EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Soybean Event DP-305423-1 Using Real-time PCR

Validated Method

19 January 2009

Corrected version 1 - 29/03/2010 (see page 2)

Corrected version 2 - 27/08/2013 (see page 2)

Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Method development:

Pioneer Overseas Corporation

Collaborative trial:

European Reference Laboratory for GM Food and Feed (EURL-GMFF)
Molecular Biology and Genomics Unit

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 apply 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 IHCP Institute provided by CERMET

Correction from the previous version:

Corrected version 1 - 29/03/2010

Page 10 §4.2 :
TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437 changed by
TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No **4324018**

Corrected version 2 - 27/08/2013

Page 1 and 2: New layout

Page 4, 5, 6, 7, 8, 9, 10:
The PCR set-up for the taxon specific target sequence (lec) changed by
*The PCR set-up for the taxon specific target sequence (***Le1***)*

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Content

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of soybean event DP-305423-1 DNA to total soybean DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction plates.

Template DNA extracted by means of suitable methods (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) 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 DP-305423-1 DNA, a 93 bp fragment of the integration region of the construct inserted into the plant genome (3' insert-to-plant junction) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM dye and TAMRA as quencher dye.

For the relative quantification of soybean event DP-305423-1 DNA, a soybean-specific reference system amplifies a 74-bp fragment of the soybean endogenous *lectin* gene (*Le1, GeneBank Accession No K00821*), using a pair of specific primers and a *Le1* gene-specific probe labelled with FAM and TAMRA as described above.

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 event DP-305423-1 DNA in a test sample, Ct values for the DP-305423-1 and the *Le1* system are determined for the sample. Standard curves are then used to estimate the relative amount of soybean event DP-305423-1 DNA to total soybean DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from homogenised soybean seeds.

The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GMO contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve participating laboratories in September 2007.

Each participant received twenty blind samples containing DP-305423-1 genomic DNA at five GM contents, ranging from 0.09 % to 5.0 %.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM DP-305423-1 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

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% in 100 ng of total soybean DNA. The relative LOD was not assessed in a collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.08% in 100 ng of total soybean DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.09 %.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to soybean event DP-305423-1 and thus imparts event-specificity to the method.

The specificity of the event-specific assay was experimentally tested by the method developer in real-time PCR against samples containing 100 ng of 1% GM content or 20 ng non-GM gDNA from: soybean DP-305423-1, EAFS 3040.6.2, EAFS 3044.1.15, RoundUp Ready; maize MON 810, Bt176, Bt11, GA21, T25, MON 863; rapeseed RoundUp Ready; rice LL62 and conventional soybean, rapeseed, rice, wheat, maize, potato, sugar beet, cotton.
According to the method developer, none of the lines tested, except the positive control soybean line DP-305423-1, yielded detectable amplification.

The specificity of the soybean reference assay *Le1* was experimentally tested by the method developer against DNA extracted from plant materials containing at least 1000 genomic copies of soybean, rapeseed, rice, wheat, maize, potato, sugar beet, cotton, sugar pea, navy bean, broad bean. According to the method developer, none of the samples tested, except the positive control soybean, yielded detectable amplification.

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
- 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 used 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 DP-305423-1

3.2.1 General

The PCR set-up for the taxon specific target sequence (*Le1*) and for the GMO (event DP-305423-1) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 100 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 25 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of four samples. The first point of the calibration curves is a 10% DP-305423-1 soybean DNA for a total of 100 ng of DNA (corresponding to 86,580 soybean genome copies with one genome assumed to correspond to 1.155 pg of haploid soybean genomic DNA) [(1)].

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

The copy number measured for the unknown sample DNA is obtained by interpolation from the standard curves.

The ratio of transgene copy number to reference gene copy number multiplied by 100 gives the % GM contents of the samples.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. **Keep thawed reagents at 1-4°C on ice**.
2. In two reaction tubes (one for the DP-305423-1 system and one for the *Le1* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the amplification reaction mixtures.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the DP-305423-1 specific system.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.50
DP305-f1 (10 µM)	800 nM	2.00
DP305-r5 (10 µM)	500 nM	1.25
DP305-p (10 µM)	220 nM	0.55
Nuclease free water	#	3.70
Template DNA (max 100 ng)	#	5.0
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the soybean *Le1* reference system.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
GMO3-126 Rev (10 µM)	550 nM	1.375
Le1 for2 (10 µM)	550 nM	1.375
Le1 probe (10 µM)	100 nM	0.25
Nuclease free water	#	4.5
Template DNA (max 100 ng)	#	5.0
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the DP-305423-1 and one for the *Le1* reaction mixes) 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 (e.g. 20 x 3 = 60 µL reaction mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 x 3 = 15 µL DNA for three PCR repetitions). Vortex each tubes 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 microcentrifuge. 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 3000 rpm for 1 minute) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Cycling program for DP-305423-1/*Le1* systems.

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	Initial denaturation		95	600	No	1
2	Amplification	Denaturation	95	15	No	45
		Annealing & Extension	60	60	Yes	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

a) Set the threshold: display the amplification curves of one system (e.g. DP-305423-1) 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. 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 geometric 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) and b) on the amplification plots of the other system (e.g. *Le1* system).

e) Save the settings and export all the data to a text file 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 DP-305423-1 specific systems 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 copy numbers in the unknown sample DNA.

For the determination of the amount of event DP-305423-1 DNA in the unknown sample, the DP-305423-1 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 to obtain the percentage value (GM% = DP-305423-1/*Le1* x 100).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction plates (glass capillaries are not recommended for the described buffer composition)

- Plastic reaction plates 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
- Centrifuge for plates
- Micropipettes
- Vortex
- Rack for reaction tubes
- 0.5/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4324018

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
DP-305423-1 target sequence	
DP305-f1	5' – CGT GTT CTC TTT TTG GCT AGC – 3'
DP305-r5	5' – GTG ACC AAT GAA TAC ATA ACA CAA ACT A – 3'
DP305-p	6-FAM 5' – TGA CAC AAA TGA TTT TCA TAC AAA AGT CGA GA – 3' TAMRA
Reference gene *Le1* target sequence	
Le1 for2	5' – CCA GCT TCG CCG CTT CCT TC – 3'
GMO3-126 Rev	5' – GAA GGC AAG CCC ATC TGC AAG CC – 3'
Le1 Probe	6-FAM 5' – CTT CAC CTT CTA TGC CCC TGA CAC – 3' TAMRA