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Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

EURL European Union Reference Laboratory for GM Food & Feed

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

Validated Method

6 May 2013

Method development:

Monsanto Company

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

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. 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 (re-)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 qPCR (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 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 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. 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 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.