# Event-specific Method for the Quantification of Soybean MON 87701 Using Real-time PCR

## Protocol

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# Content

# 1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative PCR (polymerase chain reaction) procedure for the determination of the relative content of soybean event MON 87701 DNA to total soybean DNA in a sample.

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

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 detection of soybean event MON 87701, an 89 bp fragment of the integration region of the construct inserted into the plant genome (5' insert-to-plant junction) is amplified using specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as a reporter dye and TAMRA (carboxytetramethylrhodamine) as a quencher dye.

For the relative quantification of soybean event MON 87701, a soybean-specific reference system amplifies a 74 bp fragment of the soybean *lectin* gene (*Le1*), using specific primers and a *Le1* specific probe labelled with FAM and TAMRA as described above.

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 87701 DNA in a test sample, Ct values for the MON 87701 and *Le1* system are determined for the sample. Standard curves are then used to estimate the relative amount of MON 87701 DNA to total soybean DNA.

# 2. Validation and performance characteristics

## 2.1 General

The method was optimised for suitable DNA extracted from homogenised soybean grains.
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 a collaborative study coordinated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF). The study was undertaken with twelve participating laboratories in December 2009.

Each participant received twenty blind samples containing MON 87701 genomic DNA at five GM contents, ranging from 0.085% to 8.1%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each GM level 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 at least 0.04% in 200 ng of total soybean DNA. The relative LOD was not assessed in a 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 soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.085%.

## 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 87701 and thus imparts event-specificity to the method.

The event-specificity of the assay was tested by the method developer in real-time PCR against 200 ng of genomic DNA of oilseed rape RT73, RT200, maize GA21, NK603, MON 810, MON 863, MON 88017, LY038, MON 89034, MON 87460, cotton MON 531, MON 15985, MON 1445, MON 88913, soybean 40-3-2, MON 89788, wheat MON 71800 and against 200 ng genomic DNA of non-GM oilseed rape, maize, cotton, soybean and wheat.
According to the method developer, none of the materials tested, except the positive control soybean event MON 87701 yielded detectable amplification.

The specificity of the soybean reference assay *Le1* was tested by the method developer in real-time PCR against DNA extracted from plant materials containing 200 ng genomic DNA of oilseed rape RT73, RT200, maize GA21, NK603, MON 810, MON 863, MON 88017, LY038, MON 89034, MON 87460, cotton MON 531, MON 15985, MON 1445, MON 88913, soybean 40-3-2, MON 89788, MON 87701, wheat MON 71800 and against 200 ng genomic DNA of non-GM oilseed rape, maize, cotton, soybean, wheat.
According to the method developer, none of the materials tested, except the soybean materials, yielded detectable amplification.

# 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 the guidelines given by relevant authorities, e.g. ISO 24276
- 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 be removed. All materials 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 hypochlorite 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 87701

### 3.2.1 General

The PCR set-ups for the taxon-specific target sequence (*Le1*) and for the GMO (event MON 87701) are 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 standard curves consist of five samples. The first point of the calibration curves is a 9 % MON 87701 soybean DNA in a total of 200 ng of soybean DNA (corresponding to approximately 176,991 soybean genome copies with one genome assumed to correspond to 1.13 pg of haploid soybean genomic DNA) [(1)]. The other four standard samples 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 e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly using the options available with the sequence detection system software.

The copy number measured for the 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 at 1-4 °C 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 87701 assay and one for the *Le1* assay) on ice in the order mentioned below (except DNA).

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 87701 assay

| Component | Final concentration | µL / reaction |
|---|---|---|
| TaqMan® Universal PCR Master Mix (2x) | 1x | 25 |
| MON 87701 primer 1 (20 µM) | 600 nM | 1.50 |
| MON 87701 primer 2 (20 µM) | 600 nM | 1.50 |
| MON 87701 probe (5 µM) | 250 nM | 2.50 |
| Nuclease-free water | # | 15.50 |
| 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* reference 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 | 0.50 |
| Nuclease-free water | # | 19 |
| Template DNA (max 200 ng) | # | 4.0 |
| Total reaction volume: | | 50 |

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the MON 87701 and one for the *lec* reaction mixes) 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 master mix (e.g. 46 x 3 = 138 µL master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 4 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 250x *g* for 1 minute at 4 °C) to spin down the reaction mixture.
7. Place the plate in the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Cycling program for MON 87701/*lec* assays

| Step | Stage | | T°C | Time (sec) | Acquisition | Cycles |
|---|---|---|---|---|---|---|
| 1 | UNG | | 50 °C | 120 | No | 1 |
| 2 | Initial denaturation | | 95 °C | 600 | No | 1 |
| 3 | Amplification | Denaturation | 95 °C | 15 | No | 45 |
| | | Annealing & Extension | 60 °C | 60 | Yes | |

## 3.3 Data analysis

Subsequent to 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 87701) 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. 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 87701 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 copy numbers in the unknown sample DNA.

To obtain the percentage value of event MON 87701 DNA in the unknown sample, the MON 87701 copy number is divided by the copy number of the soybean reference gene (*lec*) and multiplied by 100 (GM% = MON 87701/*Le1* x 100).

# 4. Materials

## 4.1 Equipment

- Real-time PCR instrument for plastic reaction plates (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction plates 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
- Centrifuge for plates
- Micropipettes
- Vortex
- Rack for reaction tubes
- 0.5/1.5/2.0 mL reaction tubes

## 4.2 Reagents

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

## 4.3 Primers and Probes

Table 4. Primer and probe sequences

| Name | Oligonucleotide DNA Sequence (5' to 3') |
|---|---|
| MON 87701 target sequence | |
| MON 87701 1 (reverse) | 5' – CGT TTC CCG CCT TCA GTT TAA A – 3' |
| MON 87701 2 (forward) | 5' – TGG TGA TAT GAA GAT ACA TGC TTA GCA T – 3' |
| MON 87701 | 6 - FAM 5' – TCA GTG TTT GAC ACA CAC ACT AAG CGT GCC – 3' TAMRA |
| Taxon-specific *Le1* target sequence | |
| Le1 (forward) | 5' – CCA GCT TCG CCG CTT CCT TC – 3' |
| Le1 (reverse) | 5' – GAA GGC AAG CCC ATC TGC AAG CC – 3' |
| *Le1* | 6-FAM 5' – CTT CAC CTT CTA TGC CCC TGA CAC – 3' TAMRA |

FAM: 6-carboxyl-fluorescein; TAMRA: tetramethyl-6-carboxyrhodamine

# 5. References

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