



Event-specific Method for the Quantification of Soybean Event A5547-127 Using Real-time PCR

Protocol

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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 A5547-127 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 A5547-127 DNA, a 75-bp fragment of the region that spans the 5' plant-to-insert junction in soybean A5547-127 event is amplified using two 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, as a reporter at its 5' end, and with the non-fluorescent quencher TAMRA at its 3' end.

For the relative quantification of soybean event A5547-127 DNA, a soybean-specific reference system amplifies a 102-bp fragment of the soybean endogenous *lectin* gene (*Le1*), using two specific primers and a *Le1* gene-specific probe labelled with VIC, as a reporter at its 5' end, and with the non-fluorescent quencher TAMRA 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 event A5547-127 DNA in a test sample, soybean A5547-127 and *Le1* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of soybean event A5547-127 DNA to total soybean DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from soybean leaves, seeds and grains containing mixtures of genetically modified and conventional soybean.

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 Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve participating laboratories in June 2008.

Each participant received twenty blind samples containing soybean A5547-127 genomic DNA at five GM contents, ranging from 0.08% 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 A5547-127 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 0.023% 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 at least 0.08% in 200 ng of total soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.08%.

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 A5547-127 and thus imparts event-specificity to the method.

The specificity of event-specific and the soybean-specific assays were experimentally tested by the applicant in real-time PCR against DNA extracted from plant materials containing the specific targets of rice LLRice62; maize T25, MON810, Bt11, GA21, NK603; oilseed rape Ms1, Ms8, Rf1, Rf2, Rf3, Topas 19-2, T45, OXY-235, RT73; soybean A2704-12, Round-Up Ready[®]; cotton lines LLCotton25, GHB614, T304-40, GHB119, MON1445 and conventional soybean.

According to the applicant, the A5547-127 system did not react with any of the plant materials tested, except the positive control soybean line A5547-127; the soybean-specific reference system reacted only with conventional soybean and with all the soybean GM varieties tested.

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 A5547-127

3.2.1 General

The PCR set-up for the taxon specific target sequence (*Le1*) and for the GMO (event A5547-127) 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 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 10% A5547-127 in non-GM soybean DNA for a total of 300 ng of DNA (corresponding to approximately 265,487 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. In two reaction tubes (one for the A5547-127 system and one for the *Le1* system) on ice, add the following components (Table 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the A5547-127 specific system.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
SHA003 primer (10 µM)	400 nM	1
SHA004 primer (10 µM)	400 nM	1
TM058 TaqMan® probe (10 µM)	200 nM	0.5
Nuclease free water	#	5
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the soybean *Le1* reference system.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM164 primer (10 µM)	200 nM	0.5
KVM165 primer (10 µM)	200 nM	0.5
TM021 TaqMan® probe (10 µM)	200 nM	0.5
Nuclease free water	#	6
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the soybean A5547-127 and one for the *Le1* 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 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 s. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
6. Spin down the tubes in a micro-centrifuge. 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.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 3.

Table 3. Cycling program for A5547-127 specific system and for the soybean *Le1* reference system.

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. A5547-127) 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 A5547-127 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.

For the determination of the amount of event A5547-127 DNA in the unknown sample, the A5547-127 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 to obtain the percentage value (GM% = A5547-127/*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')
A5547-127 target sequence	
SHA003	5' – GCT ATT TGG TGG CAT TTT TCC A – 3'
SHA004	5' – CAC TGC GGC CAA CTT ACT TCT – 3'
TM058 (probe)	FAM - 5' – CCG CAA TGT CAT ACC GTC ATC GTT GT – 3' TAMRA
Reference gene <i>Le1</i> target sequence	
KVM164	5' – CTT TCT CGC ACC AAT TGA CA – 3'
KVM165	5' – TCA AAC TCA ACA GCG ACG AC – 3'
TM021 (probe)	VIC 5' – CCA CAA ACA CAT GCA GGT TAT CTT GG – 3' TAMRA

5. References

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