

# JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

## Event-specific Method for the Quantification of Soybean MON 87751 Using Real-time PCR

*Validation report*  
Corrected version 1

European Union Reference Laboratory for  
Genetically Modified Food and Feed

2016



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**Contact information**

Molecular Biology and Genomics Unit

Address: Joint Research Centre, Via Enrico Fermi 2749, TP 201, 21027 Ispra (VA), Italy

E-mail: [eurl-gmff@jrc.ec.europa.eu](mailto:eurl-gmff@jrc.ec.europa.eu)

Tel.: +39 0332 78 5165

Fax: +39 0332 78 9333

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EUROPEAN COMMISSION  
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection  
Molecular Biology and Genomics Unit



# Event-specific Method for the Quantification of Soybean MON 87751 Using Real-time PCR

## Validation Report

27 June 2016

Corrected version 1 - 01/08/2016 (see page 2)

European Union Reference Laboratory for GM Food and Feed

### Executive Summary

In line with its mandate<sup>a</sup> the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), validated an event-specific real-time polymerase chain reaction (qPCR) method for detecting and quantifying the soybean event MON 87751 (unique identifier MON-87751-7). The validation study was conducted according to the EURL GMFF validation procedure [<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>] and the relevant internationally accepted guidelines<sup>(1-5)</sup>.

In accordance with current EU legislation<sup>b</sup>, Monsanto Company (represented by Monsanto Europe S.A.) provided the detection method and the positive and negative control samples (genomic DNA from soybean seeds harbouring the MON 87751 event as positive control DNA, genomic DNA from conventional soybean seeds as negative control DNA). The EURL GMFF verified the method performance data provided by the applicant, where necessary experimentally, prepared the validation samples (calibration samples and blind samples at different GM percentage [copies GM/total soybean genome copies]), organised an international collaborative study and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method, in line with the provisions of Annex III-3.C.2 to Regulation (EU) No 503/2013, meets most of the method performance requirements, as established by the EURL GMFF and the ENGL, and it fulfils the analytical requirements of Regulation (EU) No 619/2011<sup>c</sup>. In agreement with the Steering Committee of the ENGL, the EURL GMFF concludes that the method is by all means fit for purpose

<sup>a</sup> Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed"

<sup>b</sup> Regulation (EC) No 503/2003 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".

<sup>c</sup> Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired.

and can be used for regulatory control of food and feed. Laboratories using it for the purposes of Regulation (EU) No 619/2011 are asked to provide the EURL GMFF with their experimental data and results in order to allow further verification of the performance of the method in that part of the dynamic range.

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

## Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR)].

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.

Modification from the previous version:.

Page 28 table 5: probe sequence

Probe	MON 87751 probe	5' – 6FAM – TgA CTg gAg ATC TCC AAA gTg Agg ggA AAA – TAMRA – 3'	29
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changed to:

Probe	MON 87751 probe	5' – 6FAM – TgA CTg gAg ATC TCC AAA gTg Agg ggA AA – TAMRA – 3'	29
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## Address of contact laboratory:

European Commission, Joint Research Centre (JRC)  
Institute for Health and Consumer Protection (IHCP)  
Molecular Biology and Genomics Unit (MBG)  
European Union Reference Laboratory for GM Food and Feed  
Via E. Fermi 2749, 21027 Ispra (VA) – Italy

Functional mailbox: [eurl-gmff@jrc.ec.europa.eu](mailto:eurl-gmff@jrc.ec.europa.eu)

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## 1. Introduction

In line with Regulation (EC) No 1829/2003, Monsanto Company provided the EURL GMFF with an event-specific method for detection and quantification of soybean event MON 87751 (unique identifier MON-87751-7) together with genomic DNA as positive and negative control samples.

The EURL GMFF started its step-wise validation procedure with step 1: dossier reception and concluded that the submitted dossier was complete.

The scientific dossier assessment (step 2) assessed the reported method performance characteristics against the ENGL method acceptance criteria<sup>d</sup> and concluded that these were adequately met.

In step 3 of the procedure (experimental testing), the EURL GMFF verified the purity of the control samples provided and conducted an in-house testing of samples and method. The positive and negative control DNA, submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Regulation (EC) No 1829/2003, were found of good quality.

Step 3 was completed with the conclusion that the method could be submitted to an international collaborative study (step 4).

The collaborative study (step 4) demonstrated that the method is suited for analysing DNA of GM soybean MON 87751, appropriately extracted from food or feed.

The preparation of the report (step 5) was aligned with the timelines communicated by EFSA for its risk assessment.

## 2. Step 1 (dossier acceptance) and step 2 (scientific dossier assessment and bioinformatics analysis)

Documentation and data supplied by the applicant were evaluated by the EURL GMFF for completeness (step 1) and compliance with the ENGL method acceptance criteria (step 2).

The specificity of the event-specific assay was verified by the applicant and confirmed by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

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<sup>d</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>)

## 2.1 Specificity assessment by the applicant

The specificity of the event-specific assay was assessed by the applicant in duplicate real-time PCR reactions, according to the method described, using genomic DNA extracted from 100% oilseed rape RT73 and MON88302; maize NK603, MON 810, MON 863, MON 88017, MON 89034, MON 87460, MON87427; cotton MON 531, MON 15985, MON 1445, MON 88913, MON88701; soybean 40-3-2, MON 89788, MON 87701, MON 87769, MON87705, MON87708, MON87712 and conventional oilseed rape, cotton, soybean, wheat, millet, lentil, sunflower, peanut (shelled), and quinoa.

According to the method developer, the MON 87751 method did not react with any sample except the positive control.

The soybean -specific system lectin (*le1*) was assessed by the method developer, according to the method described, with genomic DNA extracted from 100% oilseed rape RT73 and MON 88302; maize NK603, MON 810, MON 863, MON 88017, MON 89034, MON 87460, MON87427; cotton MON 531, MON 15985, MON 1445, MON 88913, MON88701, soybean 40-3-2, MON 89788, MON 87701, MON 87769, MON87705, MON87708, MON87712 and conventional alfalfa, cotton, maize, soybean, wheat.

According to the method developer the soybean-specific reference system did not react with any sample except the positive control soybean lines.

## 2.2 Bioinformatics analysis

The EURL GMFF performed bioinformatics analysis and confirmed that the detection method spans the junction between the transgenic insert and the 5' genomic region. The forward primer "MON 87751 primer 2" binding site was found in the genomic border adjacent to the insertion. The reverse primer "MON 87751 primer 1" binds in the insert, in a region that corresponds to T-DNA border region. The probe "MON 87751 probe" binds in a region overlapping the genomic border and the insert. The amplicon size is expected to be 87 bp, consistent to what was reported by the applicant. The sequence of the amplicon was analysed by BLAST (NCBI<sup>e</sup>) against local copies of the "nt" and "patents" databases, and no significant similarity was found with any other published sequence except the related parental sequences. In addition, the primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System of the JRC, as well as the whole genomes of more than 80 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays*) using the e-PCR prediction tool (NCBI<sup>f</sup>), and no potential amplicon was identified.

<sup>e</sup> Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990 Oct 5;215(3):403-10. PubMed PMID: 2231712.

<sup>f</sup> Schuler GD. Sequence mapping by electronic PCR. Genome Res. 1997 May;7(5):541-50.3: Rotmistrovsky K, Jang W, Schuler GD. A web server for performing electronic PCR. Nucleic Acids Res. 2004 Jul 1;32(Web Server issue):W108-12.



## 2.3 ENGL method acceptance criteria

The parameters of the calibration curve (slope,  $R^2$  coefficient) were determined by the applicant by quantifying three test samples at different GM levels (see Table 1).

Table 1. Summary of the average slope and  $R^2$  values obtained by the applicant

MON 87751		<i>Le1</i>	
Slope	$R^2$	Slope	$R^2$
-3.44	1.00	-3.47	1.00

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  $R^2$  coefficient shall be  $\geq 0.98$ .

Table 1 indicates that the slope and  $R^2$  coefficient of the standard curve for the GM-system (MON 87751) and the soybean-specific *lectin* (*Le1*) system, as established by the applicant, was within the ENGL acceptance criteria.

Also precision and trueness of the method were established by the applicant and 15 values for each of 3 GM levels (expressed as mass fraction of GM-material) were provided. Table 2 reports the resulting precision and trueness values for the three GM-levels as provided by the applicant. Both parameters were within the ENGL acceptance criteria (trueness  $\pm 25\%$ , RSDr  $\leq 25\%$  across the entire dynamic range).

*Note: Numerical values presented in the following tables were rounded keeping two digits for values  $\leq 1$ , one digit for values between 1 and 10 and no digit for values  $\geq 10$ , 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 2. Mean %, precision and trueness values provided by the applicant, based on duplicate measurements

Expected GMO%	Test results		
	0.085	1.0	10.0
Measured mean GMO %	0.088	1.1	11
Precision (RSDr %)	8.1	4.3	4.8
Trueness (bias %)	3.2	11	6.8

### 3. Step 3 (experimental testing of the samples and method)

#### 3.1 DNA extraction

Genomic DNA was isolated from ground soybean seeds using a "CTAB-based" protocol coupled with PEG purification.

This protocol had been already validated in-house by the EURL GMFF. The protocol for DNA extraction and a report on testing were published in 2008 at <http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-05-06-XP-Corrected-version-1.pdf>.

In agreement with the ENGL position, endorsing the modularity principle (see also Annex III to Regulation (EU) No 503/2013), and given the similarity in the matrix, the EURL GMFF considers the above mentioned DNA extraction protocol applicable in the context of this validation of the method for soybean event MON 87751.

#### 3.2 Method protocol for the PCR analysis

The PCR method provided by the applicant and validated by the EURL GMFF is an event-specific, quantitative, real-time TaqMan<sup>®</sup> PCR procedure for the determination of the relative content of GM event MON 87751 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean specific assay targeting the endogenous gene *lectin* (*Le1*), and the GM target assay for MON 87751 are performed in separate wells. The detailed protocol for the validated Method is published by the EURL GMFF at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

For the detection of GM event MON 87751, an 87-bp fragment of the region spanning the 5' insert-to-plant junction in soybean MON 87751 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 TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of GM event MON 87751, a soybean taxon-specific system amplifies a 74-bp fragment of a soybean *lectin* (*Le1*) endogenous gene, using *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with FAM as reporter dye at its 5' end and TAMRA as quencher dye at its 3' end.

Standard curves are generated for both the MON 87751 and the *lectin* systems by plotting the C<sub>q</sub> 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 MON 87751 DNA in a test sample, the MON 87751 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 to obtain the percentage value (GM% = MON 87751/*Le1* x 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) <sup>(6)</sup>. The copy number values used in the quantification, the GMO contents of the calibration samples, and the total DNA quantity used in the PCR reactions 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 soybean DNA in the reaction (ng)	220	73	18	4.6	1.1
Target taxon <i>Le1</i> copies	194690	64897	16224	4056	1014
Target MON 87751 copies	19469	6490	1622	406	101

### 3.3 EURL GMFF experimental testing

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

Annex II of Regulation (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 EURL GMFF.” This requires knowledge of the zygosity of the event, i.e. the ratio of the GM-target copies to the reference target copy in the genome.

The copy number of the GM-target for MON 87751 and of the *Le1* reference target in the positive control sample were determined by digital PCR (dPCR) performed on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan<sup>®</sup> Universal PCR Master Mix (Applied Biosystems, Cat. number 4304437), 1X GE sample loading reagent (Fluidigm PN 85000746), primers and probes at concentrations indicated in the corresponding Validated Method (MON 87751 primer 1 and MON 87751 primer 2 at 500 nM each, MON 87751 probe at 300 nM; *lec* F and *lec* R at 150 nM each, *lec* P at 50 nM), 1 µL of DNA at a concentration of 1.2 ng/µL (concentration chosen to avoid panel saturation, optimal between 200<positive partitions<700).

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 4.6 µL were distributed into the 765 partitions (or chambers) constituting one panel.

The experiment was repeated three times for a total number of fifteen data sets for both targets. ‘No template controls’ were included. Amplification conditions were as reported in Annex 1 or in the Validated Method document (<http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). Data analysis and copy number calculations were performed using the BioMark digital PCR Analysis software. The range of Cq retention was from 15 to 35.

Calculations of means and variances were carried out 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>9</sup>.

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

The method performance characteristics were verified by quantifying on a copy number basis five blinded test samples containing a range of GM levels (0.1%-10%). The experiments were performed on an ABI 7500 and ABI 7900 real-time platform under repeatability conditions and followed the protocol described in the material and method section. Test samples with GM levels 0.1%, 0.9%, 2.5%, 5.0% and 10% 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 two additional runs. Tests were conducted on ABI 7900 and ABI 7500 as a test of robustness.

Average values of the slope and of the  $R^2$  coefficient of the standard curves and method trueness and precision over the dynamic range were evaluated against the ENGL method acceptance criteria.

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

## **3.4 International collaborative trial (step 4)**

The international collaborative study involved twelve randomly selected laboratories, all being "National Reference Laboratories, assisting the EURL GMFF for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 1981/2006 who had expressed their interest in participation. 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) <sup>(1)</sup>
- ISO 5725 "Accuracy (trueness and precision) of measurement methods and results", Part 1 and Part 2 (ISO, 1994); ISO 5725-1:1994/Cor 1 (ISO, 1998) and ISO 5725-2:1994/Cor 1 (ISO, 2002) <sup>(2-5)</sup>

The objective of the international collaborative study was to verify in experienced laboratories the trueness and precision of the PCR analytical method provided by the applicant and verified by the EURL GMFF in step 3 of its validation procedure.

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<sup>9</sup> Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods. European Network of GMO Laboratories (ENGL), 2011.  
<http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf>

### 3.4.1 List of participating Laboratories

The twelve laboratories participating in the MON 87751 validation study were randomly selected from 29 National Reference Laboratories (NRL) that offered to participate. The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the validation of the detection method for soybean event MON 87751

Laboratory	Country
Ministry of Finance, Secretariat General for Public Revenue, General Chemical State Laboratory (GCSL), Food Division - Athens	GR
Institute for Diagnosis and Animal Health, Molecular Biology and GMOs Unit - National Reference Laboratory for GMOs in food and feed	RO
Croatian National Institute for Public Health - GMO Quantification and risk assessment Unit	HR
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE
BioGEVES - Groupement d'Intérêt Public – Groupe d'Etude et de contrôle des Variétés et des Semences	FR
National Health Laboratory, Food Control Department	LU
Veterinary Public Health Institute for Lazio and Toscana Regions; National Reference Centre for GMO Analysis	IT
Institute for Hygiene and Environment- Hamburg	DE
Laboratorio Arbitral Agroalimentario	ES
Saxon State Company of Environmental and Agriculture - Radebeul, Business Division Laboratories Agriculture	DE
National Food and Veterinary Risk Assessment Institute, Molecular Biology and GMO Department	LT
Hellenic Agricultural Organisation, Demetra	GR

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

Laboratories involved in the collaborative study used the following real-time PCR equipment: four laboratories used the ABI 7900HT, two used the ABI 7900HT FAST, three used ABI 7500, one laboratory used ABI 5700 and two laboratories used Stratagene Mx3005p.

This variability of equipment, with its known potential influence on PCR results, reflects the real-life situation in control laboratories and provides additional assurance that the method is robust and usable under real conditions.

### 3.4.3 Materials used in the international collaborative study

For the validation of the quantitative event-specific method, test samples were provided by the EURL GMFF to the participating laboratories. They were derived from:

- i) genomic DNA extracted by the applicant from homozygous soybean seeds harbouring event MON 87751, and
- ii) genomic DNA extracted by the applicant from conventional soybean seeds genetically similar to those harbouring MON 87751 event.

The control samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11<sup>h</sup>

The control samples were used by the EURL GMFF to prepare standards (of known GMO content) and test samples (of undisclosed GM content = blinded samples) by mixing MON 87751 soybean DNA and non-GM soybean DNA.

The calibration sample S1 was prepared by mixing the appropriate amount of MON 87751 DNA with control non-GM soybean DNA to obtain a 10% (copy no/copy no) GM-sample. Calibration samples S2-S5 were prepared by one 3-fold and three 4-fold serial dilutions from the S1 sample.

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

- ✓ Five calibration samples with known concentrations of GM-event (140 µL of DNA solution each) labelled from S1 to S5 (Table 3).
- ✓ Twenty blinded test DNA samples (70 µL of DNA solution, each at 50 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 5)

Table 5. MON 87751 GM contents

MON 87751 GM%
GM copy number/soybean genome copy number x 100
10
5.0
2.5
0.90
0.10

- ✓ Reaction reagents:
  - TaqMan<sup>®</sup> Universal PCR Master Mix (2x), three vials: 15 mL
  - distilled sterile water, one vial: 10.8 mL
- ✓ Primers and probes (1 tube each) as follows:
  - lectin* taxon-specific assay
  - lec F (10 µM): 240 µL
  - lec R (10 µM): 240 µL
  - lec probe (10 µM): 80 µL

<sup>h</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).

## MON 87751 assay

- MON 87751 primer 1 (10  $\mu$ M): 800  $\mu$ L
- MON 87751 primer 2 (10  $\mu$ M): 800  $\mu$ L
- MON 87751 probe (10  $\mu$ M): 480  $\mu$ L

### 3.4.4 Design of the collaborative study

Participating laboratories received a detailed validation protocol that included the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the MON 87751 event-specific system and for the *Le1* taxon-specific system. In total, two plates were run by each participating laboratory.

Clear guidance was given to the participating laboratories for strictly following the validation protocol.

The laboratories prepared the PCR master-mixes for the two assays in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per pre-determined plate layout.

The amplification reaction followed the cycling program specified in the validation protocol. Participants determined the GM% in the test samples according to the instructions and also reported the raw data to the EURL GMFF on an Excel sheet that was designed, validated and distributed by the EURL GMFF. All data are stored by the EURL GMFF on a dedicated and protected server.

The EURL GMFF analysed the data against the parameters and the limits set by the ENGL, i.e. trueness, precision, amplification efficiency and linearity.

### 3.4.5 Deviations reported from the protocol

Seven laboratories reported no deviations from the method protocol. Two laboratories set the total PCR reaction volume to 30  $\mu$ L instead of 50  $\mu$ L. One laboratory did not provide results since a wrong gain setting for FAM was used; this was too high, causing, from the start of the run, saturation of the FAM signal in the top half of the plate. One laboratory excluded one Cq value of the reference gene due to an error message for ROX. One laboratory applied a denaturation of 25" for one run and a wrong cycling program.

## 4. Results

### 4.1 EURL GMFF experimental testing

#### 4.1.1 Zygoty ratio in the positive control sample

The results of the digital PCR analysis conducted by the EURL GMFF on the positive control samples to determine the zygoty of MON 87751 and *Le1* targets are shown in Table 6.

Table 6. Zygoty of the MON 87751 and *Le1* targets in the positive control sample

Mean zygoty (MON 87751/ <i>Le1</i> )	1.03
Standard deviation	0.08
RSD <sub>r</sub> (%)	7.7
Standard error of the mean	0.021
Upper 95% CI of the mean	1.075
Lower 95% CI of the mean	0.99

The mean zygoty (MON 87751/*Le1*) equals 1.03%. The 95% confidence interval (CI) spans around 1.03% and therefore the mean zygoty is not significantly different from the expected zygoty for a soybean homozygous control sample assuming a single-copy endogenous gene target, for an  $\alpha = 0.05$ .

Hence:

$$0.1 \text{ GM\% in DNA copy number ratio} = 0.1 \text{ GM \% in mass fraction}$$

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

The standard curve parameters and the results of efficiency, linearity, trueness and precision obtained in the two real-time PCR runs with the test samples are shown in Tables 7, 8 and 9.

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  $R^2$  coefficient shall be  $\geq 0.98$ . Table 7 documents that the slopes of the standard curves and the  $R^2$  coefficients were within the limits established by the ENGL. The EURL GMFF in-house results confirm the data provided by the applicant.



Table 7. Standard curve parameters of the real-time PCR tests carried out on ABI7900 and ABI 7500

	<b>MON 87751 system</b>			<b><i>Le1</i> system</b>		
	Slope	PCR efficiency*	R <sup>2</sup>	Slope	PCR efficiency*	R <sup>2</sup>
Run A	-3.40	97	1.00	-3.30	101	1.00
Run B	-3.40	97	1.00	-3.32	100	1.00
Run C	-3.33	100	1.00	-3.32	100	1.00
Run D	-3.39	97	1.00	-3.35	99	1.00
Run E	-3.38	98	1.00	-3.36	98	1.00
Run F	-3.45	95	1.00	-3.40	97	1.00

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

Runs A-B were used to quantify GM-levels from 0.90 to 10% on ABI 7900; Runs C-D were used to quantify GM-levels from 0.90 to 10% on ABI 7500; Run E was used to quantify GM-level 0.1% on ABI 7900; Run F was used to quantify GM-level 0.1% on ABI 7500.

According to the ENGL method acceptance criteria the method trueness (measured as bias in % of the target GM level) should be within  $\pm 25\%$  of the accepted reference value over the entire dynamic range and the precision, expressed as RSDr % (relative standard deviation of repeatability), should be  $\leq 25\%$ , also over the entire dynamic range.

Tables 8 and 9 show that trueness and precision of quantification were within the limits established by the ENGL for both PCR machines used.

Table 8. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI 7900

<b>Target GM-levels %</b>	<b>Measured GM level %</b>	<b>Bias % of the target GM-level</b>	<b>Precision (RSDr %)</b>
10	9.8	-1.8	3.9
5.0	4.7	-6.1	3.4
2.5	2.5	1.9	2.3
0.90	0.84	-6.3	4.6
0.10	0.1	3.76	11

Table 9. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI 7500

<b>Target GM-levels %</b>	<b>Measured GM level %</b>	<b>Bias % of the target GM-level</b>	<b>Precision (RSDr %)</b>
10	10	0.26	2.9
5.0	4.7	-6.8	5.2
2.5	2.5	1.8	5.8
0.90	0.80	-12	3.2
0.10	0.11	5.8	10

## 4.2 Results of the international collaborative study

### 4.2.1 PCR efficiency and linearity

The PCR efficiency (%) and R<sup>2</sup> values (expressing the linearity of the regression) for the standard curve, reported by participating laboratories are displayed in Table 10. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

$$\text{Efficiency} = (10 * (-1/\text{slope})) - 1 \times 100$$

Table 10. Values of slope, PCR efficiency and R<sup>2</sup> obtained during the international collaborative trial

Lab	Plate	MON 87751			Le1		
		Slope	PCR Efficiency (%)	R <sup>2</sup>	Slope	PCR Efficiency (%)	R <sup>2</sup>
1	A	-3.54	92	1.00	-3.53	92	1.00
	B	-3.48	94	1.00	-3.35	99	1.00
2	A	-3.51	93	1.00	-3.41	97	1.00
	B	-3.36	99	1.00	-3.36	98	1.00
3	A	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*
	B	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*
4	A	-3.76	85	0.99	-3.65	88	0.99
	B	-3.63	89	1.00	-3.61	89	1.00
5	A	-3.66	87	0.99	-3.49	93	1.00
	B	-3.57	91	1.00	-3.43	96	1.00
6	A	-3.63	89	0.97	-3.44	95	0.99
	B	-3.50	93	0.98	-3.62	89	0.98
7	A	-3.35	99	1.00	-3.25	103	1.00
	B	-3.47	94	1.00	-3.44	95	1.00
8	A	-3.45	95	0.99	-3.58	90	1.00
	B	-3.50	93	1.00	-3.50	93	1.00
9	A	-3.55	91	1.00	-3.45	95	1.00
	B	-3.53	92	1.00	-3.49	93	1.00
10	A	-3.49	93	1.00	-3.42	96	1.00
	B	-3.46	95	1.00	-3.40	97	1.00
11	A	-3.22	105	1.00	-3.32	100	1.00
	B	-3.40	97	1.00	-3.52	93	1.00
12	A	-3.58	90	0.96	-3.16	107	0.97
	B	-3.97	79	0.98	-3.42	96	0.98
Mean		-3.53	92	0.99	-3.45	95	0.99

\*n.d: not determined (i.e. no data provided)

Table 10 indicates that the efficiency of amplification for the MON 87751 system ranges from 79 to 105 and the linearity from 0.96 to 1.00; the amplification efficiency for the soybean-specific system ranges from 88% to 107% and the linearity from 0.97 to 1.00. The mean PCR efficiency was 92%

for MON 87751 assay and 95% for *Le1* assay. The average  $R^2$  of the methods was 0.99 for both the MON 87751 and *Le1* assays. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

These results confirm the appropriate performance characteristics of the methods tested in terms of efficiency and linearity.

#### 4.2.2 GMO quantification

Table 11 reports the values of quantification for the four replicates of each GM level as reported by each of the participating laboratories.

Table 11. GM% values determined by laboratories for test samples

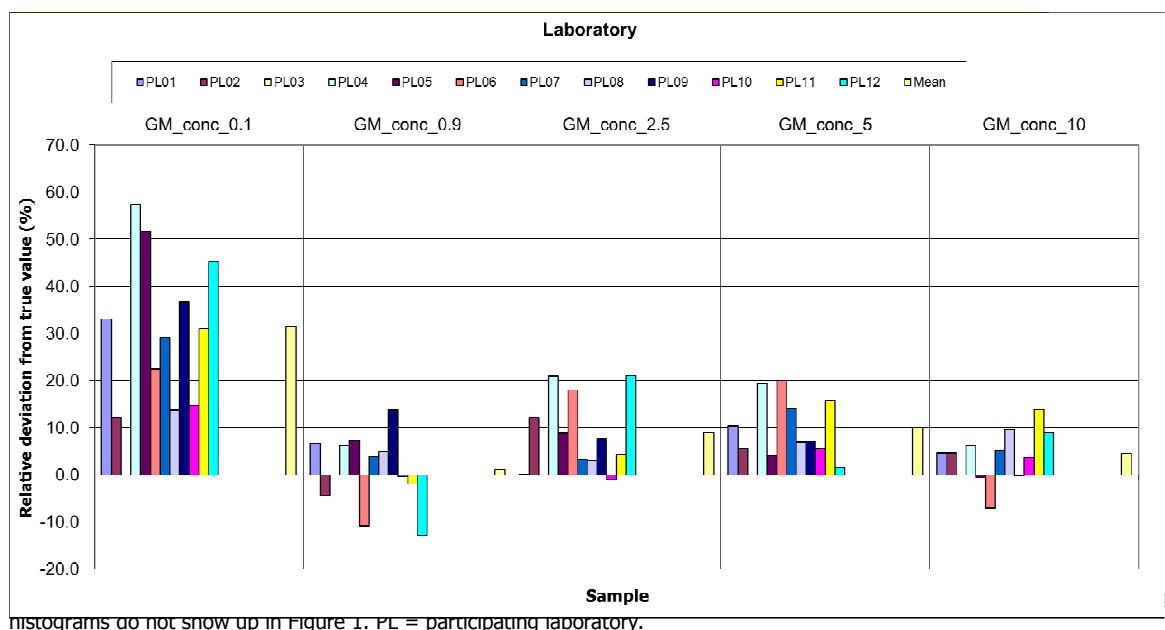
LAB	GMO content (%) **															
	0.10				0.90				2.5				5.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
<b>1</b>	0.12	0.12	0.14	0.15	0.85	0.86	1.14	0.99	2.91	2.27	2.32	2.51	5.72	5.40	5.18	5.77
<b>2</b>	0.10	0.11	0.12	0.11	0.83	0.84	0.98	0.79	2.71	3.04	2.76	2.70	5.40	5.91	4.41	5.40
<b>3</b>	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*	n.d*
<b>4</b>	0.14	0.16	0.17	0.16	0.96	0.98	1.07	0.82	3.37	2.96	2.96	2.80	6.09	6.31	5.40	6.10
<b>5</b>	0.14	0.15	0.15	0.16	0.89	0.96	0.97	1.04	2.89	2.70	2.64	2.64	5.69	5.44	4.45	5.24
<b>6</b>	0.11	0.16	0.15	0.07	1.00	1.04	0.54	0.63	2.50	3.85	3.56	1.89	8.44	6.69	4.26	4.59
<b>7</b>	0.15	0.15	0.12	0.11	0.94	0.96	1.01	0.83	2.73	2.73	2.72	2.15	6.45	6.11	4.52	5.76
<b>8</b>	0.12	0.11	0.11	0.11	0.83	0.94	1.06	0.93	3.05	2.04	2.41	2.80	5.30	4.93	5.11	6.03
<b>9</b>	0.14	0.13	0.13	0.15	0.93	0.99	1.09	1.09	2.81	2.68	2.46	2.81	5.65	n.d*	5.14	5.26
<b>10</b>	0.12	0.12	0.11	0.11	0.91	0.87	0.99	0.82	2.64	2.47	2.46	2.32	5.65	5.48	4.75	5.22
<b>11</b>	0.16	0.12	0.15	0.09	0.83	0.91	1.07	0.72	2.45	2.89	2.35	2.74	5.43	5.66	5.65	6.42
<b>12</b>	0.14	0.17	0.16	0.12	0.49	0.66	0.86	1.11	2.55	2.70	3.06	3.79	3.40	3.67	6.39	6.85

\*\* GMO% = (GMO copy number/soybean genome copy number) x 100

n.d\* not determined (i.e. no data provided)

A graphical representation of the data reported in Table 11 is provided in Figure 1 that shows the relative deviation from the true value for each GM level tested for each laboratory. The coloured bars represent the deviation of the GM level measured in % of the true GM level; the yellow bar on the right represents the mean relative deviation over all eleven participating laboratories for each true GM level.

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



Overall a trend for over-estimation of the amount of the GM-target over the dynamic range can be observed. The mean bias generated by all laboratories ranges between 1% and 32% at the 0.1% level. The method is within the accepted limits established by the ENGL ( $\pm 25\%$ ), with the exception of the 0.1%.

At 0.1% GM level the method shows a trueness value slightly outside the required range but at that low levels this is deemed to be acceptable because the precision is within the limits, the positive bias would determine an overestimation of the GM content and not an underestimation which would instead cause a problem and because the true value falls within the 95% CI around the mean ( $0.13\% \pm 0.036$ ). All data were retained for the statistical analysis and for tests of outliers (Cochran and Grubbs) whose results are reported in Table 12.

#### 4.2.3 Method performance requirements

Among the performance requirements established by ENGL and adopted by the EURL GMFF (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>), repeatability and reproducibility are to be assessed through an international collaborative trial. Table 12 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study (see Table 4 for a list of the participant laboratories). Four outliers were identified further to the application of the Cochran's and Grubbs' tests for identification and removal of outliers.

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 12, the method satisfies this requirement at all GM levels tested. Indeed, the highest value of  $RSD_R$  is 17% at the 5.0% GM level, thus within the acceptance criterion.

Table 12. Summary of validation results for the MON 87751 method, expressed as GM copy numbers in relation to target taxon copy numbers

	Test Sample Expected GMO %				
	0.10	0.90	2.5	5.0	10
Laboratories having returned valid results	11	11	11	11	11
Samples per laboratory	4	4	4	4	4
Number of outliers	1		1		2
Reason for exclusion	C*		C		CC
Mean value	<b>0.13</b>	<b>0.91</b>	<b>2.7</b>	<b>5.5</b>	<b>11</b>
Relative repeatability standard deviation, $RSD_r$ (%)	12	16	11	17	7.1
Repeatability standard deviation	0.02	0.14	0.30	0.93	0.75
Relative reproducibility standard deviation, $RSD_R$ (%)	16	16	12	17	7
Reproducibility standard deviation	0.02	0.14	0.32	0.93	0.79
Bias** (absolute value)	0.03	0.01	0.20	0.50	0.53
Bias (%)	33	1.1	8	10	5.3

\* C= Cochran's test; 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 12 also documents the relative repeatability standard deviation ( $RSD_r$ ) estimated for each GM level. In order to accept methods for a collaborative study, the EURL GMFF and ENGL require that the  $RSD_r$  value indicated by the applicant and confirmed by the EURL GMFF through in-house experiments, is below 25% (see ENGL document "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 repeatability standard deviation is below 25% at all GM levels, with the highest value of 17% at the 5.0% 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 most of the tested dynamic range, including the 0.9% GM level (legal threshold for labelling of adventitious presence of GM material, as per Regulation (EC) No 1829/2003). However, the method overestimates the true GM content at the 0.1% level, with a bias of +33%, in absolute terms an average overestimation by 0.033%.

## 5. Compliance of the method for detection and quantification of event MON 87751 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 13:

- at step 2 of the validation process (scientific assessment of the dossier), the EURL GMFF acknowledged that the RSDr value at the 0.085% level shown by the applicant's dossier (expressed as mass fraction of GM-material) was 8.11%, based on 15 replicates (Table 2), hence below the maximum value of 25% required by the ENGL. The EURL GMFF therefore concluded that it could accept the applicant's data on method performance;
- at step 3 of the validation process (experimental testing of samples and methods), the EURL GMFF determined the RSDr value at the level of 0.1% in mass fraction of GM-material (corresponding to 0.1% expressed in terms of copy number ratio). The experiments were carried out under repeatability conditions on fifteen replicates. The RSDr resulted to range between 10% and 11% (Table 8 and 9) depending on the qPCR platform applied, hence also below 25%;
- the collaborative study (step 4 of the validation process) established that over the eleven participating laboratories that provided data at the level of 0.1% related to mass fraction of GM-material the RSDr of the method was 11.5%, therefore also below 25% and well in line with the previous data.

The outcome of the different steps is summarised Table 13.

Table 13. Precision of the event-specific method for quantitative detection of MON 87751 at or around 0.1% level related to mass fractions of GM material.

Source	RSDr %	GM %
Applicant' method optimisation*	8.11%	0.085%
EURL GMFF tests	10-11%	0.1%
Collaborative study	12%	0.13%

Based on the results of the EURL GMFF in-house verification and of the international collaborative study, it is concluded that the method RSDr % is lower than 25% at the level of 0.1% related to mass fraction of GM material, hence the method meets the requirement laid down in Regulation (EU) No 619/2011.

## 6. Conclusion

The method provided by the applicant has been validated in accordance to the EURL GMFF validation scheme, respecting all requirements of the relevant EU legislation and international standards for method validation.

This validation study confirmed that the method is applicable to the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex I-3.C.2 to Commission Regulation (EU) No 503/2013 and (EU) No 619/2011, and meets the method performance requirements established by the ENGL, with the exception of a higher bias (33%) at the level of 0.1%. At 0.1% GM level the method shows a trueness value slightly outside the required range but at that low levels this is deemed to be acceptable for the following reasons: a) the precision is within the limits b) the positive bias would determine an overestimation of the GM content and not an underestimation which would instead cause a problem c) the true value falls within the 95% CI around the mean ( $0.13\% \pm 0.036$ ).

The method is therefore valid to be used for regulatory purposes, including the quantification of low level presence of 0.1% (m/m) of the GM event where a slight positive bias (0.03%) has to be expected. It can be assumed that it is applicable to any appropriately extracted soybean DNA.

Regarding the method performance requirements that were not met by the results of the collaborative study, i.e. trueness at the 0.1% level, the EURL GMFF, in collaboration with the European Network of GMO Laboratories (ENGL), will conduct a monitoring by asking laboratories using the methods for the purpose of Regulation (EU) No 619/2011 to provide their experimental data to the EURL GMFF in order to clarify if the found minor deviations are confirmed in practice and of any significance.

However, user of the method should always verify the quality of the extracted genomic DNA prior to the PCR experiment, in particular if the DNA is extracted from more complex food or feed products potentially harbouring the GM-event.

A detailed description of the method is available as "Validated Method" at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx> and in Annex 1 to this report.

## 7. References

1. Horwitz W. Protocol for the design, conduct and interpretation of method- performance studies, *Pure and Appl. Chem.* 1995; 67: 331-343.
2. International Standard (ISO) 5725-1, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 1: General principles and definitions. International Organization for Standardization, Genève, Switzerland.
3. ISO 5725-1:1994/Cor 1:1998.
4. International Standard (ISO) 5725-2, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method. International Organization for Standardization, Genève, Switzerland.
5. ISO 5725-2:1994/Cor 1:2002.
6. Plant DNA C-values Database, <http://data.kew.org/cvalues/>



# **Annex 1: Event-specific Method for the Quantification of Soybean MON 87751 Using Real-time PCR**

## **Validated Method**

### **Method development:**

Monsanto Company

## 1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan<sup>®</sup> PCR (polymerase chain reaction) procedure for the determination of the relative content of soybean event MON 87751 DNA to total soybean DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assays. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are also recommended to ensure suitability of the extracted DNA.

For the specific detection of soybean event MON 87751, a 87-bp fragment of the region spanning the 5' plant-to-insert junction in soybean MON 87751 event is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein), as a reporter at its 5' end, and the dye TAMRA (6-carboxytetramethylrhodamine) as a quencher at its 3' end.

For the relative quantification of soybean event MON 87751 DNA, a soybean taxon-specific system amplifies a 74-bp fragment of a *lectin (Le1)* soybean endogenous gene (Accession number, GeneBank: K00821.1), using gene-specific primers and probe labelled with FAM as reporter dye at its 5' end, and TAMRA as a quencher at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "C<sub>q</sub>" value. For quantification of the amount of MON 87751 DNA in a test sample, C<sub>q</sub> values for the MON 87751 and the *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON 87751 DNA to total soybean DNA.

## 2. Validation and performance characteristics

### 2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional soybean seeds. Precision and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

### 2.2 Collaborative trial

The method was validated in an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF). The study was undertaken with twelve participating laboratories in June 2015.

A detailed validation report can be found at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

### 2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.04% (related to mass fraction of GM material) in 200 ng of total suitable soybean DNA. The relative LOD was not assessed in the collaborative study.

### 2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is below 0.09% (related to mass fraction of GM material) in 200 ng of total suitable soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1% (mass fraction of GM-material).

### 2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' plant -to- insert junction in soybean MON 87751 and is therefore event-specific for the event MON 87751. This was further verified *in silico* by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

According to the method developer the soybean-specific reference system did not react with any sample except the positive control soybean lines.

## 3. Procedure

### 3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. "forward flow direction" during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.
- PCR reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment should be sterilised prior to use and any residue of DNA should have been removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed regularly

- Laboratory benches and equipment should be cleaned periodically, with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps, unless specified otherwise, should be carried out at room temperature.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

## 3.2 Real-time PCR for quantitative analysis of soybean event MON 87751

### 3.2.1 General

The qPCR set-up for the taxon (*Le1*) and the GMO (event MON 87751) target sequences are carried out in separate vials. Multiplex qPCR (using differential fluorescent labels for the probes) has not been tested or validated by the EURL GMFF.

The method is developed for a total volume of 50 µL per reaction mixture with the reagents as listed in Table 2 and Table 3.

### 3.2.2 Calibration

The calibration curves have to be established on at least five samples. The first point of the calibration curve (S1) should be established for a sample containing 10% soybean MON 87751 DNA in a total of 220 ng of soybean DNA (corresponding to 194690 soybean genome copies with one haploid genome assumed to correspond to 1.13 pg of soybean genomic DNA) <sup>(1)</sup>. Standards S2 to S5 are to be prepared by serial dilutions according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of soybean DNA in the reaction (ng)	220	73	18	4.6	1.1
Target taxon <i>Le1</i> copies	194690	64897	16224	4056	1014
Target MON 87751 copies	19469	6490	1622	406	101

A calibration curve is to be produced by plotting the C<sub>q</sub> values against the logarithm of the target copy number for the calibration points. This can be done by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the software.

The copy number measured for each unknown sample DNA is obtained by interpolation from the standard curves.

### 3.2.3 Real-time PCR set-up

1. Thaw, mix and spin down the components needed for the run. Keep thawed reagents on ice.

2. To prepare the amplification reaction mixtures add the components listed in Table 2 and 3 in two reaction tubes (one for the MON 87751 assay and one for the *Le1* assay) on ice in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 87751 assay.

Component	Final concentration	µL/reaction
TaqMan <sup>®</sup> Universal PCR Master Mix (2x)	1x	25
MON 87751 primer 1 (10 µM)	500 nM	2.5
MON 87751 primer 2 (10 µM)	500 nM	2.5
MON 87751 probe (10 µM)	300 nM	1.5
Nuclease free water	-	14.5
DNA (max 200 ng)	-	4
Total reaction volume:		50 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the soybean *Le1* assay.

Component	Final concentration	µL/reaction
TaqMan <sup>®</sup> Universal PCR Master Mix (2x)	1x	25
Lec F (10 µM)	150 nM	0.75
Lec R(10 µM)	150 nM	0.75
Lec P (10 µM)	50 nM	0.25
Nuclease free water	-	19.25
DNA (max 200 ng)	-	4
Total reaction volume:		50 µL

3. Vortex for approx. 5 seconds and spin down.
4. Prepare two reaction tubes (one for the soybean MON 87751 and one for the *Le1* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of reaction mix for 3.5 PCR repetitions (161 µL for the *Le1* system and for the MON 87751 soybean system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (14 µL DNA). The volume for the additional 0.5 repetition will ensure adequate volume when loading the samples. Vortex each tube for approx. 5 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.

6. Spin down the tubes in a micro-centrifuge. Aliquot 50 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4 °C) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for MON 87751/*Le1* assays.

Step	Stage	T (°C)	Time (s)	Acquisition	Cycles
1	UNG *	50	120	No	1
2	Initial denaturation	95	600	No	1
3	Amplification	95	15	No	40
		60	60	Yes	

\*UNG: Uracil-N-glycosylase

### 3.3 Data analysis

After the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold: display the amplification curves of one assay (e.g. MON 87751) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the “update” button to ensure changes affect C<sub>q</sub> values (only needed for some analysis software). Switch to the linear view mode by clicking on the Y axis of the amplification plot and check that the threshold previously set falls within the exponential phase of the curves.
- b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest C<sub>q</sub> = 25, set the baseline crossing at C<sub>q</sub> = 25 – 3 = 22).
- c) Save the settings.
- d) Repeat the procedure described in a), b) and c) on the amplification plots of the other system (e.g. *Le1*).
- e) Save the settings and export all the data for further calculations.

### 3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument’s software calculates the C<sub>q</sub> values for each reaction.

The standard curves are generated both for the *Le1* and the MON 87751 specific assays by plotting the Cq values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the DNA copy number in the unknown samples.

To obtain the percentage value of event MON 87751 DNA in the unknown sample, the MON 87751 copy number is divided by the copy number of the soybean endogenous gene *Le1* and multiplied by 100 (GM% = MON 87751/*Le1* x 100).

## 4. Equipment and Materials

### 4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for analysis of the runs (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Centrifuge for PCR plates
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

### 4.2 Reagents

- TaqMan® Universal PCR Master Mix. Applied Biosystems Cat. No 4304437.

### 4.3 Primers and Probes

Table 5. Primers and probes for the MON 87751 and *Le1* methods

		DNA Sequence (5' to 3')	Length (nt)
MON 87751			
Forward primer	MON 87751 primer 2	5' – CTA AAT TgC TCT TTg gAg TTT ATT TTg Tag – 3'	30
Reverse primer	MON 87751 primer 1	5' – ggC CTA ACT TTT ggT gTg Atg ATg – 3'	24
Probe	MON 87751 probe	5' – 6FAM – TgA CTg gAg ATC TCC AAA gTg Agg ggA AA – TAMRA – 3'	29
Lectin (Le1)			
Forward primer	lec F	5' – CCA gCT TCg CCg CTT CCT TC – 3'	20
Reverse primer	lec R	5' – gAA ggC Aag CCC ATC TgC Aag CC – 3'	23
Probe	lec P	5' – 6FAM – CTT CAC CTT CTA TgC CCC TgA CAC – TAMRA – 3'	24

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine;

## 5. References

1. Plant DNA C-values Database. Royal Botanic Gardens, Kew, <http://data.kew.org/cvalues/>



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