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JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection  
Molecular Biology and Genomics Unit



# Event-specific Method for the Quantification of Soybean DAS-81419-2 by Real-time PCR

## Validated Method

**13 March 2015**

**Method development:**

Dow AgroSciences LLC

## Quality assurance

The EURL 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](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 EURL GMFF quality system.

The EURL 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 EURL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection (IHCP) provided by SGS.

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## 1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan<sup>®</sup> PCR (polymerase chain reaction) procedure for the determination of the relative content of soybean (*Glycine max*) event DAS-81419-2 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 a 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-81419-2, a 105 bp fragment of the region spanning the 5' plant-to-insert junction in soybean event DAS-81419-2 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 TAMRA (carboxytetramethylrhodamine) as a quencher at its 3' end.

For the relative quantification of soybean event DAS-81419-2 DNA, a *Glycine max* specific system amplifies a fragment of the *lectin (Le1)* endogenous gene, using (*Le1*) gene-specific primers and a (*Le1*) gene-specific probe, labelled with FAM as reporter dye at its 5' end, and TAMRA as quencher at its 3' end. The amplified *Le1* fragment is 74 bp long.

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-81419-2 DNA in a test sample, Ct values for the DAS-81419-2 and *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DAS-81419-2 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 grains. The reproducibility 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 (EURL GMFF). The study was undertaken with 12 participating laboratories in June-July 2014.

Each participant received twenty blind samples containing mixtures of soybean DAS-81419-2 genomic DNA and non-GM soybean genomic DNA at five GM contents, ranging from 0.1% to 5% (copy/copy). Each laboratory received each GM level in four blind replicates. Each test sample was analysed by PCR in three repetitions. Two replicates of each GM level were analysed on the same PCR plate.

The validation report can be found at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

### **2.3 Limit of detection (LOD)**

According to the method developer, the relative LOD of the method is at least 0.04% (GM DNA copy numbers to target taxon-specific DNA copy numbers) in 100 ng of total soybean DNA. The relative LOD was not assessed in the collaborative study.

### **2.4 Limit of quantification (LOQ)**

According to the method developer, the relative LOQ of the method is at least 0.085% (GM-DNA copy numbers to target taxon-specific DNA copy numbers) 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/mass) equivalent to level 0.1% expressed in terms of copy number ratio).

### **2.5 Molecular specificity**

The method exploits a unique DNA sequence in the region spanning the 5' plant-to-insert junction in soybean event DAS-81419-2; the sequence is specific to event DAS-81419-2 and thus imparts event-specificity to the method.

The specificity of the event-specific assay was assessed by the method developer in real-time PCR using genomic DNA (100 ng) containing 1% of the GMO extracted from DAS-81419-2 soybean (positive control sample) and from maize events GA21, Bt176, Bt11, NK603, MON863, TC1507, MIR604, MON810, DAS59122, 3272, MON88017, MON89034, T25, MIR162, 98140; soybean DAS-28129-1, DAS-81615-9, DAS-28151-5, DAS-68416-4, A2704-12, A5547-127, DP305423, DP356043, GTS 40-3-2, MON89788, FG72, MON87701, CV127, DAS-44406-6; oilseed rape Ms8, RF3, T45, GT73/RT73, Topas 19/2, Ms1, Rf1, Rf2; cotton GHB614, MON1445, MON531, MON15985, 281-24-236 x 3006-210-23, LLCotton25, GHB119, T304-40, MON88913; sugar beet H7-1; potato EH92-527-1 and AM04-1020; rice LLRICE62 and conventional soybean, oilseed rape, rice, maize, potato, sugar beet, wheat, cotton.

According to the method developer, the forward and reverse oligonucleotide primers and the TaqMan<sup>®</sup> probe of the DAS-81419-2 method showed no amplification signals in the quantitative PCR analysis of the samples, apart from the soybean event DAS-81419-2. Specificity was

further verified *in silico* by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

The detection method reported spans the junction between the 5' genomic region and the transgenic insert.

The DAS81419-f2 primer binding site was found in the genomic border adjacent to the insertion. The DAS81419-r1 primer and the DAS81419-p3 probe bind in the insert, in a region that corresponds to recombined fragments of the Cry1Ac gene.

It should be noted that the reverse primer has two additional almost perfect binding sites (1 and 2 bases different, according to the submitted sequence). However, these binding sites are found far (about 5kb and 8kb) from the amplicon, and in the wrong orientation, so no secondary amplicon should be produced.

The amplicon size is expected to be 105 bp, consistent with data reported by the applicant. The sequence of the amplicon was analysed by BLAST<sup>(1)</sup> against local copies of the Nucleotide collection (nr/nt) and patents (patnt) databases available at ftp://ftp.ncbi.nih.gov/blast/db/ on 20 June 2013, and no significant similarity was found with any other published sequence.

In addition, the primers were tested against the sequences of the other GM events present in the Central Core Sequence Information System of the JRC, as well as the whole genomes of *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays* using the e-PCR prediction tool<sup>(2, 3)</sup>, and no potential amplicon was identified.

## 3. Procedure

### 3.1 General instructions and precautions

- The procedure requires 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 employed previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All 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-81419-2**

#### *3.2.1 General*

The PCR set-up for the taxon-specific target sequence (*Le1*) and for the GMO (event DAS-81419-2) target sequence is 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 (S1) contained 10% soybean event DAS-81419-2 DNA (GM-DNA copy numbers to target taxon-specific DNA copy numbers) in a total of 125 ng of soybean DNA (GM% calculated considering the 1C value for soybean genome as 1.13 pg)<sup>(4)</sup>. Standards S2 to S5 were prepared by serial four-fold dilution of the S1 sample.

The copy number values of the calibration samples and total amount of DNA/reaction are reported in Table 1.

Table 1. Total amount of DNA in PCR reaction and copy number values of the standard curve samples.

<b>Sample</b>	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	125	31.2	7.8	1.95	0.49
Target taxon <i>Le1</i> copies	110619	27655	6914	1728	432
DAS-81419-2 soybean GM copies	11062	2765	691	173	43

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.

### 3.3 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-81419-2 assay and one for the *Le1* assay) on ice in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the DAS-81419-2 assay.

Component	Final concentration	µL/reaction
2x TaqMan® Universal Master Mix, No UNG	1x	12.5
DAS81419-f2 forward primer (10 µM)	400 nM	1.00
DAS81419-r1 reverse primer (10 µM)	400 nM	1.00
DAS81419-p3 probe (10 µM)	120 nM	0.30
Nuclease free water	/	5.20
Template 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* assay.

Component	Final concentration	µL/reaction
2x TaqMan® Universal Master Mix, No UNG	1x	12.5
Lec for2 forward primer (10 µM)	650 nM	1.625
GMO3-126 Rev reverse primer (10 µM)	650 nM	1.625
Lec probe (10 µM)	180 nM	0.45
Nuclease free water	/	3.80
Template DNA	/	5.0
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.
4. Prepare two reaction tubes (one for the soybean DAS-81419-2 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-81419-2 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 for minimising the variability among the repetitions of each sample.

6. Spin down the tubes in a micro-centrifuge. Aliquot 25  $\mu$ L 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-81419-2/*LeI* assays.

Step	Stage	T (°C)	Time (s)	Acquisition	Cycles	
1	Initial denaturation	95	600	No	1x	
2	Amplification	Denaturation	95	15	No	40x
		Annealing & Extension	60	60	Yes	

### 3.4 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-81419-2) 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. *LeI*).
- e) Save the settings and export all the data to a text file for further calculations.

### **3.5 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-81419-2 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-81419-2 DNA in the unknown sample, the DAS-81419-2 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 ( $GM\% = \text{DAS-81419-2} / \text{Le1} \times 100$ ).

## **4. Equipment and 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 instruments (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<sup>®</sup> Universal PCR Master Mix, No AmpErase<sup>®</sup> UNG. Applied Biosystems Part No 4324020.

### 4.3 Primers and Probes

Oligonucleotide	Name	DNA Sequence (5' to 3')	Length (nt)
Event DAS-81419-2			
Forward primer	DAS81419-f2	5'-TCT AgC TAT ATT Tag CAC TTg ATA TTC AT-3'	29
Reverse primer	DAS81419-r1	5'-gCT TCA AgA TCC CAA CTT gCg-3'	21
Probe	DAS81419-p3	5'-FAM-ATC AAC Agg CAC CgA TgC gCA CCg-TAMRA-3'	24
<i>Le1</i>			
Forward primer	Lec F	5'-CCA gCT TCg CCg CTT CCT TC-3'	20
Reverse primer	Lec R	5'-gAA ggC AAg CCC ATC TgC AAg CC-3'	23
Probe	Lec P	5'-FAM-CTT CAC CTT CTA TgC CCC TgA CAC-TAMRA-3'	24

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine

## 5. References

1. Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*, 1997; 25: 3389-3402
2. Schuler G.D. Sequence mapping by electronic PCR. *Genome Res*, 1997; 7(5):541-50
3. Rotmistrovsky K., Jang W., Schuler G.D. A web server for performing electronic PCR. *Nucleic Acids Res*, 2004; 32(Web Server issue):W108-112
4. Plant DNA C-values Database, <http://data.kew.org/cvalues/>