Event-Specific, Quantitative PCR Method for CV127 Soybean

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1 General information on the method

1.1 Purpose and Scope

This protocol describes a quantitative event-specific real-time PCR method for determination of the relative content of CV127 soybean DNA to total soybean DNA in food and feed samples. The event- specific PCR method for CV127 soybean should be used in conjunction with a DNA extraction method which yields DNA of sufficient purity and quantity.

1.2 Principle of the method

The PCR assay has been optimized for use on an ABI 7500 Fast Real-Time PCR System (standard mode). PCR product formation is measured during each cycle (real-time) by means of a target- specific oligonucleotide probe labeled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end. The 5′-nuclease activity of *Taq* DNA polymerase cleaves the probe and liberates the fluorescent moiety during the amplification process. The resulting increase in fluorescence during amplification is monitored and recorded.

A preexisting soybean-specific PCR system which detects the *le*1 gene from *Glycine max* [1] (GenBank Accession No. K00821) is used as the reference system.

The event-specific system for CV127 soybean was established at the 3' insert-to-genomic soybean DNA junction. The forward primer binding site is located in the CV127 insert, the binding site of the reverse primer is within genomic soybean DNA, and the binding site of the probe spans the junction between the CV127 insert and genomic DNA.

The assay format makes use of standard curves for each of the two PCR systems; each standard curve is comprised of four standard points each derived from triplicate measurements. The standards are produced by preparing solutions of 20 ng/µl of total genomic DNA containing 10% CV127 soybean DNA (standard 1) and subsequent serial 1:5 dilutions with (standards 2 to 4).

Three no-template controls (NTC) per detection system are run to verify the purity of reagents. Each sample (unknown) is analyzed using 100 ng genomic DNA per reaction.
Analysis is performed in triplicate: three reactions per sample in the reference detection system and three reactions in the GMO-specific detection system. Thus, there are six reactions per sample in total for both PCR detection systems.) The relative content of CV127 soybean target to total soybean DNA is subsequently calculated by determining the mean of the copy numbers

[1] Goldberg,R.B., Hoschek,G. and Vodkin,L.O. (1983): An insertion sequence blocks the expression of a soybean lectin gene. Cell 33 (2), 465-475.

based on the standard curves (linear regression of CT-values versus log[copy numbers]) and calculating the ratio of CV127 soybean copy number/total copy number of haploid soybean genomes.

2 Details on samples and standard curve

2.1 DNA concentration of samples

The recommended assay format makes use of 100 ng template DNA per reaction. This corresponds to approximately 86580 haploid copies of the Glycine max genome, assuming a genome weight of 1.155 pg[2].

2.2 DNA concentration of calibration standards

Each of the two standard curves is comprised of four standard points, each derived from triplicate measurements. A primary DNA solution containing 10% Event 127 soybean in non-GM soybean DNA at a concentration of 20 ng/µl is prepared. Subsequently, five-fold serial dilutions of this primary DNA solution are produced by diluting in 0.1x TE (pH 8.0) containing 10 ng/µl salmon sperm DNA, resulting in DNA solutions of 4 ng/µl, 0.8 ng/µl and 0.16 ng/µl soybean DNA. For the standard reactions, 5 µl of each of these DNA solutions is used in triplicate reactions resulting in reactions containing 100 ng, 20 ng, 4 ng and 0.8 ng DNA, respectively.

Remark: Preparation of the dilution requires careful and thorough mixing after each dilution step; dilutions shall be freshly prepared in dilution buffer.
The total DNA content as well as the copy numbers is listed in the following table.

Table 1. Calibration standards

Genomic DNA	Standard 1	Standard 2	Standard 3	Standard 4
10% CV127 soybean DNA in non-GM soybean DNA	100 ng (20 ng/µl x 5µl)	20 ng (4 ng/µl x 5 µl)	4 ng (0.8 ng/ µl x 5 µl)	0.8 ng (0.16 ng/µl x 5
CV127 copies (haploid)	8658	1732	346	69
bean genome copies (haploid)	86580	17316	3463	693

[2] Arumuganathan & Earle (1991): Nuclear DNA Content of Some Important Plant Species. Plant Molecular Biology Reporter 9(3), 208-218.

3 Details on composition and preparation of reaction mixes

Be sure to thoroughly mix each reagent before use. Two reaction mixes (one for the soybean-specific reference PCR system and one for the CV127-specific PCR system) must be prepared in sufficient quantities for all reactions (including those for the standard curves). These reaction mixes consist of all components of the PCR amplification, except template DNA. For template DNA, 5 µl of 20 ng/µl experimental (unknown) DNA solutions are used per reaction.

Table 2. Preparation of the master mix for the soybean-specific reference PCR system

Chemicals	Concen-tration	Final concentration	µl/rxn	µl/50 runs
TaqMan Universal PCR Master Mix with UNG	2 x	1 x	12.500	**625.00**
lec F	10 µM	150 nM	0.375	**18.75**
lec R	10 µM	150 nM	0.375	**18.75**
lec P	10 µM	50 nM	0.125	**6.25**
Sterile water			6.625	**331.25**
Total volume			**20.000**	**1000.00**

***) Total PCR reaction is 25 µl (20 µl master mix and 5 µl genomic DNA template)**

Table 3. Preparation of the master mix for the CV127-specific PCR system

Chemicals	Concen-tration	Final concentration	µl/rxn	µl/50 runs
TaqMan Universal PCR Master Mix with UNG	2 x	1 x	12.500	**625.00**
SE-127-f4	10 µM	400 nM	1.000	**50.00**
SE-127-fr2	10 µM	400 nM	1.000	**50.00**
SE-127-p3	10 µM	100 nM	0.250	**12.50**
Sterile water			5.250	**262.50**
Total volume			**20.000**	**1000.00**

***) Total PCR reaction is 25 µl (20 µl master mix and 5 µl genomic DNA template)**

4 Cycling parameters

Run the assay with the following cycling conditions.

Table 4. Cycling parameters

Step	Stage		T (°C)	Time (sec)	Data collection	Cycles
1	UNG		50°C	120''	no	1x
2	Initial enzyme activation		95°C	600''	no	1x
3	Amplification	Denaturation	95°C	15"	no	45x
4		Annealing & Extension	60°C	60"	yes	

Please note: The assay was developed on an ABI 7500 Fast Real-Time PCR System, using the standard mode (not the fast mode) in the instrument setup.

5 Primers and probes

The following table contains the sequences of the primers and probes of the two PCR systems:

Table 5. Sets of primers and probes

Name	Sequence (5' to 3')
lec F	CCAGCTTCGCCGCTTCCTTC
lec R	GAAGGCAAGCCCATCTGCAAGCC
lec P	6FAM-CTTCACCTTCTATGCCCCTGACAC-TAMRA
SE-127-f4	AACAGAAGTTTCCGTTGAGCTTTAAGAC
SE-127-r2	CATTCGTAGCTCGGATCGTGTAC
SE-127-p3	6FAM–TTTGGGGAAGCTGTCCCATGCCC-TAMRA

6 Equipment and materials

Table 6. Equipment and materials

Equipment	Number/Specification
ABI 7500 Fast Real-Time PCR System including Software: 7500 Fast Sequence Detection System version 1.3.	Applied Biosystems PartNo. 4351106
Vortex	NeoLab Vortex VM-300 or equivalent
Thermo-Fast 96 well plate	Abgene PartNo. AB-1900 or equivalent
Clear seal foil	Abgene PartNo. AB-0812 or equivalent
Thermo Sealer	Abgene 0384/240 or equivalent
Pipettes with adjustable volume	Eppendorf Research, 2–20µl, 20–200µl, 100–1000µl or equivalent
Filter Tips	fitting the pipette models used
Reaction tubes 1.5 ml	Roth, 4182.1 or equivalent
Reaction tubes 1.5 ml, screw lids	Sarstedt PartNo. 72.692.005 or

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 shall not adsorb protein or DNA.

7 Reagents, buffers and solutions

Table 7. Reagents, buffers and solutions

Reagent	Specification
TaqMan Universal PCR Master Mix, with AmpErase UNG	Applied Biosystems Part No. 4326708
1x TE buffer pH 8.0	Applichem PartNo. A2575,1000 or equivalent
Salmon sperm DNA	Sigma PartNo. D7656 or equivalent
Dilution buffer	10 ng salmon sperm DNA /µl 0.1x TE (pH 8.0)
Water HPLC Gradient Grade	Rotisolv®HPLC Gradient Grade Prod Nr. A511.1 or equivalent

All handling of reagents and controls shall be carried out under sterile conditions and in a manner that precludes contamination of reagents or controls with exogenous DNA or undesired enzymatic activities (e.g. DNase).

8 Experimental procedure and evaluation of results

8.1 Experimental procedure and assay format

The assay format makes use of standard curves for both PCR systems; the standard curves are comprised of four standard points each derived from triplicate measurements. Three no-template controls (NTC) per system are run to verify the purity of reagents. Each experimental sample (UK 1-11) is analyzed at 100 ng genomic DNA per reaction.

The figure below summarizes the plate layout:

Figure 1. PCR plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1			Std2			Std3			Std4		
B	Std1			Std2			Std3			Std4		
C	UK1			UK2			UK3			UK4		
D	UK1			UK2			UK3			UK4		
E	UK5			UK6			UK7			UK8		
F	UK5			UK6			UK7			UK8		
G	UK9			UK10			UK11			NTC		
H	UK9			UK10			UK11			NTC		

Std1-4: Calibration standards, **UK 1-11**: Unknown samples, **NTC**: No template control
White cells: soybean specific reference system, **grey cells**: CV127-specific system

8.2 Data analysis and evaluation of results

8.2.1 Data analysis

Subsequent to PCR, the results are exported using the baseline setting ‘automatic baseline’. The threshold should be placed in the region of exponential amplification across all of the amplification plots. This region is recognized in the log view of the amplification plots as the portion of the plot which is linear. The threshold line should neither be placed in the plateau phase nor in the initial linear phase of amplification. Make sure that the threshold line is placed clearly above the background fluorescence and above the level where splitting or fork effects between replicates can be observed.

Ct values obtained for the four calibration standards with both PCR systems (the soybean-specific reference system and the CV127-specific detection system) are used to establish two calibration functions, one for the reference system and one for the CV127-specific detection system. As there is a linear relationship between CT values and the logarithm of target copy numbers, linear regression analysis is carried out to find the best straight lines through the data. The linear regression equations calculated are subsequently used to evaluate the copy numbers of the target sequences in the unknown samples for the CT values experimentally determined. The relative amount of CV127 soybean in each sample is calculated by dividing the copy number of the respective event target by the copy number evaluated with the soybean specific reference system.

8.2.2 Analytical quality control measures

Master mix performance

If the CT values of the DNA standards are plotted against the log [copy numbers], the resulting regression line may indicate failure of the master mix, e.g. due to improper handling or storage or incorrect use of the instrument. Acceptance: The regression line ideally should show a slope of -3.321 (which corresponds to an amplification efficiency 100%). A slope value between -3.1 and -3.6 is acceptable. The R^2 value of the regression line should be > 0.985. If the R^2 value of the regression line is ≤ 0.985, the analysis should be repeated.

Inhibition

After DNA extraction, the absence of PCR inhibition must be assured, e.g., by performing a preliminary real-time PCR run. If PCR inhibition is detected, further purify the DNA extract or repeat DNA extraction before quantitative analysis.

No-Template Controls

All NTCs must be negative (CT= 45). Otherwise, the entire run must be repeated.