



Event-specific Method for the Quantification of Soybean CV127 Using Real-time PCR

Protocol

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Method validation:

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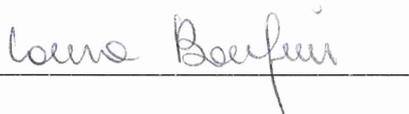
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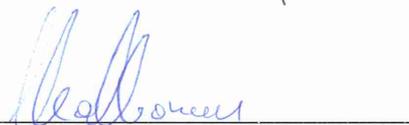
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1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan[®] PCR procedure for the determination of the relative content of soybean event CV127 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 CV127 DNA, an 88-bp fragment of the region spanning the 3' plant-to-insert junction in soybean CV127 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 TAMRA (carboxytetranethylrhodamine) as quencher at its 3' end.

For the relative quantification of soybean event CV127 DNA, a soybean-specific reference system amplifies a 74-bp fragment of *lectin1 (le1)*, a soybean 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 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 CV127 DNA in a test sample, Ct values for the CV127 and *le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of CV127 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 EURL-GMFF. The study was undertaken with twelve participating laboratories in July 2009.

Each participant received twenty blind samples containing soybean CV127 genomic DNA at five GM contents, ranging from 0.09% to 4.5%.

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 CV127 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 $\leq 0.04\%$ in 100 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 $\leq 0.08\%$ in 100 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 event CV127 and thus imparts event-specificity to the method.

The specificity of the soybean taxon-specific assay was assessed by the method developer in real-time PCR against 100 ng DNA extracted from plant materials of rapeseed, rice, wheat, potato, sugar beet, cotton, maize, common bean, mung bean, lentil, chickpea and soybean line CV127. According to the method developer the soybean-specific reference system did not react with any target DNA except the positive control soybean line CV127.

The specificity the GMO assay was assessed by the applicant in real-time PCR against DNA (100 ng) extracted from plant materials containing 1% of CV127, 40-3-2, A2704-12, 305423, 356043 soybean; GA21, Bt176, Bt11, NK603, MON863, TC1507, MIR604, MON810, 59122, T25 maize; Ms8, Rf3 rapeseed; MON531, MON15985, 281-24-236x3006-210-23 cotton; LL62 rice; H7-1 sugar beet; EH 92-527-1 potato; and in real-time PCR against 100 ng DNA extracted from plant materials of soybean, cotton, potato, rapeseed, rice, sugar beet, maize, mouse ear cress.

According to the method developer, the CV127 system did not react with any of the plant materials tested, except the positive control soybean line CV127.

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 CV127

3.2.1 General

The PCR set-up for the taxon specific target sequence (*le1*) and for the GMO (event CV127) target sequence is carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 100 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 25 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 sample containing 5% CV127 in non-GM soybean DNA for a total of 100 ng of DNA (corresponding to approximately 86960 soybean genome copies with one genome assumed to correspond to 1.15 pg of haploid soybean genomic DNA) ⁽¹⁾. The other four standards are prepared by serial 2.8-fold 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 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 (Table 1 and 2) in two reaction tubes (one for the CV127 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 CV127 assay.

Component	Final concentration	µL/reaction
TaqMan [®] Universal PCR Master Mix (2x) with UNG	1x	12.5
SE-127-f4 (10 µM)	400 nM	1.0
SE-127-r2 (10 µM)	400 nM	1.0
SE-127-p3 (10 µM)	100 nM	0.25
Nuclease free water	#	5.25
Template DNA (max 100 ng)	#	5
Total reaction volume:		25

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) with UNG	1x	12.5
<i>Lec F</i> (10 µM)	150 nM	0.375
<i>Lec R</i> (10 µM)	150 nM	0.375
<i>Lec P</i> (10 µM)	50 nM	0.125
Nuclease free water	#	6.625
Template DNA (max 100 ng)	#	5
Total reaction volume:		25

- Mix gently and centrifuge briefly.
- Prepare two reaction tubes (one for the soybean CV127 and one for the *le1* reaction mix) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of reaction mix (e.g. 20 µL x 3 = 60 µL reaction mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 µL x 3 = 15 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.
- Spin down the tubes in a microcentrifuge. Aliquot 25 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.
- Place the plate into the instrument.
- Run the PCR with the cycling program described in Table 3.

Table 3. Cycling program for CV127/*le1* assays.

Step	Stage	T (°C)	Time (s)	Acquisition	Cycles
1	UNG	50	120	No	1
2	Initial denaturation	95	600	No	1
3	Denaturation	95	15	No	45
	Amplification 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 assay (e.g. CV127) 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 assay (e.g. *le1*).
- 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 CV127 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 numbers in the unknown sample.

To obtain the percentage value of event CV127 DNA in the unknown sample, the CV127 copy number is divided by the copy number of the soybean reference gene (*le1*) and multiplied by 100 (GM% = CV127/*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 4326708.

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
CV127 target sequence	
SE-127-f4	5' – AAC AGA AGT TTC CGT TGA GCT TTA AGA C- 3'
SE-127-r2	5' – CAT TCG TAG CTC GGA TCG TGT AC - 3'
SE-127-p3	6-FAM - 5' – TTT GGG GAA GCT GTC CCA TGC CC-TAMRA - 3'
<i>le1</i> target sequence	
<i>Lec F</i>	5' – CCA GCT TCG CCG CTT CCT TC - 3'
<i>Lec R</i>	5' – GAA GGC AAG CCC ATC TGC AAG CC - 3'
<i>Lec P</i>	6-FAM - 5' – CTT CAC CTT CTA TGC CCC TGA CAC - TAMRA -3'

5. References

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