EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Soybean SYHT0H2 by Real-time PCR

Validated Method

Method development:

Syngenta Crop Protection AG

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 SYHT0H2 (unique identifier SYN-ØØØH2-5) genomic DNA to total soybean genomic DNA in a sample.

Template genomic DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay, in particular in case of complex and difficult matrices. 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 SYHT0H2, an 88-bp fragment of the region spanning the 3′ insert-to-plant junction in soybean SYHT0H2 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 BHQ-1 (Black Hole Quencher-1) as a quencher dye at its 3′ end.

For the relative quantification of soybean event SYHT0H2 DNA, a soybean-specific reference system amplifies a 74-bp fragment of soybean endogenous genes *lectin (Le1)*, (GenBank accession number K00821.1), using *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with FAM as reporter dye at its 5′ end, and TAMRA (carboxytetramethylrhodamine) 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 "Cq" value. For quantification of the amount of SYHT0H2 genomic DNA in a test sample, Cq values for the SYHT0H2 and *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of SYHT0H2 genomic DNA to total soybean genomic DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable genomic DNA extracted from mixtures of genetically modified and conventional soybean seeds. The method was fully validated by the EURL GMFF and its precision and trueness were tested through an international collaborative trial, using genomic DNA samples at different GM contents.

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

2.3 Limit of detection (LOD)

The relative LOD of the method is at least 0.04% (related to mass fraction of GM-material) in 100 ng of total soybean genomic DNA. The relative LOD was not assessed in the international collaborative study.

2.4 Limit of quantification (LOQ)

The relative LOQ of the method is at least 0.080% (related to mass fraction of GM-material) in 100 ng of total soybean genomic DNA. The lowest relative GM content of the target sequence included in the international collaborative study was 0.10% (mass fraction of GM-material).

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 3' insert-to-plant junction in soybean SYHT0H2; the sequence is specific to event SYHT0H2 and thus imparts event-specificity to the event specific assay, which was confirmed in the EURL GMFF validation study (page 6 of the validation report).

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 require dedicated equipment, especially pipettes.
- All the equipment 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 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 SYHT0H2

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*Le1*) and for the GMO (event SYHT0H2) target sequence is to be carried out in separate vials. The method is developed and validated for a total volume of 25 µL per reaction mixture with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

To establish the calibration curve five samples should be prepared and analysed. The range of GM contents in the calibration curve should be equal or included in the range validated during the international collaborative study, as given in table 1.

Table 1. Copy number values of the standard curve samples.

Sample	**S1**	**S2**	**S3**	**S4**	**S5**
Total amount of DNA in reaction (ng)	100	30	9.2	2.8	0.80
% GM (DNA/DNA)	10	10	10	10	10
Target taxon Le1 copies	88496	26817	8126	2463	704
SYHT0H2 soybean GM copies	8850	2682	813	246	70

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 e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available within the sequence detection system software of the method user.

3.2.3 Real-time PCR set-up

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.

2. To prepare the amplification reaction mixtures, add the following components (Table 2 and 3) in two reaction tubes (one for the event specific assay and one for the *taxon specific* assay) on ice and in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the SYHT0H2 event specific assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix 2x (no UNG)	1x	12,5
FE08316-F (10 µM)	600 nM	1.5
FE08317-R (10 µM)	600 nM	1.5
FE08318-P (10 µM)	200 nM	0.5
Nuclease free water Nuclease free water	#	4.0
DNA	#	5.0
Total reaction volume:		25 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the soybean *Le1* taxon specific assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix 2X (no UNG)	1x	12.5
Lec for 2 (10 µM)	650 nM	1.625
GMO3-126 Rev (10 µM)	650 nM	1.625
Lec probe (10 µM)	180 nM	0.45
Nuclease free water	#	3.8
DNA	#	5.0
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.

4. Prepare two reaction tubes (one for the event specific and one for the taxon specific 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 (70 µL for the *Le1* reference system and 70 µL for the SYHT0H2 soybean system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (e.g. 17,5 µL DNA). The additional 0.5 repetition included will ensure adequate volume when loading the samples. 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 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) 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 SYHT0H2/*Le1* assays.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	Initial denaturation		95	600	No	1X
2	Amplification	Denaturation	95	15	No	45X
		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 following the automatic or the manual mode. In the manual mode display the amplification curves of the event specific assay 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 following the automatic or the manual mode. In the manual mode: 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 taxon specific system.

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 for both 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 sample.

To obtain the percentage value of event DNA in the unknown sample, the event copy number is divided by the copy number of the reference gene and multiplied by 100 (GM% = number of event copies/number of reference gene copies 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
- 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 (no UNG), Applied Biosystems Cat. No. 4326614.
- Nuclease free water

4.3 Primers and Probes

Oligonucleotides	**Name**	**DNA Sequence (5' to 3')**	**Length (nt)**
SYHT0H2			
Forward primer	FE08316-F	5' GGG AAT TGG GTA CCA TGC C 3'	19
Reverse primer	FE08317-R	5' TGT GTG CCA TTG GTT TAG GGT 3'	21
Probe	FE08318-P	5'-6FAM™- CCA GCA TGG CCG TAT CCG CAA –BHQ™-1-3'	21
Le1			
Forward primer	Lec for 2	5' CCA GCT TCG CCG CTT CCT TC 3'	20
Reverse primer	GMO3-126 Rev	5' GAA GGC AAG CCC ATC TGC AAG CC 3'	23
Probe	Lec probe	5' 6-FAM™- CTT CAC CTT CTA TGC CCC TGA CAC -TAMRA™-3'	24

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine; BHQ™-1:Black Hole Quencher 1

5. References

1. Plant DNA C-values Database, http://data.kew.org/cvalues/