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

Validated Method

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

Method development:

Monsanto Company

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 AA**A** – TAMRA – 3′	29

changed to:

Probe	MON 87751 probe	5′ – 6FAM – TgA CTg gAg ATC TCC AAA gTg Agg ggA AA – TAMRA – 3′	29

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 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 "Cq" value. For quantification of the amount of MON 87751 DNA in a test sample, Cq 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) [(1)]. 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 *Le1* 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 Cq 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® 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® 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	Denaturation	95	15	No	40
		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. 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 Cq 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 Cq = 25, set the baseline crossing at Cq = 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 Cq 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/