EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Soybean DAS-68416-4 Using Real-time PCR

Validated Method

13 May 2014

Method development:

Dow AgroSciences LLC

Method validation:

European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF)

Quality assurance

The EU-RL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EU-RL GMFF quality system.

The EU-RL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EU-RL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection IHCP provided by CERMET.

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Content

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 DAS-68416-4 (unique identifier DAS-68416-4) DNA to total soybean DNA in a sample.

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 DAS-68416-4, a 130-bp fragment of the region spanning the 3' insert-to-plant junction in soybean DAS-68416-4 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 the compound MGBNFQ (Minor Groove Binding Non-Fluorescent Quencher) as a quencher dye at its 3' end.

For the relative quantification of soybean event DAS-68416-4 DNA, a soybean-specific reference system amplifies a 74-bp fragment of *lectin (Le1)*, a soybean endogenous gene (Accession number, GeneBank: K00821 and M30884), using *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with FAM as reporter dye at its 5' end, and TAMRA (6-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 "Ct" value. For quantification of the amount of DAS-68416-4 DNA in a test sample, Ct values for the DAS-68416-4 and *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DAS-68416-4 DNA to total soybean DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional soybean seeds. Precision and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF).

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 0.04% (related to mass fraction of GM material) in 100 ng of total soybean DNA. The relative LOD was not assessed by the EU-RL GMFF 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% (related to mass fraction of GM material) in 100 ng of total soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1% (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 DAS-68416-4; the sequence is specific to event DAS-68416-4 and thus imparts event-specificity to the method.

The specificity of the event-specific assay was assessed by the applicant in real-time PCR using genomic DNA samples (100 ng) containing 1% of the GMO, extracted from DAS-68416-4 (positive control sample) and from maize DAS 40278-9, DAS-40474-7, GA21, Bt176, Bt11, NK603, MON863, TC1507, MIR604, 59122, T25, MON810, MON88017, 3272, MON89034; sugar beet H7-1; oilseed rape Ms8, Rf3, T45, RT73; cotton MON531, MON15985, MON1445, GHB614, LLcotton25, 281-24-236x3006-210-23; potato EH92-527-1; soybean DAS-64209-9, A2704-12, DP305423, DP356043, GTS 40-3-2, MON89788, A5547-127; rice LLRICE62 and conventional soybean, maize, oilseed rape, rice, cotton, wheat, potato and sugarbeet.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the TaqMan® probe of the DAS-68416-4 event showed no amplification signals following quantitative PCR analysis (45 cycles).

The specificity of the soybean taxon-specific assay was not assessed by the method developer because the *Le1* assay had been previously validated by the EU-RL GMFF. For details see http://gmo-crl.jrc.ec.europa.eu/summaries/DP356043_validated_Method_correctedversion1.pdf).

Specificity was further verified and confirmed *in silico* by the EU-RL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

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 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 DAS-68416-4

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*Le1*) and for the GMO (event DAS-68416-4) target sequence is to be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated and is therefore not recommended.

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 trial.

For the collaborative trial, the calibration curve was established on the basis of five samples. The first point of the calibration curve contained 10% soybean DAS-68416-4 DNA in a total of 120 ng of soybean DNA (corresponding to approximately 106195 soybean genome copies and to 10619 copies of DAS-68416-4, with one genome assumed to correspond to 1.13 pg of haploid soybean genomic DNA) [(1)].

Standards S2 and S3 were prepared by serial 3-fold dilution of the S1 sample. Standard S4 and S5 were prepared by a 4-fold dilution of the standard S3.

The copy number values of the calibration samples and total DNA quantity used in PCR are reported 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)	120	40	13.3	3.35	0.85
Target taxon *Le1* copies	106195	35398	11799	2950	737
DAS-68416-4 soybean GM copies	10619	3540	1180	295	74

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 software of the real-time PCR equipment.

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 DAS-68416-4 assay and one for the *Le1* 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 DAS-68416-4 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix no AmpErase® UNG 2x	1x	12.5
DAS-68416-4-3f5 (10 µM)	550 nM	1.375
DAS-68416-4-3r3 (10 µM)	550 nM	1.375
DAS-68416-4-3p3 (10 µM)	150 nM	0.375
AmpliTaq Gold® DNA Polymerase (5U/µl)	1U	0.2
Nuclease free water	#	4.175
DNA	#	5
Total reaction volume:		25 µL

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

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix no AmpErase® UNG 2X	1x	12.5
Lec for2 (10 µM)	650 nM	1.625
GMO3-126 Rev (10 µM)	650 nM	1.625
Lec probe (10 µM)	180 nM	0.450
Nuclease free water	#	3.800
DNA	#	5
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.

4. Prepare two reaction tubes (one for the soybean DAS-68416-4 and one for the *Le1* 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 (e.g. (70 µL for the *Le1* reference system and 70 µL for the DAS-68416-4 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 at 4 °C) 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 DAS-68416-4/*Le1* methods.

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: display the amplification curves of one assay (e.g. DAS-68416-4) 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 (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: 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), b) and c) on the amplification plots of the other system (e.g. *Le1*).

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 Ct values for each reaction.

The standard curves are generated both for the *Le1* and the DAS-68416-4 specific assays by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the DNA copy number in the unknown samples.

To obtain the percentage value of event DAS-68416-4 DNA in the unknown sample, the DAS-68416-4 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 (GM% = DAS-68416-4/*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
- 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 AmpErase® UNG. Applied Biosystems Part No 4326614.
- AmpliTaq Gold® DNA Polymerase, Applied Biosystems, Cat N8080248

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5′ to 3′)	Length (nt)
DAS-68416-4			
Forward primer	DAS-68416-4_3f5	5′ gTA CAT TAA AAA CgT CCg CAA TgT gT 3′	26
Reverse primer	DAS-68416-4_3r3	5′ gTT TAA gAA TTA gTT CTT ACA gTT TAT TgT TAg 3′	33
Probe	DAS-68416-4_3p3	5′-6FAM- TTA AgT TgT CTA AgC gTC AAT A –MGBNFQ-3′	22
Le1			
Forward primer	Lec for2	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′ 6FAM- CTT CAC CTT CTA TgC CCC TgA CAC-TAMRA 3'	24

FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine; MGBNFQ: Minor Groove Binding Non-Fluorescent Quencher

5. References

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