EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Soybean MON87708 Using Real-time PCR

Validation Report

6 May 2013

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate[1] 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 real-time polymerase chain reaction (qPCR) method for detecting and quantifying the soybean event MON87708 (unique identifier MON-877Ø8-9). The validation study was conducted according to the EU-RL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and the relevant internationally accepted guidelines[2, 3].

In accordance with current EU legislation[1], Monsanto Company has provided the detection method and the positive and negative control samples (genomic DNA from soybean seeds harbouring the MON87708 event as positive control DNA, genomic DNA from conventional soybean seeds as negative control DNA). The EU-RL GMFF verified the method performance data provided by the developer, prepared the validation samples (calibration samples and blind samples at different 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 GMFF and the ENGL and laid down in Annex I-2.C.2 to Regulation (EC) No 641/2004 and it fulfils the analytical requirements of Regulation (EU) No 619/2011[4].

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

Quality assurance

The EU-RL GMFF is accredited ISO 17025:2005 [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://gmo-crl.jrc.ec.europa.eu/accredited_methods.html.

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EU-RL GMFF quality system.

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Content

1. Timeline

In line with Regulation (EC) No 1829/2003, Monsanto provided the EU-RL GMFF with a copy of the official application for authorisation of an event-specific method for detection and quantification of soybean event MON87708 (unique identifier MON-877Ø8-9) together with genomic DNA as positive and negative control samples (January 2011). The submission was found to be complete (step 1).

The scientific dossier assessment (step 2) compared the method performance characteristics reported by the applicant in the dossier with the ENGL method acceptance criteria[5] (see 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). In August 2011 it was terminated, concluding that the reported performance was in line with the requirements.

In step 3 of the validation procedure (experimental testing), the EU-RL GMFF verified the purity of the control samples provided and conducted an *in-house* verification of the method performance.

The positive and negative control DNA, submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Reg. (EC) No 1829/2003, were verified with regard to quantity and quality of the DNA and found of good quality.

The method performance characteristics were verified by quantifying blinded samples of unknown GM levels within the range 0.1%-8% (copy number basis, verified by means of digital PCR). The experiments were performed under repeatability conditions and demonstrated that the efficiency, linearity, trueness and precision of the method were within the limits established by the ENGL.

In addition, and in line with the requirements of Reg. (EU) No 619/2011 demanding the assessment of the conversion factor between genome copy numbers and mass fractions, the EU-RL GMFF investigated by digital PCR the ratio between GM and reference targets and determined the zygosity ratio of the positive control sample submitted. Moreover, the EU-RL GMFF verified on fifteen replicates the method's precision (relative repeatability standard deviation, RSDr %) at the 0.1% related to mass fraction of GM-material. Step 3 was concluded in September 2011 with the conclusion that the method shows the required performance.

The collaborative trial, involving 12 randomly selected laboratories (step 4) was organised and took place in December 2011-January 2012. It verified the precision and trueness of the method. It demonstrated that the method is well suited for identifying the presence of GM event MON87708 in DNA samples and is therefore applicable for this purpose.

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

Documentation and data provided by the applicant were evaluated (desk study) by the EU-RL GMFF for compliance with the ENGL method acceptance criteria. The parameters of the calibration curves (slope, R^2 coefficient) were determined by the applicant by quantifying three test samples in one run at different GM-levels (see Table 2). This procedure was found to be adequate.

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

MON87708		***le1***	
Slope	**R^2**	**Slope**	**R^2**
-3.47	1.00	-3.38	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 ≥ 0.98.

Table 1 indicates that the slope and R^2 coefficient of the standard curves for the GM-system (MON87708) and the soybean–specific *lectin* (le1) system, as established by the applicant, were 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 were provided. Table 2 reports precision and trueness values for the three GM-levels as provided by the applicant. Both parameters were within the ENGL acceptance criteria (trueness ± 25%, RSDr ≤ 25% across the entire dynamic range).

Table 2. Mean %, precision and trueness values provided by the applicant

	0.085	**1.0**	**10.0**
Mean %	0.10	1.2	10.1
Precision (RSDr %)	10	8.8	5.0
Trueness (bias %)	19	16	0.6

3. Step 3 (experimental testing of the samples and methods)

Please note that results of step 3 and of the international collaborative trial (step 4) are reported in chapter 5 of this validation report.

3.1 DNA extraction

Genomic DNA was isolated from ground soybean seeds and grains using a "CTAB-based" protocol coupled with PEG purification previously submitted for detection of soybean event MON89788.

This protocol has already been in-house tested by the EU-RL GMFF. The protocol for DNA extraction and a report on testing were published in 2008 at http://gmo-crl.jrc.ec.europa.eu/summaries/MON89788_Soya_DNAExtrSampl_report.pdf.

In agreement with the ENGL position, which endorses the modularity principle (see also Annex I to Reg. (EC) No 641/2004), and given the similarity in the matrix, the EU-RL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for soybean event MON87708.

3.2 Method protocol for the PCR analysis

The PCR method provided by the applicant and subsequently validated by the EU-RL GMFF (see http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event MON87708 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 MON87708 are performed in separate wells. A detailed validated Method Protocol is published by the EU-RL GMFF at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

For the detection of GM event MON87708, a 91-bp fragment of the region spanning the 3' plant-to-insert junction in soybean MON87708 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 (carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of GM event MON87708, a soybean taxon-specific system amplifies a 74-bp fragment of a soybean *lectin* (*le1*), an 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 MON87708 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 MON87708 DNA in a test sample, the MON87708 copy number is divided by the copy number of the soybean reference gene (*le1*) and multiplied by 100 to obtain the percentage value (GM% = MON87708/*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)[6]. 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)	200	50	12.52	3.13	1.04
Target taxon *le1* copies	176991	44248	11062	2765	922
Target MON87708 copies	17699	4425	1106	277	92

3.3 EU-RL GMFF experimental testing

3.3.1 Determination of the zygosity ratio in the positive control sample

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." This requires knowledge of the zygosity of the species in question. The EU-RL GMFF verified this by conducting an experimental assessment of the zygosity (GM-target to reference-target ratio) in the positive control sample that was submitted by the applicant, as follows.

The copy number of the MON87708 and of the *le1* targets in the positive control sample were determined by digital PCR (dPCR) performed on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Eight micrograms of genomic DNA control sample for soybean event MON87708 were digested at 37 ºC overnight with 20 units of the four-base cutter restriction enzyme TaqI. The latter does not cleave within the annealing sites for the primers of the MON87708 or *le1* amplification systems. Further to digestion, the DNA was precipitated with two volumes of absolute ethanol and ammonium acetate at a final concentration of 2.5 M. The outcome of the enzymatic digestion was controlled by running in 1% agarose-gel electrophoresis approximately 200 ng of Taq I digested and undigested DNA in comparison with DNA molecular markers. 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) (Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probes at the final concentrations indicated in the corresponding Validated Method document

(http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) (MON87708 primer 1 and MON87708 primer 2 at 300 nM each, MON87708 probe at 150 nM; *lec* F and *lec* R primers at 150 nM each, *lec* P probe at at 50 nM), 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). The reaction mixes were loaded into each well of which only approximately 4.6 µL were distributed into the 765 partitions 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 the Validated Method (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). Data analysis and copy number calculations were performed using the BioMark digital PCR Analysis software. The range of Ct retention was from 15 to 40.

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' [7].

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%-5% copy no/copy no). The experiments were performed on an ABI 7900 real-time platform under repeatability conditions and followed the protocol described above and published at (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). In addition test samples with GM-levels 0.45%, 0.9%, 4.0% and 8.0% 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^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 Reg. (EU) No 619/2011, the EU-RL GMFF also determined the zygosity of the GM-insert in the positive control sample. It also 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 trial involved twelve randomly selected 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 who had expressed their interest in participation. The study was carried out in accordance with the following internationally accepted guidelines:

- ISO 5725 (1994)[2]
- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995)[3]

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 described under 3.2, above.

3.4.1 List of participating laboratories

The participants in the MON87708 validation study were randomly selected from 24 national reference laboratories (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 event MON87708

Laboratory	Country
BioGEVES	FR
Central Agricultural Office	HU
Crop Research Institute	CZ
Danish Veterinary and Food Administration	DK
Environment Agency Austria	AT
Hessian State Laboratory	DE
Laboratory for the Detection of GMO in Food	DE
National Institute of Biological Resources	PT
National Institute of Biology	SI
National Research Institute of Animal Production, National Feed	PL
National Veterinary Research Institute	PL
Veterinary Public Health Institute for Lazio and Toscana Regions	IT

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipments: five laboratories used the ABI 7500, four used the ABI 7900, one used the Stratagene Mx 3000, one the Rotor-Gene 6000Q and one the ABI 7000.

This variability of equipment, with its known potential influence on PCR results, reflects the real-life situation in the 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, the following control samples were provided by the applicant. They consisted of:

i) genomic DNA extracted by the applicant from homozygous soybean seeds harbouring the event MON87708, and

ii) genomic DNA extracted by the applicant from conventional soybean seeds genetically similar to those harbouring the MON87708 event.

The control samples were used by the EU-RL GMFF to prepare blinded standard and test samples (of different GM-content) as mixtures of MON87708 soybean DNA and non-GM soybean DNA. The content, which was only known to the EURL, was expressed in terms of haploid genomes ratios between the calculated GM-DNA and target taxon-specific DNA copy numbers.

The calibration sample S1 was prepared by mixing the appropriate amount of MON87708 DNA with control non-GM soybean DNA to obtain a 10% (copy no/copy no) GM-sample. Calibration samples S2-S4 were prepared by 4-fold dilution from the S1 sample and the S5 sample was prepared by 3-fold dilution from the S4 sample.

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

- ✓ Five calibration samples with known concentrations of GM-event (160 µL of DNA solution each) labelled from S1 to S5 (Table 3).
- ✓ Twenty blinded test DNA samples (80 µL of DNA solution, each at 45 ng/µL) labelled from U1 to U20, representing 5 GM levels (information not known by the participants), each in 4 replicates (Table 5)

Table 5. MON87708 GM contents

MON87708 GM% GM copy number/soybean genome copy number x 100
8.0
4.0
0.90
0.45
0.10

- ✓ Reaction reagents:
  - TaqMan Universal PCR Master Mix (2x), three vials: 5 mL
  - distilled sterile water, one vial: 12 mL

- ✓ Primers and probes (1 tube each) as follows:

  le1 taxon-specific assay
  - LecF (10 µM): 240 µL
  - LecR (10 µM): 240 µL
  - LecP (5 µM): 160 µL

  MON87708 assay
  - MON87708 primer 1 (10 µM): 480 µL
  - MON87708 primer 2 (10 µM): 480 µL
  - MON87708 probe (5 µM): 480 µL

3.4.4 Design of the collaborative study

In total, two plates were run per laboratory. On each PCR plate, the samples were analysed for the MON87708-specific and for the *le1* taxon-specific systems.
The laboratories prepared the master-mixes for the MON87708 and the *le1* assays in accordance with the description provided in the method protocol (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). Calibration and test samples were loaded on the PCR plates as per plate lay-out provided to the participants. The amplification reactions followed the cycling program provided. Raw data were reported by the participating laboratory on an excel sheet designed, validated and distributed by the EU-RL GMFF. Participants determined the GM% in the test samples according to the instructions and using the excel sheet provided. The raw 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 data were stored by the EU-RL GMFF on a dedicated and protected server.

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

3.4.5 Deviations reported from the protocol

The ring-trial parcels were delivered to the twelve participating laboratories on 14th December 2011 via DHL courier. Due to causes independent of the EU-RL GMFF, the courier delayed the parcel consignments, otherwise expected to happen within 48 hours from delivery. The EU-RL GMFF monitored the delivery and informed the laboratories about the delay. Parcels were delivered between the 15th and 23rd December 2011. As per procedure, laboratories were asked to report the sample conditions at reception. Ten kilograms of dry-ice had been introduced into each parcel to preserve the reagents (3.4.3). Most laboratories reported that samples were still cold but not necessarily frozen with little dry-ice left in the parcel. Instructions were provided to laboratories to nevertheless proceed with the testing of the method as per protocol.

After the analyses, ten laboratories reported no deviations from the method protocol. One laboratory performed the qPCR reactions in a total volume of 20 µL because a 384-well plate configuration of the ABI 7900HT instrument was used. However, the final concentrations of the PCR reagents and the volume of DNA loaded per reaction remained unchanged. One laboratory adjusted the volumes of all reagents, except the DNA aliquot, proportionally to the maximum capacity of the 25 µL-wells.

4. Results

4.1 EU-RL GMFF experimental testing

4.1.1 Zygosity and conversion factor copy/copy to mass/mass

The results of the digital PCR analysis conducted by the EU-RL GMFF on the MON87708 and *le1* targets to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Zygosity ratio of the MON87708 and *le1* targets in the positive control sample

Mean ratio (MON87708/*le1*)	1.033
Standard deviation	0.088
RSD_r (%)	8.5
Standard error of the mean	0.023
Upper 95% CI of the mean	1.082
Lower 95% CI of the mean	0.985

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 homozygous GMO and a single-copy endogenous gene target, for an alpha = 0.05.

Hence:

GM % in DNA copy number ratio = GM % in mass fraction

4.1.2 Method performance

Test samples with GM-levels from 0.45% to 8.0% 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 sample at 0.1% GM-level was tested in 15 replicates in one run.
The corresponding standard curve parameters and the results of efficiency, linearity, trueness and precision are shown in tables 7 and 8.

Table 7. Standard curve parameters

	MON87708 system			***le1* system**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.42	96	1.00	-3.29	101	1.00
Run B	-3.38	98	1.00	-3.42	96	1.00
Run C	-3.40	97	1.00	-3.31	100	1.00
Run D	-3.35	99	1.00	-3.35	99	1.00
Run E	-3.40	97	1.00	-3.31	100	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$
Runs A-B were used to quantify GM-levels 0.45%, 4.0% and 8.0%; Runs C-D were used to quantify GM-level 0.9%; Run E was used to quantify GM-level 0.1%.

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 that the slopes of the standard curves and the R^2 coefficients were within the limits established by the ENGL. The EU-RL GMFF in-house results confirm the data provided by the applicant.

Table 8. Values of trueness and precision as established by the EURL GMFF in its *in-house* verification

Target GM-levels %	**Measured GM level %**	**Bias % of the target GM-level**	**Precision (RSDr %)**
8.0	8.8	10.3	7.3
4.0	3.9	-1.8	6.3
0.9	0.99	10.2	10.3
0.45	0.42	-6.1	10
0.10	0.09	-7.8	18

According to the ENGL method acceptance criteria the method trueness (measured as bias in % of the target GM level) should be within ±25% of the accepted reference value over the entire dynamic range and the method's precision, expressed as RSDr % (relative standard deviation of repeatability), should be ≤25%, also over the entire dynamic range. Table 8 documents that trueness and precision 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 Efficiency = $(10^{(-1/slope)}) - 1) \times 100$], and R^2 values (expressing the linearity of the regression) reported by participating laboratories for the MON87708 and the *le1* assays are provided in Table 9.

Table 9. Values of slope, PCR efficiency and R^2 obtained during the international collaborative trial

		MON87708			*le1*		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.21	105	1.00	-3.36	99	1.00
	B	-3.13	109	0.99	-3.36	99	1.00
2	A	-3.49	93	1.00	-3.51	93	1.00
	B	-3.41	96	1.00	-3.40	97	1.00
3	A	-3.46	94	1.00	-3.47	94	1.00
	B	-3.41	97	1.00	-3.38	97	1.00
4	A	-3.49	93	1.00	-3.47	94	1.00
	B	-3.56	91	0.99	-3.47	94	1.00
5	A	-3.54	92	1.00	-3.52	92	1.00
	B	-3.56	91	1.00	-3.54	92	1.00
6	A	-3.54	92	1.00	-3.53	92	1.00
	B	-3.44	95	0.99	-3.48	94	1.00
7	A	-3.58	90	1.00	-3.45	95	1.00
	B	-3.41	96	1.00	-3.58	90	1.00
8	A	-3.32	100	1.00	-3.37	98	1.00
	B	-3.40	97	1.00	-3.39	97	1.00
9	A	-3.26	103	1.00	-3.30	101	1.00
	B	-3.34	99	1.00	-3.30	101	1.00
10	A	-3.48	94	0.98	-3.34	99	1.00
	B	-3.25	103	0.99	-3.78	84	0.99
11	A	-3.35	99	1.00	-3.41	96	1.00
	B	-3.36	98	1.00	-3.39	97	1.00
12	A	-3.53	92	1.00	-3.60	90	1.00
	B	-3.49	93	1.00	-3.53	92	1.00
	Mean	-3.42	96	1.00	-3.46	95	1.00

Table 9 indicates that the efficiency of amplification for the MON87708 system ranges from 90 to 109 and the linearity from 0.98 to 1.00; the amplification efficiency for the soybean-specific system ranges from 84% to 101% and the linearity is about 1.00. The mean PCR efficiency was 96% for the MON87708 assay and 95% for the *le1* assay. Both values were within the ENGL acceptance criteria. The average R^2 of the methods was 1.00 for both the MON87708 and *le1* assays.

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

4.2.2 GMO quantification

Table 10 reports the values of quantification for the four replicates of each GM level as reported by each of the twelve participating laboratories.

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

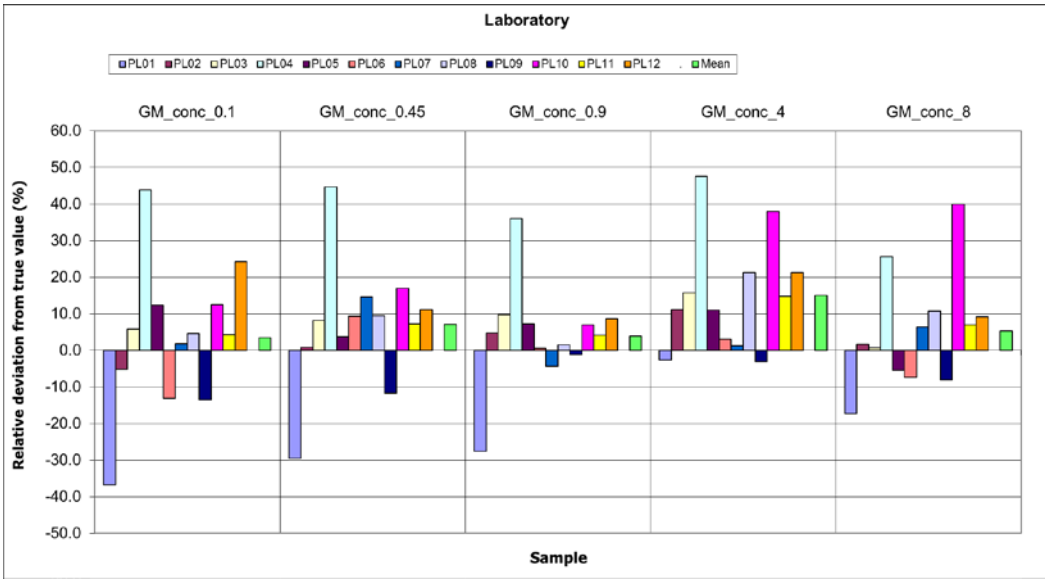
	GMO content (%) *																			
LAB	0.1				0.4				0.9				2.0				5.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.07	0.05	0.06	0.07	0.33	0.28	0.33	0.32	0.73	0.63	0.63	0.62	4.04	3.34	4.83	3.36	6.32	7.96	6.32	5.89
2	0.08	0.10	0.08	0.11	0.41	0.39	0.51	0.51	0.99	0.86	0.96	0.96	4.86	4.30	4.22	4.40	9.19	7.98	8.02	7.33
3	0.09	0.11	0.11	0.11	0.39	0.46	0.54	0.56	1.04	0.93	0.97	1.01	4.51	4.74	4.76	4.51	8.37	8.72	7.76	7.35
4	0.10	0.12	0.18	0.18	0.47	0.50	0.78	0.85	1.00	0.97	1.48	1.45	6.74	4.99	7.18	4.71	8.13	7.98	11.57	12.49
5	0.10	0.12	0.12	0.11	0.52	0.41	0.46	0.47	1.05	1.03	0.91	0.87	4.26	4.52	4.20	4.78	8.07	7.65	7.25	7.28
6	0.09	0.05	0.10	0.10	0.48	0.46	0.54	0.50	0.95	0.91	0.83	0.93	3.99	3.75	4.63	4.11	7.65	6.86	7.79	7.33
7	0.09	0.11	0.13	0.08	0.55	0.44	0.48	0.59	0.93	0.78	0.84	0.91	3.24	4.41	4.20	4.33	9.97	8.57	9.43	6.06
8	0.09	0.11	0.11	0.12	0.48	0.54	0.43	0.52	0.89	0.94	0.86	0.96	5.34	4.34	4.47	5.27	8.18	9.79	8.52	8.92
9	0.08	0.09	0.10	0.08	0.42	0.37	0.38	0.42	0.85	0.94	0.85	0.92	3.88	4.02	3.99	3.62	7.58	6.49	8.65	6.70
10	0.08	0.13	0.12	0.12	0.70	0.43	0.51	0.47	1.44	0.90	0.78	0.73	4.50	5.66	5.13	6.79	15.52	11.68	7.24	10.32
11	0.10	0.12	0.10	0.10	0.43	0.45	0.55	0.51	0.95	1.03	0.89	0.88	4.68	4.55	4.87	4.27	8.84	8.99	7.93	8.45
12	0.10	0.12	0.13	0.14	0.48	0.44	0.54	0.54	1.02	0.89	0.98	1.01	4.69	5.06	4.72	4.94	9.12	9.16	8.45	8.21

* GMO% = (GMO copy number/soybean genome copy number) x 100 and (GM DNA mass/soybean DNA mass) x 100

A graphical representation of the data reported in Table 10 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 green bar on the right represents the mean relative deviation over all twelve participating laboratories for each true GM level.

Data of all replicates were retained for the statistical analysis and for application of the Cochran and Grubbs tests, which according to ISO 5725 are performed for identifying outlying values. (Cochrane and Grubbs). The results of the Cochrane and Grubbs tests are reported in Table 11.

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



*PL6 at GM level 0.9% and PL 3 at GM level 8.0% had very small relative deviations from the true value and the corresponding histograms do not show up in Figure 1. PL = participating laboratory.

Overall a trend for overestimation of the amount of the GM-target over the dynamic range can be observed. The mean bias generated by all laboratories is slightly over-predictive, indeed it is +7.0% and +15.0% at the 0.45% and 4.0% GM-level respectively, while for the other GM-levels the other values of bias are lower. The method is nonetheless well within the accepted limits established by the ENGL (± 25% over the entire dynamic range).

4.2.3 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. Table 11 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).

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. Indeed, the highest value of RSD_R is 25% at the 0.1% GM level, thus within the acceptance criterion.

Table 11. Summary of validation results for the MON87708 method.

	Test Sample Expected GMO %				
	0.1	**0.45**	**0.9**	**4.0**	**8.0**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	2	3	0	2
Reason for exclusion	-	1C, 1DG	2C, 1G	-	2C
Mean value	0.10	0.48	0.93	4.6	8.0
Relative repeatability standard deviation, RSD_r (%)	17	13	6.6	13	11
Repeatability standard deviation	0.02	0.06	0.06	0.58	0.84
Relative reproducibility standard deviation, RSD_R (%)	25	14	7.3	17	13
Reproducibility standard deviation	0.03	0.07	0.07	0.80	1.02
Bias (absolute value)	0.00	0.03	0.03	0.60	-0.02
Bias (%)	3.4	7.0	3.4	15	-0.3

C= Cochran's test; G= Grubbs' test; DG= Double Grubbs 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 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 and ENGL requires that the RSD_r value indicated by the applicant and confirmed by the EU-RL 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 0.1% 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 ± 25% across the entire dynamic range. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 15% at the 4.0% GM level.

5. Compliance of the method for detection and quantification of event MON87708 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 acknowledged that the RSDr value at the 0.08% level shown by the applicant's dossier (expressed as ratio between GM- and target taxon-specific DNA copy numbers) was 9.5%, based on 16 replicates (Table 2), hence below the maximum value of 25% required by the ENGL. The EU-RL therefore concluded that it could accept the applicant's data on method performance;

- at step 3 of the validation process (in-house testing of the method), the EU-RL GMFF determined the RSDr at the 0.1% level expressed as mass fraction of GM-material. The RSDr was assessed on the basis of fifteen replicates carried out under repeatability conditions (single run) and resulted equal to 18% (Table 8), hence also below 25% and, while being higher, still confirming the applicants data;

- the collaborative study (step 4 of the validation process) established that over the twelve participating laboratories at the level of 0.1% the RSDr of the method related to mass fraction of GM-material was 17 %, therefore also below 25% and well in line with the previous data.

A summary of the precision data obtained in the different steps of the process is presented in Table 12.

Table 12. Precision of method for quantitative detection of MON87708

Source	**RSDr %**	**GM %**
Applicant' method optimisation*	9.5 %	0.085 %
EU-RL GMFF tests	18 %	0.1 %
Collaborative study	17 %	0.1 %

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

The results of the EU-RL GMFF in-house verification and of the collaborative study indicate that the method RSDr is less than 25% at the level of 0.1% expressed as mass fraction of GM-material. It is concluded therefore, that the method for quantitative detection of soybean event MON87708 meets the requirements laid down in Regulation (EU) No 619/2011.

6. Conclusion

The method provided by the applicant and described in detail under 3.2 (and available as "Validated Method" at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm and in Annex 1) has been validated in accordance to the EU-RL GMFF validation scheme, respecting all requirements of the relevant EU legislation and international standards for method validation.

The dossier was found complete (step 1) and its scientific assessment (step 2) concluded that the method could meet the ENGL minimum performance criteria for entering into validation.

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

The data obtained in the international collaborative study (step 4) also indicated that the method meets all acceptance criteria and the performance requirements recommended by the ENGL (as detailed at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

In conclusion, the 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-2.C.2 to Commission Regulation (EC) No 641/2004[8] and (EU) No 619/2011. The EU-RL further concludes that the method, if carried out in accordance with the validated method protocol (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm), and in line with the modular approach established by the ENGL, is applicable to appropriately extracted soybean DNA.

7. References

1 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”.

2. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
3. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method - performance studies, *Pure & Appl. Chem.* 67, 331-343.

4. Commission 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 Text with EEA relevance. *OJ L 166, 25.6.2011, p. 9–15.*

5. EURL/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing” (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).
6. Arumuganathan K. and Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9, 208-218.
7. 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.
8. Commission Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation (Text with EEA relevance). OJ L 102, 7.4.2004, p. 14–25.

Annex 1: Event-specific Method for the Quantification of Soybean MON87708 Using Real-time PCR

Validated Method

Method development:

Monsanto Company

Method validation:

European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF)

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR (polymerase chain reaction) procedure for the determination of the relative content of soybean event MON87708 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 MON87708, a 91-bp fragment of the region spanning the 3' insert-to-plant junction in soybean MON87708 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 MON87708 DNA, a soybean taxon-specific system amplifies a 74-bp fragment of *lectin (le1)*, a soybean endogenous gene (Accession number, GeneBank: K00821), 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 at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of MON87708 DNA in a test sample, Ct values for the MON87708 and the *le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON87708 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. Its performance characteristics were verified by the EURL-GMFF and its precision and trueness were validated through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The international collaborative ring trial was organised in December 2011-January 2012 by the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF) and involved twelve randomly selected laboratories.

Each laboratory received twenty blind samples containing soybean MON87708 genomic DNA at five levels of GM contents, ranging from 0.1% to 5%.
Each test sample was analysed by each laboratory in accordance to the method protocol by PCR in three repetitions. The trial was designed as a blind quadruplicate collaborative trial; each laboratory received each GM level of event MON877089 in four replicates. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.04% 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 at least 0.085% 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%.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 3' insert-to-plant junction in soybean MON87708 and is therefore event-specific for the event MON87708.

The specificity of the event-specific assay was assessed by the applicant in real-time PCR, according to the method described (Tables 1, 2, 3 and 4), using genomic DNA extracted from oilseed rape RT73, RT200; maize GA21, NK603, MON810, MON863, MON88017, LY038, MON89034, MON87460; cotton MON531, MON15985, MON1445, MON88913; soybean 40-3-2, MON89788, MON87701, MON87769, MON87708; wheat MON71800 and conventional oilseed rape, maize, cotton, soybean, wheat, millet, lentil, sunflower, peanut (shelled), and quinoa.

According to the method developer the MON87708 method did not react with any sample except the positive control.

The specificity of the soybean taxon-specific assay was assessed by the method developer using real-time PCR, according to the method described (Tables 1, 2, 3 and 4), with genomic DNA extracted from oilseed rape RT73, RT200; maize GA21, NK603, MON810, MON863, MON88017, LY038, MON89034, MON87460; cotton MON531, MON15985, MON1445, MON88913; soybean 40-3-2, MON89788, MON87701, MON87708; wheat MON71800 and conventional oilseed rape, maize, cotton, soybean, wheat, millet, lentil, sunflower, peanut (shelled) and quinoa.

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 MON87708

3.2.1 General

The qPCR set-up for the taxon-specific target sequence (*le1*) and for the GMO (event MON87708) target sequence is carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated by the EU-RL GMFF.
The method is developed for a total volume of 50 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

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 MON87708 DNA in a total of 200 ng of soybean DNA (corresponding to 176991 soybean genome copies with one haploid genome assumed to correspond to 1.13 pg of soybean genomic DNA) [(1)]. Standards S2 to S4 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 reaction (ng)	200	50	12.52	3.13	1.04
le1 copies	176991	44248	11062	2765	922
MON87708 copies	17699	4425	1106	277	92

A calibration curve is to be produced by plotting the Ct 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 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 MON87708 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 MON87708 assay.

Component	Final concentration	µL / reaction
TaqMan Universal PCR Master Mix (2x)	1x	25
MON87708 primer 1 (10 µM)	300 nM	1.5
MON87708 primer 2 (10 µM)	300 nM	1.5
MON87708 probe (5 µM)	150 nM	1.5
Nuclease free water	-	16.5
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 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 (5 µM)	50 nM	0.50
Nuclease free water	-	19
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 MON87708 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 161 µL for the MON87708 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. 10 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 MON87708/*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	Denaturation	95	15	No	45
		Annealing & Extension	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. MON87708) 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 Ct 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 Ct = 25, set the baseline crossing at Ct = 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 Ct values for each reaction.

The standard curves are generated both for the *le1* and the MON87708 specific assays by plotting the Ct 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 MON87708 DNA in the unknown sample, the MON87708 copy number is divided by the copy number of the soybean endogenous gene *le1* and multiplied by 100 (GM% = MON87708/*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 Part No 4304437.

4.3 Primers and Probes

Table 4. primers and probes for the MON87708 and *lectin (le1)* methods

		DNA Sequence (5' to 3')	Length (nt)
MON87708			
Forward primer	MON87708 primer 1	5' – TCA TAC TCA TTG CTG ATC CAT GTA G – 3'	25
Reverse primer	MON87708 primer 2	5' – AGA ACA AAT TAA CGA AAA GAC AGA ACG – 3'	27
Probe	MON 87708 probe	6-FAM 5' – TCC CGG ACT TTA GCT CAA AAT GCA TGT A – 3' TAMRA	28
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 probe	6-FAM 5' – CTT CAC CTT CTA TGC CCC TGA CAC – 3' TAMRA	24

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine;

5. References

1. Arumuganathan K., Earle E.D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter,* 9: 208-218.