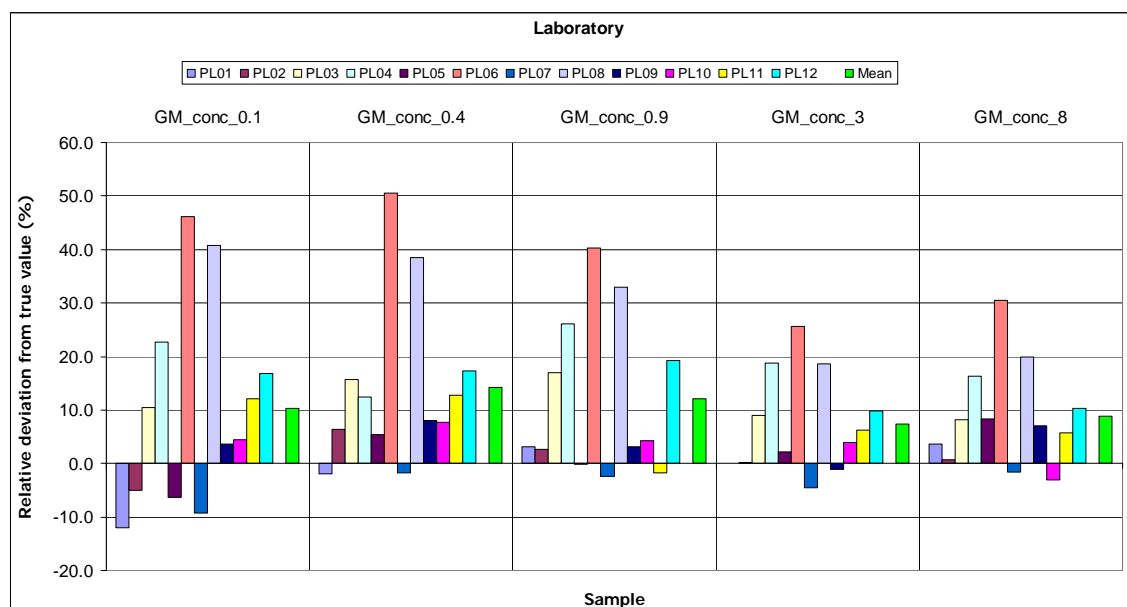
GMO content GMO% = GMO copy number/soybean genome copy number x 100 and GM DNA mass/soybean DNA mass x 100																				
LAB	0.1				0.4				0.9				3.0				8.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.09	0.11	0.07	0.09	0.45	0.34	0.39	0.39	0.93	0.92	0.87	0.99	1.98	2.94	2.82	4.26	10.19	7.78	7.93	7.27
2	0.09	0.10	0.08	0.11	0.43	0.43	0.39	0.46	0.86	0.84	1.04	0.95	2.74	-	3.25	3.03	7.95	8.07	8.24	7.99
3	0.11	0.11	0.10	0.12	0.44	0.48	0.47	0.46	0.97	1.02	1.24	0.99	3.07	3.68	3.18	3.15	8.99	8.84	8.49	8.29
4	0.14	0.11	0.14	0.11	0.61	0.39	0.42	0.38	1.30	1.12	1.03	1.09	3.67	4.13	3.53	2.93	9.96	10.56	7.67	9.04
5	0.10	0.08	0.10	0.10	0.42	0.46	0.39	0.42	0.91	0.84	0.87	0.98	3.10	3.10	3.13	2.94	8.93	9.63	7.17	8.93
6	0.13	0.14	0.14	0.18	0.58	0.66	0.69	0.49	1.04	1.16	1.65	1.20	4.23	4.30	3.58	2.97	14.83	11.21	6.23	9.51
7	0.09	0.09	0.09	0.09	0.40	0.39	0.38	0.40	0.89	0.79	0.93	0.91	2.80	2.76	3.23	2.67	7.78	7.95	7.97	7.81
8	0.17	0.13	0.12	0.14	0.52	0.66	0.43	0.61	1.03	1.14	1.38	1.25	3.71	3.76	3.65	3.11	9.99	10.25	8.41	9.74
9	0.10	0.10	0.10	0.11	0.44	0.42	0.43	0.43	0.90	0.91	1.00	0.90	3.15	2.70	3.05	2.97	-	8.36	8.41	8.91
10	0.12	0.10	0.10	0.10	0.42	0.39	0.45	0.46	0.91	0.92	1.01	0.91	3.00	2.98	3.46	3.02	7.84	7.77	7.85	7.55
11	0.12	0.10	0.11	0.12	0.45	0.38	0.50	0.46	0.86	0.82	0.86	0.99	3.24	3.36	3.15	3.00	7.96	10.26	7.50	8.11
12	0.12	0.12	0.11	0.12	0.46	0.46	0.44	0.51	1.00	1.03	1.16	1.11	3.16	3.32	3.43	3.26	9.50	9.68	7.94	8.18

Table 10 indicates that most of the single measurements per laboratory (replicates) showed that trueness was within the ENGL limits. At 0.1% GM level, thirty-eight replicate values out of forty-eight were within the 25% upper limit of quantification (bias %), forty at the 0.4% GM level, thirty-nine at the 0.9% GM level, forty-two at the 3.0% GM level, forty-two at the 8.0% GM-level. Data of all the replicates were retained to feed the statistical analysis whose results are reported in Table 11.

In Figure 1 the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the deviation of the GM level measured by the respective laboratory in % of the true GM level; the green bar on the right represents the mean relative deviation over all 12 participating laboratories for each true GM level.



Figure 1. Relative deviation (%) from the true value of FG72 for all laboratories\*



\*PL05 at GM level 0.9%, PL 01 and 02 at GM level 3.0% had very small relative deviations from the true value and the corresponding histograms do not show up in Figure 1.

Overall, most mean relative deviations from the true values are within the ENGL acceptance criterion of maximum 25% for all GM levels, in particular, 10 laboratories were within the limits at the 0.1% GM-level, 10 at the 0.4%, 9 at the 0.9%, 11 at the 3.0% and 11 at the 8.0%. Two laboratories overestimated the DNA content of sample 0.1% by more than 40% and one of those laboratories also overestimated the DNA content of samples 0.4 and 0.9% by almost 40% and over 30% respectively. All the data were retained to feed the statistical analysis whose results are reported in Table 11.

The bias generated by all laboratories is about +10% or below, i.e. the method is slightly over-predictive but well within the accepted limits.

## 5. Method performance requirements

Among the performance requirements established by ENGL and adopted by the EU-RL GMFF (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>), repeatability and reproducibility are to be assessed through an international collaborative trial, carried out with the support of twelve ENGL laboratories (see Table 3). Table 11 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the collaborative trial.

According to the ENGL method performance requirements, the relative reproducibility standard deviation ( $RSD_R$ ), that describes the inter-laboratory variation, should be below 35% at the target

concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 11, the method satisfies this requirement at all GM levels tested. In fact, the highest value of  $RSD_R$  is 20% at the 0.1% GM level, thus within the acceptance criterion.

Table 11. FG72, summary of validation results expressed as GM- DNA copy numbers in relation to target taxon specific DNA copy numbers

	Test Sample Expected GMO %				
	0.1	0.4	0.9	3.0	8.0
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	1	1	1	1
Reason for exclusion	-	DG	C	C	C
Mean value	0.11	0.44	0.99	3.24	8.55
Relative repeatability standard deviation, $RSD_r$ (%)	12	12	9	10	10
Repeatability standard deviation	0.01	0.05	0.09	0.31	0.84
Relative reproducibility standard deviation, $RSD_R$ (%)	20	14	14	12	11
Reproducibility standard deviation	0.02	0.06	0.13	0.39	0.92
Bias (absolute value)	0.010	0.044	0.086	0.242	0.549
Bias (%)	10.4	11.0	9.5	8.1	6.9

C= Cochran's test, DG= Double Grubbs; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2. Bias is estimated according to ISO 5725 data analysis protocol.

Table 11 also documents the relative repeatability standard deviation ( $RSD_r$ ) estimated for each GM level. In order to accept methods for collaborative study, the EU-RL GMFF requires that the  $RSD_r$  value is below 25%, as indicated by the ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>). As it can be observed from the values reported, the method showed a repeatability standard deviation below 25% at all GM levels, with the highest value of  $RSD_r$  (%) of 12% at 0.1% and 0.4% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be  $\pm 25\%$  across the entire dynamic range. In this case, the method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 11% at the 0.4% GM level.

## 6. Compliance of the method of detection of event FG72 with the requirements of Regulation (EU) No 619/2011

To verify the compliance of the method under validation with the requirements of Regulation (EU) No 619/2011, the following steps were carried out and their outcome is summarised in Table 12:

- at step 2 of the validation process (scientific assessment of the dossier), the EU-RL GMFF concluded that it could accept the applicant's data on method performance and therefore accepted that the RSDr at the level of 0.08% in terms of GM- DNA copy numbers in relation to target taxon specific DNA copy numbers was 15% on 18 replicates (Table 4), hence below 25%;
- at step 3 of the validation process (in-house testing of the method), the EU-RL GMFF determined the RSDr at the level of 0.1% related to mass fraction of GM-material by fifteen replicates in repeatability conditions (single run). The RSDr was 18% (Table 8), hence within the range indicated by the applicant;
- further to the conclusion of step 4 of the validation process (ring trial), the EU-RL GMFF analysed the data generated by the 12 participating laboratories for determining the method performance parameters. It found that the RSDr over all laboratories of the method at the level of 0.1% related to mass fraction of GM-material was 12%.

Table 12. Precision of the event-specific method for quantitative detection of FG72 at or around 0.1% level related to mass fractions of GM material

Source	RSDr %	GM %
Applicant' method optimisation*	15 %	0.08 %
EU-RL GMFF tests	18 %	0.1 %
Collaborative study	12 %	0.1 %

\* GM- DNA copy numbers in relation to target taxon specific DNA copy numbers

Based on the results of the EU-RL GMFF in-house verification and of the collaborative study, it is concluded that the method RSDr is equal or less than 25% at the level of 0.1% related to mass fraction of GM-material, hence the method for quantitative detection of event FG72 soybean meets the requirement laid down in Regulation (EU) No 619/2011.

## 7. Conclusion

The method provided by the applicant and described in detail under 3.2 (available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>) has been validated in accordance to the EU-RL GMFF validation scheme; respecting all requirements of the relevant EU legislation and international standards.

The dossier was found complete (step 1) and the scientific dossier analysis (step 2) concluded that the method met the ENGL minimum performance criteria for a quantitative PCR method for detection and quantification of GM events for entering into validation.

The subsequent in-house verification of the method (step 3) by the EU-RL GMFF confirmed this conclusion.

The international collaborative study (step 4) yielded data that also indicated that the method meets all acceptance criteria and method performance requirements recommended by the ENGL (as detailed at <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>) for a valid quantitative PCR method for detection and quantification of GM-events.

In conclusion, the method is considered applicable to the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004 and (EU) No 619/2011. The method is also applicable to appropriately extracted soybean DNA

## 8. Quality assurance

The EU-RL GMFF is ISO 9001:2008 certified (certificate number: CH-32232) and 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](http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7)].

## 9. References

1. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem.* 67, 331-343.
2. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
3. Arumuganathan K. and Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9, 208-218.