Appendix 6

Event-Specific, Quantitative PCR Detection Method for CV127 Soybean

Event-Specific, Quantitative PCR Detection Method for CV127 Soybean

Method Number: CV127-11172008

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CONTENTS

I. Validation of the CTAB-Anion-Exchange Method for DNA Extraction from Soybeans

1 Introduction

The purpose of the small-scale DNA extraction method described below is to provide high-quality DNA from soybeans for subsequent real-time PCR based detection methods. This report describes the method as well as validation experiments including results.

2 Method description

2.1 Applicability

The "CTAB-Anion-Exchange" method for DNA extraction described below is suitable for the isolation of genomic DNA from a wide variety of matrices. However, validation data presented here are restricted to ground soybeans. Application of the method to other matrices may require adaptation and needs specific validation.

2.2 Practicability

For DNA extraction using the "CTAB-Anion-Exchange" method described below only standard equipment for molecular biological work is required, e. g. a centrifuge, an incubator and pipettes (for details see section 3). Costs for reagents and consumables add up to about 8 EUR per DNA extraction. The whole procedure from sample weighing to the final purification step takes about nine hours of time in total with 2.5 hours of hands-on time.

2.3 Principle

The basic principle of DNA extraction consists of first releasing the DNA present in the sample into aqueous solution and further, concurrently or subsequently, purifying the DNA from PCR inhibitors.
The "CTAB-Anion-Exchange" method starts with a thermal lysis step using 1 g ground soybean material. The CTAB lysis buffer used in this step contains sufficient NaCl for keeping the DNA released from the sample material in solution. CTAB binds polysaccharides, cell wall debris and denatured proteins. After lysis, contaminants such as lipophilic molecules, proteins and CTAB/polysaccharide complexes are removed by extraction with chloroform-octanol and a crude DNA extract is generated by precipitation with isopropanol.

After dissolving the resulting precipitate in low-salt buffer, any remaining inhibitors are removed by anion-exchange chromatography using the commercially available gravity-flow column “Genomic-tip 20/G” (Qiagen). During this procedure, DNA fragments (polyanions) bind under low-salt conditions by electrostatic interactions between the negatively charged phosphate groups of the nucleic acid backbone and the positively charged anion-exchange resin. Subsequent washing steps remove impurities. Finally, the DNA is eluted with high-salt buffer, desalted and concentrated by a final isopropanol precipitation step.

2.4 Application Notes

Milling/grinding of soybeans not only facilitates the lysis by mechanically disrupting cellular structures and increasing the surface area, but is also indispensable for the generation of representative test portions by reducing the particle size. For details concerning requirements for particle size/particle number, generation of the test portions etc. please refer to the literature (e.g. prEN ISO 21568 sampling).

DNA extractions should be performed on at least two test portions. Duplicate extraction blanks (negative controls which are handled identically but without sample material) are mandatory throughout the extraction process and subsequent PCR.

In order to avoid any risk of contamination, suitable lab facilities with dedicated working places for different working steps are recommended. All material used (e.g.

tubes, vials, containers, pipette tips, etc.) must be suitable for DNA extraction and molecular biology applications. They must be DNase-free, sterile and shall not adsorb protein or DNA. All handling of reagents and controls shall be carried out in a manner that precludes contamination of reagents or controls with exogenous DNA or DNase. Grinding of soybeans shall be done in a separate room.

2.5 Protocol

Lysis/isopropanol precipitation of DNA

1. Transfer 15 ml CTAB lysis buffer, 60 µl proteinase K, 200 µl 2-mercaptoethanol and 10 µl RNase A to a 50 ml conical tube.
2. Weigh out 1 g *) of ground soybean and add to the tube containing CTAB lysis buffer, proteinase K, 2-mercaptoethanol and RNase; mix thoroughly.
3. Incubate for 1 hour at 65 °C with agitation. Cool down to room temperature (15 minutes).
4. Add 10 ml chloroform:octanol (24:1) and mix vigorously by vortex or inversion.
5. Spin down at room temperature for 10 minutes at 10,000 x g.
6. Transfer supernatant to a new 50 ml conical tube containing 10 ml isopropanol and invert the tube several times to mix.
7. To precipitate the DNA, spin down at 4 °C for 30 minutes at 10,000 x g.
8. Discard supernatant and add 5 ml 70% ethanol and mix carefully.
9. Spin down at 4 °C for 20 minutes at 10,000 x g.
10. Carefully remove and discard ethanol.
11. Repeat centrifugation step for 1 minute at 4 °C at 10,000 x g.
12. Remove residual ethanol by pipetting.
13. Resuspend the pellet in 500 µl 1x TE buffer pre-warmed to 50 °C.

***) Depending on the particle size of the ground soybean material used for DNA extraction, it might be advisable to adapt the amount of starting material. In case of very fine material, a reduction of the amount of starting material might be advantageous.**

Purification using Genomic-tip 20/G

14. Add 5 ml G2 buffer (containing 10 µl RNase A and 25 µl Proteinase K) and mix thoroughly.
15. Incubate 1 hour at 50 °C with agitation.
16. Spin down at room temperature for 5 minutes at 10,000 x *g.*
17. Transfer the supernatant to a 15 ml conical tube.
18. Equilibrate a QIAGEN Genomic-tip 20/G with 2 ml QBT.
19. Apply the sample to the equilibrated Genomic-tip 20/G.
20. Wash the Genomic-tip 20/G twice with 2 x 1.5 ml buffer QC.

21. Elute the genomic DNA with 1 ml QF buffer (pre-warmed to 50 °C) and collect the DNA in a 2 ml microcentrifuge tube.
22. Repeat the elution with 1 ml QF buffer and collect the DNA in a second 2 ml microcentrifuge tube.
23. Add 700 µl isopropanol to each tube; mix carefully by inverting (about 10 times).
24. Spin down at 4 °C for 30 minutes at 10,000 x g.
25. Discard the supernatant and wash the DNA pellets with 1 ml 70% ethanol each.
26. Spin down at 4 °C for 10 minutes at 15,000 x g.
27. Discard the supernatant and air-dry the pellets for 10 minutes.
28. Dissolve DNA pellets in 100 µl 1x TE buffer pre-warmed to 50 °C.
29. Incubate 30 minutes at 50 °C with agitation.
30. Allow DNA to dissolve overnight at 4 °C.
31. Spin down at room temperature for 5 minutes at 15,000 x g.
32. Combine the first and second elutions which were obtained from the same Genomic-tip 20/G in one microcentrifuge tube for a final volume of about 200 µl.

3 Equipment/Reagents/Buffers/Plastic ware

3.1 Equipment

The following equipment is used in the DNA extraction procedure described above (equivalents may be substituted):

Equipment	Example of appropriate apparatus
Pipettes with adjustable volume	e.g. Eppendorf Research, 2 - 20 µl, 20 - 200 µl, 100 - 1000 µl
Incubator with shaker or shaking water bath	e.g. Heraeus Function Line B12 in combination with shaker GFL 3005 (Gesellschaft für Labortechnik mbH)
Balances for the preparation of buffers and solutions and for sample weigh in	e.g. Ohaus Scout II, Ohaus Adventurer
Refrigerated centrifuge with rotors for 50 ml centrifuge tubes and microcentrifuge tubes	e.g. Sigma 4 K 15C with suitable rotors
Thermo block for 2.0 ml microcentrifuge tubes	e.g. Bioblock Scientific 92333
Vortex	e.g. NeoLab Vortex VM-300

3.2 Reagents

The following materials are used in the DNA extraction procedure described above (equivalents may be substituted):

Material	Specification
CTAB	p.a. (pro analysi) quality or Molecular biology grade
Tris	p.a. quality or Molecular biology grade
EDTA · Na_2-salt	p.a. quality or Molecular biology grade
HCl	p.a. quality
NaCl	p.a. quality
Proteinase K	From *Tritirachium album*, DNases, RNases, Exonucleases not detectable, Molecular biology grade
RNase A	From bovine pancreas, salt free, protease free and chromatographically homogeneous, ca. 90 Kunitz units/mg
Chloroform	p.a. quality
Octanol	p.a. quality
Isopropanol	p.a. quality
Ethanol	p.a. quality
2-Mercaptoethanol	p.a. quality
Genomic-tip 20/G, 25 columns	Cat. #10223, QIAGEN
Genomic DNA Buffers Set including G2, QBT, QC and QF*)	Cat. #19060, QIAGEN
CTAB lysis buffer	Applichem, Cat. #4150, 2000

***) Buffers can also be prepared. Please refer to the "Qiagen Genomic DNA Handbook" supplied with the Genomic-tip 20/G columns for details.**

3.3 Buffers and Solutions

The following buffers and solutions are used in the DNA extraction procedure described above:

CTAB lysis buffer

2% (w/v) CTAB

20 mM EDTA · Na_2·$2H_2O$

1.4 M NaCl

100 mM Tris, pH 8.0

For 1 liter CTAB lysis buffer weigh out 81.8 g NaCl, 20 g CTAB, 12.1 g Tris-Base and 7.44 g EDTA in an appropriate beaker and add about 800 ml H_2O_{deion}. Adjust pH with HCl to pH 8.0; stir until all reagents are dissolved. Adjust volume to 1 liter with H_2O_{deion}. Autoclave.

Store at room temperature for up to 1 year.

(CTAB lysis buffer can alternatively be purchased from Applichem)

Chloroform:octanol (24:1)

For 100 ml chloroform:octanol (24:1) combine 96 ml chloroform and 4 ml octanol. Mix. Store at room temperature under the fume hood for up to 6 months.

Proteinase K

20 mg/ml H_2O_{deion}

For 10 ml proteinase K solution dissolve 200 mg proteinase K in 10 ml $H_2O_{deion.}$

Store at -20 °C for up to 2 years.

RNase A

91 mg/ml

Dissolve 0.5 g RNase A in 5 ml 0.01 M sodium acetate (pH 5.2), aliquot in 1 ml portions, boil for 15 minutes to inactivate DNases, cool slowly to room temperature and add 100 µl 1 M Tris-HCl (pH 7.4) to each aliquot.

Store at -20 °C for up to 2 years.

70% (v/v) Ethanol

To prepare 200 ml, combine 140 ml 100% ethanol with 60 ml H_2O_{deion}.

Store at room temperature for up to 5 years.

0.5 M EDTA, pH 8.0

For 500 ml solution dissolve 93.0 g EDTA · Na_2-salt in 350.0 ml H_2O_{deion}. Adjust pH with NaOH to pH 8.0 and the volume to 500.0 ml with H_2O_{deion}. Autoclave.
Store at room temperature for up to 1 year.

1 M Tris, pH 8.3

For 1000 ml solution dissolve 121.1 g Tris in 800.0 ml H_2O_{deion}. Adjust pH with HCl to pH 8.3 and the volume to 1000.0 ml with H_2O_{deion}. Autoclave.
Store at room temperature for up to 1 year.

1x TE buffer

10 mM Tris, pH 8.3
1 mM EDTA
To prepare 100 ml 1x TE buffer, combine 1 ml 1 M Tris (pH 8,3) and 200 µl 0.5 M EDTA (pH 8,0) and adjust the volume to 100 ml with H_2O_{deion}. Autoclave.
Store at room temperature for up to 2 years.

3.4 Plastic ware

Note: All plastic ware has to be sterile and free of DNases, RNases and nucleic acids.

Item	Specification
50 ml conical tubes	e.g. Greiner Bio One GmbH, Cat. #227270
15 ml conical tubes	e.g. Greiner Bio One GmbH, Cat. #188271
2 ml microcentrifuge tubes	e.g. Eppendorf, Cat. 0030 120.094
filter tips	fitting the pipette models used

4 Experimental Validation and Results

For experimental validation, DNA was extracted six times each from homogenized non-GM (B25-08-N00413) and CV127 (B25-08-N00412) soybeans using the method described above.

The following method parameters were determined:

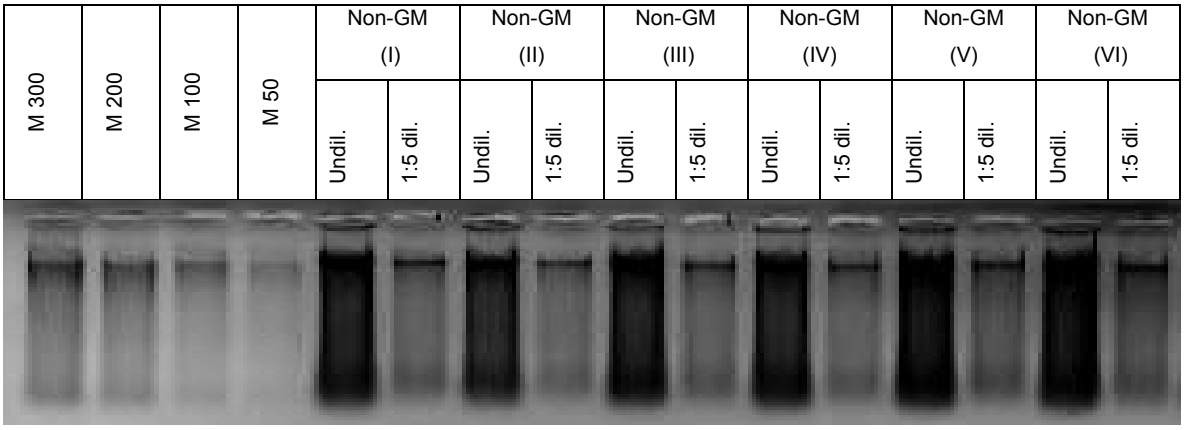
- Fragmentation state of DNA
- DNA concentration/yield, repeatability standard deviation RSD_r
- Purity/absence of PCR-inhibitors

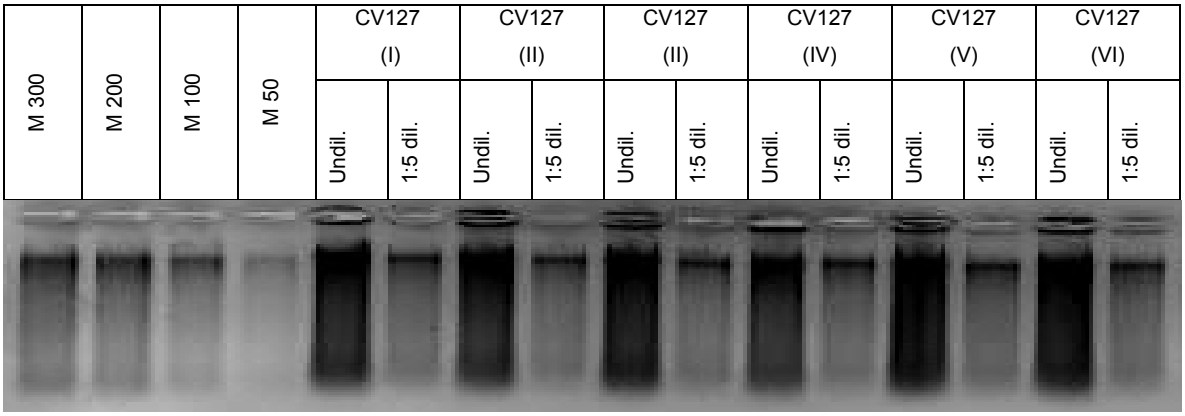
4.1 Fragmentation State of DNA

The fragmentation state of the extracted DNA was evaluated by agarose gel electrophoresis. 5.0 µl of each undiluted and 1:5-diluted DNA aliquots were analyzed on a 1.5% agarose gel (TAE buffer system). Defined amounts of high molecular weight plant genomic plant DNA (300 ng, 200 ng, 100 ng and 50 ng) were loaded as DNA quantity standards (labeled M 300, M 200, M 100 and M 50, respectively in Figure 1).

After electrophoretic separation the gel was stained in ethidium bromide solution (1 µg/ml in distilled water) for 30 min and the DNA was visualized using an UV transilluminator (Figure 1).

Figure 1. Agarose gel electrophoresis of DNA aliquots extracted from non-GM and CV127 material (undiluted and 1:5 dilution of each sample).





Acceptance criteria: Medium to high molecular weight DNA **- FULFILLED**

4.2 DNA Concentration/Yield, Repeatability Standard Deviation RSD_r

The concentration of the DNA extracts was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Invitrogen). Suitable dilutions of each DNA extract were prepared in triplicate and mixed with the PicoGreen reagent. DNA concentration was determined based on a five-point standard curve (each point measured in triplicate) ranging from 50 ng/ml to 800 ng/ml using a Stratagene Mx3005P as the fluorescence detector.

Table 1. DNA concentration / Yield

Soybean	Replicate	Concentration (ng/µl)	Yield (µg)
Non-GM	I	169	33.8
Non-GM	II	120	24.0
Non-GM	III	147	29.4
Non-GM	IV	148	29.6
Non-GM	V	178	35.6
Non-GM	VI	192	38.4
CV127	I	130	26.0
CV127	II	105	21.0
CV127	III	109	21.8
CV127	IV	115	23.0
CV127	V	122	24.4
CV127	VI	116	23.2

DNA concentration of the non-GM preparations:

Mean:	159.0 ng/µl
Standard deviation:	25.8 ng/µl
Relative standard deviation:	16.2%

Yield of the non-GM preparations:

Mean:	31.8 µg
Standard deviation:	5.2 µg
Relative standard deviation:	16.2%

DNA concentration of the CV127 preparations:

Mean:	116.2 ng/µl
Standard deviation:	9.0 ng/µl
Relative standard deviation:	7.7%

Yield of the CV127 preparations:

Mean:	23.2 µg
Standard deviation:	1.8 µg
Relative standard deviation:	7.7%

Acceptance criteria:

- Concentration ≥ 20 ng/µl **- FULFILLED**
- Yield ≥ 4 µg **- FULFILLED**
- RSD_r of DNA concentration and yield < 25% **- FULFILLED**

4.3 Purity/Absence of PCR Inhibitors

In order to assess the purity and confirm the absence of PCR inhibitors, the extracted DNA solutions were adjusted to a concentration of 40 ng/µl. Subsequently fourfold serial dilutions of each DNA solution were prepared with 0.2 x TE buffer (1:4, 1:16, 1:64, 1:256) and analyzed using a real-time PCR system detecting the soy-specific *Le*1 gene (GenBank Accession No. K00821).

To measure inhibition, the CT values of the four diluted samples were plotted against the logarithm of the dilution and the CT value for the undiluted sample (40 ng/µl, 200 ng/reaction) was extrapolated from the equation calculated by linear regression. Subsequently the coefficient of correlation was determined and the extrapolated CT for the undiluted sample was compared with the measured CT. Evaluation of the data

was performed under the assumption that PCR inhibitors are present if the measured CT value for the undiluted sample deviates by >0.5 cycles from the calculated CT value. The CT values obtained for the different dilution steps are shown in Table 2.

Table 2. CT values of 'undiluted' (200 ng/reaction) and fourfold serial diluted DNA extracts

Soybean	Replicate	undiluted	diluted			
		1:1	1:4	1:16	1:64	1:256
Non-GM	I	21.2	23.2	25.3	27.2	29.1
Non-GM	II	21.1	23.1	25.1	26.9	29.0
Non-GM	III	21.1	23.1	25.0	27.2	29.2
Non-GM	IV	21.0	23.0	25.1	27.2	29.1
Non-GM	V	21.1	23.1	25.2	27.1	29.2
Non-GM	VI	21.2	23.2	25.2	27.2	29.2
CV127	I	21.3	23.3	25.3	27.2	29.2
CV127	II	21.2	23.2	25.2	27.1	29.2
CV127	III	21.1	23.0	25.0	27.1	29.1
CV127	IV	21.0	23.0	25.1	27.1	29.0
CV127	V	21.0	23.1	25.1	27.0	28.9
CV127	VI	21.2	23.1	25.1	27.1	29.0

Table 3 summarizes the comparison of the extrapolated CT values versus the experimentally determined CT values, as well as the differences between the CT values (delta CT) and the R^2 coefficients.

Table 3. Comparison of extrapolated CT values versus measured CT values (mean of triplicates)

Soybean	Replicate	CT extrapol.	CT experim.	delta CT*	R^2
Non-GM	I	21.3	21.2	0.1	1.000
Non-GM	II	21.2	21.1	0.1	0.999
Non-GM	III	21.0	21.1	0.1	1.000
Non-GM	IV	21.1	21.0	0.1	0.999
Non-GM	V	21.1	21.1	0.0	1.000
Non-GM	VI	21.2	21.2	0.0	1.000
CV127	I	21.4	21.3	0.1	1.000
CV127	II	21.3	21.2	0.1	1.000
CV127	III	21.0	21.1	0.1	1.000
CV127	IV	21.1	21.0	0.1	0.999
CV127	V	21.2	21.0	0.2	1.000
CV127	VI	21.2	21.2	0.0	1.000

*** delta CT = abs(CT extrapol. - CT experim.)**

Acceptance criteria: Delta CT < 0.5 for all DNA solutions extracted **– FULFILLED**

R^2 of linear regression > 0.990 for all DNA solutions extracted **– FULFILLED**

5 Conclusion

The data show that the method is suitable for the intended purpose.

6 Abbreviations

CTAB	cetyltrimethylammoniumbromide
PCR	polymerase chain reaction
Tris	tris(hydroxymethyl)aminomethane
EDTA	ethylenediaminetetraacetic acid
TAE	tris-acetate
TE	tris EDTA

II. Report of the Development and Pre-Validation of an Event-Specific Real-Time PCR System for the Quantitative Detection of CV127 Soybean

1 Task

This report describes the development and pre-validation of a quantitative, event-specific PCR detection method in simplex format for CV127 soybean using FAM/TAMRA-labeled detection probes. Pre-validation of the developed method was done according to a working plan that describes conditions and acceptance criteria for each method parameter. This working plan is in accordance (where applicable) with Annex I to Regulation (EC) 641/2004 and with the technical guidance documents of the Community Reference Laboratory (CRL) on method acceptance criteria and minimum performance requirements for GMO detection methods from version 25-01-2005 (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). Validation acceptance criteria set prior to experimental verification are cited in each section together with the respective evaluation of the results of the experiments.

2 Materials

Sample	Name	Description	Amount
GM soybean	CV127	Ground soybeans	500 g
non-GM soybean	isogenic control	Ground soybeans	1500 g

Genomic DNA from an isogenic control soybean and from Event CV127 soybean was extracted according to the newly developed and pre-validated DNA extraction method described in Annex I.

3 Development summary

The quantitative PCR detection method described in this report consists of a soybean-specific reference system and an event-specific detection system for CV127 soybean.

This herbicide-tolerant soybean plants, referred to as Event CV127 or BPS-CV127-9, are derived from a single transformation event and were produced by the introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*csr1-2*) gene from *Arabidopsis thaliana* into the soybean plant genome. The *csr1-2* expression cassette was integrated at a single genetic locus in the soybean genome.

3.1 Amplicon, primer and probe design

3.1.1 Soybean-specific reference PCR system (Le1)

A preexisting soybean-specific reference PCR system which detects the *Le*1 gene[1] (GenBank Accession No. K00821) was used as the reference system for the specific detection of sequences of *Glycine max.*

3.1.2 Event-specific PCR system for CV127 soybean

Based on sequence information about the *csr1-2* cassette containing the *Arabidopsis thaliana AHASL* 5' UTR, the herbicide-tolerant *Arabidopsis thaliana ahasl* (*csr1-2*) coding sequence and the *Arabidopsis thaliana AHASL* 3'UTR and its flanking 5' and 3' border sequences, it was decided to test PCR systems derived from the 3' junction region between the insert and the flanking genomic DNA of CV127 soybean. Assisted by oligonucleotide design software (Primer Express™ V2.0) three probes and five amplification primers were designed and experimentally tested. Comparison of the CT-values, the delta Rn-values, shapes of the amplification plots and the PCR efficiencies led to one primer/probe combination which was chosen for further optimization (see below).

[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.

3.2 PCR Optimization

The following parameters have been optimized for the CV127 PCR system:

- Overall primer concentration
- Probe concentration

Based on the CT values, the delta Rn values, the shapes of the amplification plots at different concentrations of target DNA and the PCR efficiencies, the optimum system parameters were determined.

4 Results of the development

4.1 Soybean-specific reference PCR system (Le1)

The Le1 detection system was selected as soy-specific reference PCR system.

Primers:

lec F	5′- CCA GCT TCG CCG CTT CCT TC -3′	20-mer
lec R	5′- GAA GGC AAG CCC ATC TGC AAG CC -3′	23-mer

Probe:

lec P (probe)	5′-6FAM- CTT CAC CTT CTA TGC CCC TGA CAC -TAMRA-3′	24-mer

Amplicon sequence (underlined are the primer and probe binding sites):

Length: 74 bp

lec F *lec* P *lec* R

CCAGCTTCGCCGCTTCCTTCAACTTCACCTTCTATGCCCCTGACACAAAAAGGCTTGCAGATGGGCTTG
CCTTC

Table 4. Master mix for the soybean-specific reference PCR system

Chemicals	Concentration	Final concentration*	µl/rxn
TaqMan Universal PCR Master Mix with UNG (ABI)	2 x	1 x	12.500
lec F	10 µM	150 nM	0.375
lec R	10 µM	150 nM	0.375
lec P	10 µM	50 nM	0.125
Sterile water			6.625
Total volume			**20.000**

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

4.2 Event-specific PCR system for CV127 soybean

The event-specific real-time PCR system for CV127 soybean is located at the 3' insert-to-genomic soybean DNA junction. The forward primer 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.

Primers:

SE-127-f4 5′- AAC AGA AGT TTC CGT TGA GCT TTA AGA C -3′ 28-mer

SE-127-r2 5′- CAT TCG TAG CTC GGA TCG TGT AC -3′ 23-mer

Probe:

SE-127-p3: 5′-6FAM – TTT GGG GAA GCT GTC CCA TGC CC -TAMRA-3′
23-mer

Amplicon sequence (underlined are the primer and probe binding sites):

Length: 88 bp

SE-127-f4 SE-127-p3 SE-127-f2

AACAGAAGTTTCCGTTGAGCTTTAAGACGTTTGGGGAAGCTGTCCCATGCCCATCAAAGAAGACAGTAC
ACGATCCGAGCTACGAATG

Table 5. Master mix for the CV127-specific PCR system

Chemicals	Concentration	Final concentration*	µl/rxn
TaqMan Universal PCR Master Mix with UNG (ABI)	2 x	1 x	12.500
SE-127-f4	10 µM	400 nM	1.000
SE-127-r2	10 µM	400 nM	1.000
SE-127-p3	10 µM	100 nM	0.250
Sterile water			5.250
Total volume			**20.000**

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

4.3 Reaction conditions

Format: 20 µl master mix + 5 µl genomic DNA template = 25 µl per reaction

Equipment: ABI 7500 Fast Real-Time PCR System (Applied Biosystems) (standard run mode)

Cycler profile:

50 °C	120 sec	
95 °C	10 min	
95 °C	15 sec	} 45 cycles
60 °C	1 min	

Plasticware: Plastic 96-well plates (ABgene)
Heat seals (ABgene)

4.4 Assumption

One haploid *Glycine max* genome is equivalent to 1.155 pg of *Glycine max* genomic DNA [2].

5 Pre-Validation

5.1 Applicability / Practicability

Applicability

The event-specific real-time PCR method described can be applied to determine the relative content of CV127 soybean DNA in total soybean DNA using FAM/TAMRA-labeled detection probes. The method performs in a linear manner with an acceptable level of accuracy and precision over at least the whole range from 0.080% to 5.0% GMO content. The method was developed and validated with genomic DNA extracted from soybeans. However, in principle the assay should be applicable to any matrix from which genomic DNA with sufficient quantity and quality can be purified.

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

Practicability

Equipment:

The quantitative PCR system for detection of CV127 soybean DNA has been established optimized and in-house validated on an ABI 7500 Fast Real-Time PCR System (standard mode). Transferability to other platforms (ABI PRISM® 7700, ABI PRISM® 7900HT and Stratagene Mx3005P) was shown (see also 5.6 Robustness; 5.7 Interlab transferability) however extensive data have not been collected. If no ABI 7500 Fast Real-Time PCR System is available the method might also be applied on a different platform. However further optimization and adaptation might be necessary. Beyond the real-time PCR instrument, other standard laboratory equipment commonly used for quantitative PCR analyses (e. g. appropriate pipettors, centrifuges etc.) is required.

Costs and timing:

According to the assay layout specified in 5.4 ('Precision/Accuracy/Dynamic Range/LOQ/LOD'), in addition to calibration samples for both PCR systems and NTCs (no-template controls), eleven experimental samples can be analyzed in triplicate per 96-well PCR plate. Assuming consumable costs (plasticware, reagents) of about 150 EUR per plate, the cost per sample is approximately 14 EUR. The analysis process is comprised of the setup of PCR amplifications, the automated PCR amplification and real-time measurement of fluorescence signals on the ABI 7500 Fast Real-Time PCR System, and the subsequent computer-assisted data evaluation. This entire process takes four hours of time in total with two hours of hands-on time for one PCR plate containing 11 experimental DNA samples.

General aspects:

In order to ensure the correct performance of the event-specific quantitative detection assay for CV127 soybean DNA and especially in order to avoid any risk of contamination, suitable lab facilities with dedicated working places for different working steps are recommended. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, sterile and shall not adsorb protein or DNA. All handling of reagents and controls shall be carried out in a manner that precludes contamination of reagents or controls with exogenous DNA or DNases. PCR reagents shall be stored and handled in a separate room and in equipment where no nucleic acids (with the exception of

PCR primers or probes) have been handled previously. All handling of PCR reagents and controls requires dedicated equipment.

5.2 Specificity

5.2.1 BLAST database searches

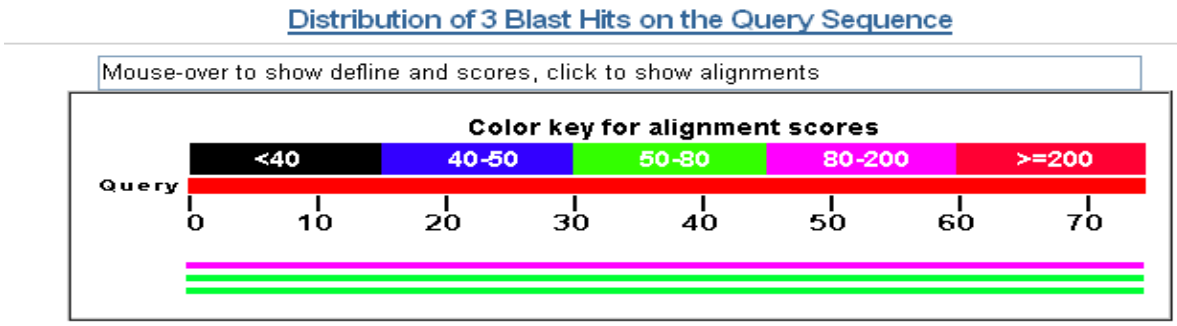
BLAST searches were performed at the National Center for Biotechnology Information (NCBI) website with the standard "nucleotide-nucleotide BLAST" (blastn) (www.ncbi.nlm.nih.gov/blast/Blast.cgi) in order to identify similarity with known sequences.

5.2.1.1. Soybean-specific reference PCR system (Le1)

Figure 2. Result of BLAST search with the amplicon sequences of the species-specific PCR system

BLASTN 2.2.18+

Reference:
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000), "A greedy algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.**Database:** All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences) 7,082,943 sequences; 24,353,626,106 total letters
Query= Length=74



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	Max ident
K00821.1	Soybean lectin (Le1) gene, complete cds	137	137	100%	100%
AY342212.1	Glycine max lectin (Le2) gene, complete cds	76.8	76.8	100%	85%
AY342213.1	Glycine max lectin (Le2) mRNA, partial cds	71.3	71.3	100%	84%

Except for *Glycine max* no significant matches found.

Please note: The BLAST database search reveals sequence similarity to another lectin gene from *Glycine max*: *Le*2. However as an alignment of the nucleotide sequences shows several sequence deviations within the 3' region of the reverse primer and within the probe binding site, amplification with the soybean-specific quantification system is specific for *Le*1.

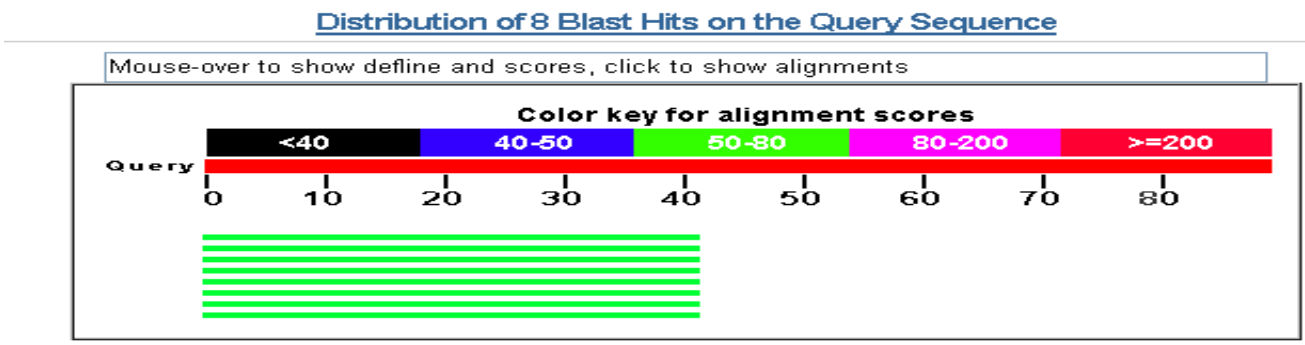
Acceptance criteria: No 100% match of the entire amplicon with other sequences except for *Glycine max* found – **FULFILLED**

5.2.1.2. Event-specific PCR system for CV127 soybean

Figure 3. Result of BLAST search with the amplicon sequence of the CV127 soybean-specific PCR system

BLASTN 2.2.18+

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS,GSS,environmental samples or phase 0, 1 or 2 HTGS sequences) 6,773,628 sequences; 23,692,698,992 total letters
Query= Length=88



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	Max ident
AM888351.1	Transformation Vector pICH13688	76.8	76.8	46%	100%
DQ991161.1	Arabidopsis thaliana acetolactate synthase catalytic subunit mRNA, complete cds	76.8	76.8	46%	100%
NM_114714.2	Arabidopsis thaliana CSR1 (CHLORSULFURON/IMIDAZOLINONE RESISTANT 1) (CSR1) mRNA, complete cds	76.8	76.8	46%	100%
AY124092.1	Arabidopsis thaliana acetolactate synthase (CSR1) gene, complete cds	76.8	76.8	46%	100%
X51514.1	Arabidopsis thaliana Csr 1.2 gene for acetolactate synthase (EC 4.1.3.18)	76.8	76.8	46%	100%
AY042819.1	Arabidopsis thaliana acetolactate synthase (T8P19.70) mRNA, complete cds	76.8	76.8	46%	100%
AL133315.1	Arabidopsis thaliana DNA chromosome 3, BAC clone T8P19	76.8	76.8	46%	100%
BT020540.1	Arabidopsis thaliana At3g48560 gene, complete cds	76.8	76.8	46%	100%

Matches of the 5' end of the amplicon with sequences derived from the *Arabidopsis thaliana* acetolactate synthase (*csr1*) gene were found. No 100% match of the entire amplicon was found.

Acceptance criteria: No 100% match of the entire amplicon with other sequences except for CV127 soybean found – **FULFILLED**

5.2.2 Specificity (experimental)

5.2.2.1. Specificity of the soybean reference PCR system

All specificity tests were done in triplicate with 100 ng genomic DNA per reaction. CT values lower than 45 were defined as positive (+), reactions where no amplification occurred (i.e. a CT value a 45) were defined as negative (-).

Table 6. Specificity tests with the soybean-specific PCR system

Reference-DNA	Result
Positive set	
CV127 soybean	**+**
Negative set	
Brassica napus (rapeseed)	**-**
Oryza sativa (rice)	**-**
Triticum aestivum (wheat)	**-**
Solanum tuberosum (potato)	**-**
Beta vulgaris (sugar beet)	**-**
Gossypium hirsutum (cotton)	**-**
Zea mays (maize)	**-**
Phaseolus vulgaris (common bean)	**-**
Vigna radiata (mung bean)	**-**
Lens culinaris (lentil)	**-**
Cicer arietinum (chickpea)	**-**

Acceptance criteria: No amplification in non-*Glycine* species **– FULFILLED**

5.2.2.2. Specificity of the CV127-specific PCR system

All specificity tests were done in triplicates (n=3). In case of GM materials, DNA with 1% GM content was used. GM and non-GM materials were used at 100 ng genomic DNA per reaction.

Reactions with CT values lower than 45 and amplification curve were defined as positive (+), reactions where no amplification occurred were defined as negative (-).

Table 7. Specificity tests with the CV127-specific PCR system

Reference-DNA	Result
Positive set	
CV127 soybean	+
Negative set	
40-3-2 soybean	-
A2704-12 soybean	-
305423 soybean	-
356043 soybean	-
GA21 maize	-
Bt176 maize	-
Bt11 maize	-
NK603 maize	-
MON863 maize	-
1507 maize	-
MIR604 maize	-
MON810 maize	-
59122 maize	-
T25 maize	-
MS8 rapeseed	-
RF3 rapeseed	-
MON531 cotton	-
MON15985 cotton	-
281-24-236 x 3006-210-23 cotton	-
LL62 rice	-
H7-1 sugar beet	-
EH92-527-1 potato	-
Glycine max (soybean)	-
Gossypium hirsutum (cotton)	-
Solanum tuberosum (potato)	-
Brassica napus (rapeseed)	-
Oryza sativa (rice)	-
Beta vulgaris (sugar beet)	-
Zea mays (maize)	-
Arabidopsis thaliana (mouseear cress)	-

Acceptance criteria: No amplification in the negative set – **FULFILLED**

5.3 Allelic variation / copy number variation

At an amount of 50 ng per reaction, the DNA of multiple soy cultivars showed uniform CT values (mean of triplicates) indicating stability of the respective PCR target in those lines. The maximum CT difference between an individual line and the mean of all lines tested was 0.3.

Table 8. Analysis of different soy cultivars at 50 ng per reaction

Cultivar name	CT (mean of triplicates)	delta CT abs (CT individual line - CT mean of all lines)
CV127	22.8	0.2
Evans	23.0	0.0
Logan	23.2	0.2
Preston	23.2	0.2
Graham	23.2	0.2
Williams 82	23.1	0.1
Hutchenson	23.1	0.0
Anderson	22.7	0.3
Jackson	23.1	0.0
Lincoln	23.0	0.1
Maple Glen	23.0	0.0
Mean of all lines:	**23.0**	
CTmin	**22.7**	
CTmax	**23.2**	
CTmax-CTmin	**0.5**	

Acceptance criteria: delta CT from mean value of all lines <1.0 for each individual line - **FULFILLED**

5.4 Precision/Accuracy/Dynamic Range/LOQ/LOD

In order to determine precision, accuracy, dynamic range, LOQ and LOD according to the definitions set forth in the guidance documents of the CRL the following experimental design was carried out in eight independent runs.

The assay format made use of standard curves for each of the two PCR systems. Each standard curve was comprised of four standard points, each derived from triplicate measurements. The standards were produced by preparing solutions of 20 ng/µl (100 ng/reaction) of total soy DNA with 10% CV127 soybean DNA in non-GM soybean DNA background (standard 1). Standards 2, 3 and 4 were produced by subsequent serial 1:5 dilutions with 0.1xTE (pH 8.0) containing 10 ng salmon sperm DNA /µl.

Three no-template controls (NTC) per system were run to verify the purity of reagents. Each reference sample (produced by preparing solutions of 20 ng/µl total soy DNA with 5.0%, 2.0%, 0.90%, 0.50%, 0.080% and 0.040% of CV127 soybean DNA in a non-GM soybean DNA background) was analyzed in triplicate reactions containing 100 ng genomic soybean DNA.

Figure 4. PCR plate layout for determination of precision, accuracy, LOQ and LOD

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Std1**			**Std2**			**Std3**			**Std4**		
B	**Std1**			**Std2**			**Std3**			**Std4**		
C	**5.0%**			**2.0%**			**0.90%**			**0.50%**		
D	**5.0%**			**2.0%**			**0.90%**			**0.50%**		
E	**5.0%**			**2.0%**			**0.90%**			**0.50%**		
F	**5.0%**			**2.0%**			**0.90%**			**0.50%**		
G	**0.080%**			**0.080%**			**0.040%**			**NTC**		
H	**0.080%**			**0.080%**			**0.040%**			**NTC**		

Std 1-4: Calibration standards; **5.0%, 2.0%, 0.90%, 0.50%, 0.080%, 0.040%**: Reference samples with given GMO level; **NTC**: No-template control; **white cells**: soybean-specific reference system; **gray cells**: CV127-specific system.

Data analysis has been accomplished by using automatic baseline setting and a threshold value of 0.1 for the reference system and 0.2 for the CV127 system on the ABI 7500 Fast Real-Time PCR system.

The quantification results were determined by calculating the mean value of triplicate reactions. Therefore the eight independent runs resulted in 16 test results for the 5.0%, 2.0%, 0.90%, 0.50% and 0.080% GMO DNA mixtures and 8 test results for the 0.040% GMO DNA mixture.

For each of the 5 samples (ranging from 5.0% down to 0.080% CV127 soybean DNA in non-GM soybean DNA), the mean value (MEAN), the relative deviation from the expected value (BIAS) as well as the standard deviation (STDEV) and the relative standard deviation (RSD_r) of the quantification results were calculated in order to determine accuracy and repeatability. The results are shown in the following table.

The relative deviation of the mean value from the expected (true) value ranged between 2.5% and –24.0% over the whole dynamic range.

Precision values for all samples with an expected value of between 5.0% and 0.080% GMO concentration ranged from 5.6% to 13.4% relative standard deviation.

Table 9. Quantification results of eight independent PCR runs under repeatability conditions for CV127

GMO-Level	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	MEAN	BIAS	STDEV	RSD_r
5.0%	4.4%	4.1%	5.1%	4.5%	4.6%	4.5%	4.6%	4.8%	4.4%	**-12.0%**	0.28%	**6.4%**
	4.4%	4.0%	4.3%	4.3%	4.1%	4.6%	4.6%	4.2%				
2.0%	2.0%	1.8%	1.8%	1.9%	1.8%	1.9%	2.0%	1.7%	1.8%	**-10.0%**	0.13%	**7.2%**
	1.7%	1.6%	1.6%	1.8%	1.6%	1.8%	1.9%	1.7%				
0.90%	0.90%	0.85%	0.81%	0.92%	0.87%	0.87%	0.96%	0.85%	0.86%	**-4.4%**	0.048%	**5.6%**
	0.85%	0.79%	0.83%	0.87%	0.78%	0.87%	0.89%	0.84%				
0.50%	0.43%	0.38%	0.38%	0.42%	0.38%	0.37%	0.39%	0.36%	0.38%	**-24.0%**	0.030%	**7.9%**
	0.41%	0.35%	0.37%	0.41%	0.33%	0.36%	0.37%	0.32%				
0.080%	0.092%	0.082%	0.070%	0.097%	0.069%	0.095%	0.093%	0.083%	0.082%	**2.5%**	0.011%	**13.4%**
(LOQ)	0.092%	0.077%	0.056%	0.086%	0.069%	0.085%	0.082%	0.084%				
0.040%	*0.038%*	*0.033%*	*0.032%*	*0.039%*	*0.028%*	*0.040%*	*0.044%*	*0.027%*	***all positive***			
(LOD)												

Precision (Repeatability Standard Deviation RSD_r)

Standard deviation of test results obtained under repeatability conditions (intra-laboratory).

Acceptance criteria: $RSD_r \leq 25\%$ over the whole dynamic range (0.080% to 5.0% GMO level) - **FULFILLED**

Accuracy (Bias)

Closeness of agreement between a test result and the accepted reference value

Acceptance criteria: The accuracy should be within +/- 25% of the accepted reference value over the whole dynamic range (0.080% - 5.0% GMO level) – **FULFILLED**

Dynamic range

Range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance criteria: The dynamic range includes 1/10th and at least 5 times the labeling threshold (0.090% - 4.5% GMO level). Acceptance criteria for accuracy and RSD_r are met over the whole dynamic range – **FULFILLED**

LOQ - Limit of quantification

Lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy. The LOQ shall not exceed $1/10^{th}$ of the labeling threshold (0.090% GMO content).

Acceptance criteria: $RSD_r \leq 25\%$ and quantification results within +/- 25% of the accepted reference value for 0.080% GMO - **FULFILLED**

LOD - Limit of detection

Lowest amount or concentration of analyte in a sample which can be detected reliably, but not necessarily quantified. LOD should be less than $1/20^{th}$ of the labeling threshold (0.045% GMO content).

Acceptance criteria: 95% positive results for 0.040% GMO – **FULFILLED**

5.5 Amplification Efficiency and R^2 Coefficient

In order to assess the amplification efficiency and R^2 coefficient of both PCR systems the standard curves of eight independent runs (see Section 5.4) have been evaluated

and the regression parameters including slope, intercept and R^2 have been determined. The efficiency of the amplification was calculated by the following equation: $E = [10^{(-1/slope)}]^{-1}$.

Table 10. Regression parameters and PCR efficiencies of the soybean-specific calibration curves

	Slope	Intercept	R^2	E
Run 1	-3.34	37.7	0.999	0.99
Run 2	-3.31	37.7	0.999	1.00
Run 3	-3.35	38.0	0.999	0.99
Run 4	-3.37	37.9	0.999	0.98
Run 5	-3.33	38.0	0.997	1.00
Run 6	-3.38	37.9	0.999	0.98
Run 7	-3.39	38.0	0.999	0.97
Run 8	-3.38	38.1	1.000	0.98
MEAN	**-3.36**	**37.9**	**0.999**	**0.99**

Acceptance criteria:

The average value of the slope should be in the range of –3.1 ≥ slope ≥ -3.6 - **FULFILLED**

The average value of R^2 should be ≥ 0.98 – **FULFILLED**

Table 11. Regression parameters and PCR efficiencies of the CV127-specific calibration curves

	Slope	Intercept	R^2	E
Run 1	-3.53	38.9	1.000	0.92
Run 2	-3.46	38.5	1.000	0.95
Run 3	-3.36	38.5	1.000	0.98
Run 4	-3.50	38.7	1.000	0.93
Run 5	-3.39	38.6	0.999	0.97
Run 6	-3.50	38.7	1.000	0.93
Run 7	-3.51	38.7	0.999	0.93
Run 8	-3.47	38.8	1.000	0.94
MEAN	**-3.47**	**38.7**	**1.000**	**0.94**

Acceptance criteria:

The average value of the slope should be in the range of –3.1 ≥ slope ≥ -3.6 - **FULFILLED**

The average value of R^2 should be ≥ 0.98 – **FULFILLED**

5.6 Robustness

5.6.1 Variation of concentration of PCR reaction mix

In order to assess the stability of both detection systems with respect to changes in the concentration of major reaction components an experiment was conducted at +20% and at -20% of the concentration of all components of the PCR reaction mix. Three samples (0.080%, 0.90% and 5.0% of CV127 soybean DNA in non-GM soybean DNA) were analyzed three times each (in triplicates). The mean value of three independent quantification results obtained for each sample is shown in the following table.

Table 12. Quantification results at +/- 20% of PCR reaction mix

	Expected (true) value (% GMO)		
	0.080%	**0.90%**	**5.0%**
Master mix +20%			
Mean quantification result	0.072%	0.91%	4.7%
Relative deviation from true value	**-10.0%**	**1.1%**	**-6.0%**
Master mix -20%			
Mean quantification result	0.078%	0.95%	4.4%
Relative deviation from true value	**-2.5%**	**5.6%**	**-12.0%**

Acceptance criteria: Results within +/- 30% of the accepted reference value – **FULFILLED**

5.6.2 Variation of annealing temperature

In order to assess the influence of varying the annealing temperature three samples (0.080%, 0.90% and 5.0% of CV127 soybean DNA in non-GM soybean DNA background) were analyzed in triplicate in three times each (in triplicates) with annealing temperatures of 58 °C and 62 °C on the ABI 7500 Fast Real-Time PCR system. The mean value of three independent quantification results obtained for each sample is shown in the following table.

Table 13. Quantification results using different annealing temperatures

	Expected (true) value (% GMO)		
	0.080%	**0.90%**	**5.0%**
58 °C			
Mean quantification result	0.094%	1.06%	4.9%
Relative deviation from true value	**17.5%**	**17.8%**	**-2.0%**
62 °C			
Mean quantification result	0.065%	0.82%	4.6%
Relative deviation from true value	**-18.8%**	**-8.9%**	**-8.0%**

Acceptance criteria: Results of the mean values within +/- 30% of the accepted reference value – **FULFILLED**

5.6.3 Performance on different instrument types

In order to assess the influence of different real-time PCR platforms, three samples (0.080%, 0.90% and 5.0% CV127 soybean DNA in non-GM soybean DNA background) were analyzed in triplicates three times at 100 ng genomic DNA per reaction each on an ABI PRISM® 7700, 7900 HT and Stratagene Mx3005P detection system. The following table shows the mean value of three independent quantification results obtained for each GM level.

Table 14. Quantification results using different platforms

	Expected (true) value (% GMO)		
	0.080%	**0.90%**	**5.0%**
ABI 7900HT			
Mean quantification result	0.098%	1.09%	4.8%
Relative deviation from true value	**22.5%**	**21.1%**	**-4.0%**
ABI PRISM® 7700			
Mean quantification result	0.086%	0.96%	5.1%
Relative deviation from true value	**7.5%**	**6.7%**	**2.0%**
Stratagene Mx3005P			
Mean quantification result	0.103%	1.16%	4.8%
Relative deviation from true value	**28.8%**	**28.9%**	**-4.0%**

Acceptance criteria: Results of the mean values are within +/- 30% of the accepted reference value – **FULFILLED**

5.7 Interlaboratory transferability

In order to assess the reproducibility of test between laboratories (interlaboratory transferability) one quantification run was performed at the following laboratory:

Eurofins Medigenomix GmbH

Frauenhoferstr. 22
D-82152 Martinsried

Different samples containing 0.080% - 5.0% GMO content were analyzed two times (each in triplicates) at 100 ng genomic DNA per reaction on an ABI 7900HT sequence detection system. The following table shows the mean quantification result obtained for each GM level.

Table 15. Quantification results under reproducibility conditions (interlaboratory)

	Expected (true) value (% GMO)				
	0.080%	**0.50%**	**0.90%**	**2.0%**	**5.0%**
Mean quantification result	0.102%	0.51%	1.09%	2.1%	4.9%
Relative deviation from true value	**27.5%**	**2.0%**	**21.1%**	**5.0%**	**-2.0%**

The results indicate good interlaboratory transferability.

Acceptance criteria: Results within +/- 35% of the accepted reference value at 0.50%, 0.90%, 2.0% and 5.0% GMO; results within +/- 50% of the accepted reference value at 0.080% GMO
– **FULFILLED**

III. Protocol for Event-Specific Quantitation of CV127 Soybean

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 detection method for CV127 soybean should be used in conjunction with a DNA extraction method which yields DNA of sufficient purity and quantity. Such a DNA extraction method is also provided (see Section I, “Validation of the CTAB-Anion-Exchange Method for DNA Extraction from Soybeans” of this document,).

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 le1 gene from Glycine max3 (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,

[3] 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.

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 dilution buffer (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 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[4].

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

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

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 16. 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 µl)
CV127 copies (haploid)	8658	1732	346	69
Soybean genome copies (haploid)	86580	17316	3463	693

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

Chemicals	Concen-tration	Final concen-tration*	µl/rxn	µl/50 runs
TaqMan Universal PCR Master Mix with UNG (ABI)	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 18. Preparation of the master mix for the CV127-specific PCR system

Chemicals	Concen-tration	Final concen-tration*	µl/rxn	µl/50 runs
TaqMan Universal PCR Master Mix with UNG (ABI)	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 19. 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 20. 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 21. 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 equivalent

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 22. 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 Part No. 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 5. 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.