Event-specific Method for the Quantification of Soybean MON 87705 Using Real-time PCR

Protocol

17 January 2012

Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Method development:

Monsanto Company

Method validation:

European Union Reference Laboratory for Genetically Modified Food and Feed
(EU-RL GMFF)
Molecular Biology and Genomics Unit

Drafted by
C. Savini (scientific officer)

Report Review
1) M. Querci

2) L. Bonfini

Scientific and technical approval
M. Mazzara (scientific officer)

Compliance to EURL Quality System
S. Cordeil (quality manager)

Authorisation to publish
G. Van den Eede (head of MBG Unit)

Address of contact laboratory:

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

Content

1. General information and summary of the methodology

This protocol describes an event-specific quantitative real-time TaqMan® PCR (polymerase chain reaction) procedure for the determination of the relative content of soybean event MON 87705 DNA to total soybean DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels.

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

For the specific detection of soybean event MON 87705 DNA, an 86-bp fragment of the region spanning the 3′ plant-to-insert junction in soybean MON 87705 event is amplified using specific primers. PCR products are measured at 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 TAMRA (carboxytetramethylrhodamine) as a quencher dye at its 3′ end.

For the relative quantification of soybean event MON 87705, a soybean-specific reference system amplifies a 74-bp fragment of *Le1*, a soybean endogenous gene encoding lectin, using specific primers and a *Le1* gene-specific probe labelled with FAM as a reporter dye at its 5′ end, and TAMRA as a quencher dye 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 MON 87705 DNA in a test sample, Ct values for the MON 87705 and *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON 87705 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.

The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GMO contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the EU-RL GMFF. The study was undertaken with twelve participating laboratories in December 2010.

Each participant received twenty blind samples containing soybean MON 87705 genomic DNA at five GM contents, ranging from 0.1% to 8.0%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of event MON 87705 in four unknown samples. 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 ≤ 0.04% in 200 ng of total 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 ≤ 0.085% in 200 ng of total soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.09%.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome; the sequence is specific to soybean event MON 87705 and thus imparts event-specificity to the method.

The specificity of both the event-specific and the soybean-specific assays was assessed by the method developer in real-time PCR against DNA extracted from plant materials containing the specific targets of maize GA21, NK603, MON810, MON863, MON88017, LY038, MON88034, MON87460 and conventional maize; canola RT73, RT200 and conventional canola; soybean GTS 40-3-2, MON89788, MON87769 and conventional soybean; wheat MON71800 and conventional wheat; rice, lentils, Buckwheat, peanuts (shelled), quinoa; cotton MON531, MON 15985, MON1445, MON88913 and conventional cotton.

According to the method developer, the MON 87705 assay did not react with any of the plant materials tested, except the MON 87705 positive control; the soybean-specific reference system reacted only with conventional soybean and with the soybean GM events tested.

The EU-RL GMFF assessed *in silico* the specificity of the event-specific assay by running similarity searches against the Central DNA Core Sequence Information System (CCSIS). The CCSIS contains non-publicly available DNA sequences of GM events from applications for authorisation according to Regulation No (EC) 1829/2003 on GM Food and Feed. The sequence records are stored locally on a dedicated server and are accessible with restricted user-access

integrated with common bioinformatics applications (BLAST, ClustalW, EMBOSS package) for immediate bioinformatics analyses.

The results of the bioinformatics analysis showed that part of the 5' flanking region is duplicated from the 3' flanking region. The reverse primer (MON 87705 primer 2) is located in this flanking region, i.e. it is complementary to both the 5' and 3' flanking region. Nevertheless no significant sequence similarity for the reverse primer was found in the CCSIS. The forward primer (MON 87705 primer 1) is complementary to a sequence of the left border region of the T-DNA, i.e. it matches many sequences of GM events containing the left border region. The probe is located on the junction between the insert and the duplicated 3' flanking region, covering just 2 nucleotides of the insert and 28 nucleotides of the duplicated flanking sequence.

The EU-RL GMFF consulted with the European Food Safety Authority (EFSA) on the findings of the bioinformatics analysis; after the assessment of the EFSA Molecular Characterisation Working Group of the GMO Panel, EFSA concluded that it could not find evidence for instability in event MON 87705.

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 used should be sterilised prior to use and any residue of DNA has to be 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 frequently.
- 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 0 – 4°C.

- In order to avoid repeated freeze/thaw cycles, aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of soybean event MON 87705

3.2.1 General

The PCR set-up for the taxon specific target sequence (*Le1*) and for the GMO (event MON 87705) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended. 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 consist of five samples. The first point of the calibration curves is a 10% MON 87705 DNA in non-GM soybean DNA for a total of 200 ng of DNA (corresponding to approximately 176991 soybean genome copies with one genome assumed to correspond to 1.13 pg of haploid soybean genomic DNA) [(1)]. The other four standards are prepared by serial dilution.

A calibration curve is 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 sequence detection system 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 gently and centrifuge the required amount of components needed for the run. Keep thawed reagents on ice.
2. To prepare the amplification reaction mixtures add the following components (Tables 1 and 2) in two reaction tubes (one for the MON 87705 assay and one for the *Le1* assay) on ice in the order mentioned below (except DNA).
3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the soybean MON 87705 and one for the *Le1* reaction mix) for each DNA sample to be tested (standard curve samples, unknown samples and controls).

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 87705 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
MON 87705 forward primer (20 µM)	450 nM	1.125
MON 87705 reverse primer (20 µM)	450 nM	1.125
MON 87705 probe (5 µM)	250 nM	2.5
Nuclease free water	#	16.25
Template DNA (max 200 ng)	#	4
Total reaction volume:		50

Table 2. 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
Le1 primer 1 (10 µM)	150 nM	0.75
Le1 primer 2 (10 µM)	150 nM	0.75
Le1 probe (5 µM)	50 nM	0.5
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4
Total reaction volume:		50

5. Add to each reaction tube the correct amount of reaction mix (e.g. 46 µL x 3 = 138 µL reaction mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 4 µL x 3 = 12 µL DNA for three PCR repetitions). Vortex each tube for approx. 10 sec. 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 room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 3.

Table 3. Cycling program for MON 87705/*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	

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 system (e.g. MON 87705) 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. 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 geometric 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) and b) on the amplification plots of the other system (e.g. *Le1* system).

e) Save the settings and export all the data to a text file 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 MON 87705 specific systems 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 numbers in the unknown sample.
To obtain the percentage value of event MON87705 DNA in the unknown sample, the MON 87705 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 (GM% = MON 87705/*Le1* x 100).

4. 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 run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437.

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')	Length (nt)
MON 87705 target sequence		
MON 87705 forward primer	5' – TTC CCg gAC ATg AAg CCA TTT AC - 3'	23
MON 87705 reverse primer	5' – ACA ACg gTg CCT Tgg CCC AAA g - 3'	22
MON 87705 probe	6-FAM 5' – AAg AgA CTC Agg gTg TTg TTA TCA CTg Cgg - 3' TAMRA	30
Taxon specific *le1* target sequence		
Le1 primer 1	5' - CCA gCT TCg CCg CTT CCT TC - 3'	20
Le1 primer 2	5' - gAA ggC AAg CCC ATC TgC AAg CC - 3'	23
Le1 probe	6-FAM 5' - CTT CAC CTT CTA TgC CCC TgA CAC -3' TAMRA	24

FAM: 6-carboxyfluorescein; TAMRA: tetramethylrhodamine

5. References

1. Royal Botanic Garden, Kew. Plant DNA c-values database. http://data.kew.org/cvalues/