EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F – Health, Consumers and Reference Materials

Food & Feed Compliance (F.5)

Event-specific Method for the Quantification of soybean GMB151 by Real-time PCR

Validated Method

Method development:

Bayer CropScience

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 GMB151 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 assays. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are also recommended to ensure suitability of the extracted DNA.

For the detection of GM event GMB151, an 84 bp fragment of the region spanning the 5' insert-to-plant junction in soybean GMB151 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and BHQ-1 (Black Hole Quencher® 1) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event GMB151, a soybean taxon-specific system amplifies a 102 bp fragment of a soybean lectin 1 (Le1) endogenous gene (Accession number, GeneBank: K00821.1 M30884), using *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with JOE as reporter dye at its 5' end and BHQ-1 (Black Hole Quencher® 1) as non-fluorescent] quencher dye at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Cq" value. For quantification of the amount of GMB151 DNA in a test sample, Cq values for the GMB151 and the *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of GMB151 DNA to total soybean DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA that can be extracted from genetically modified and conventional soybean seeds, grains and leaves. 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 (EURL GMFF). The study was undertaken with twelve participating laboratories in April 2019.

A detailed 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 0.03 % (related to mass fraction of GM material) in 200 ng of total suitable soybean DNA. The sample was analysed in six replicates in three real-time PCR runs (total eighteen values, with GM target detected in all reactions). According to the method developer, the LOD_{abs} of the GMB151 and of the Le1 systems is below 10 haploid genome copies). The 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 0.08 % (related to mass fraction of GM material) in 200 ng of total suitable 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 5' insert-to-plant junction in soybean GMB151 and is therefore event-specific for the event GMB151. This was confirmed by the applicant's specificity studies and by in silico analysis performed by the applicants and the EURL GMFF.

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 should have been 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 regularly
- Laboratory benches and equipment should be cleaned periodically, with 10% sodium hypochlorite solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.

- All handling steps, unless specified otherwise, should be carried out at room temperature.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of soybean event GMB151

3.2.1 General

The real-time PCR set-up for the taxon (*Le1*) and the GMO (event GMB151) target sequences are carried out in separate vials. Multiplex qPCR (using differential fluorescent labels for the probes) has not been tested or validated by the EURL GMFF.

The method is developed for a total volume of 25 µL per reaction mixture for the GM (event GMB151) and the taxon (*Le1*) assay with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curves have to be established on at least five samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % soybean GMB151 DNA in a total of 300 ng of soybean DNA (corresponding to 265487 soybean haploid genome copies with one haploid genome assumed to correspond to 1.13 pg of soybean genomic DNA) [(1)]. Standards S2 to S5 are to be prepared by serial dilutions (dilution factor 3 for samples S2, dilution factor 5 for sample S3, dilution factor 4 for sample S4 and dilution factor 10 for standard S5) according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of soybean DNA in reaction (ng)*	300	100	20	5	0.5
Soybean haploid genome copies	265487	88496	17699	4425	442
GMB151 copies	26549	8850	1770	442	44

* Total nanograms are rounded to the integral value

A calibration curve is to be produced by plotting the Cq 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.

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

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. In two tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mixes for the GMB151 soybean specific system (Table 2) and the *Le1* reference gene system (Table 3). Please note that additional volume is included in the total to cover pipetting variability due to the viscosity of the solution.

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the GMB151 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
PRIM1040 (10 µM)	400 nM	1.0
PRIM1041 (10 µM)	400 nM	1.0
TM1789* (10 µM)	200 nM	0.5
Nuclease free water	-	5
DNA	-	5
Total reaction volume:		25 µL

*TaqMan® probe labelled with 6-FAM at its 5'-end and BHQ-1 at its 3'-end

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 (2x)	1x	12.5
KVM164 (10 µM)	200 nM	0.5
KVM165 (10 µM)	200 nM	0.5
TM1242* (10 µM)	200 nM	0.5
Nuclease free water	-	6
DNA	-	5
Total reaction volume:		25 µL

*TaqMan® probe is labelled with JOE at its 5'-end and BHQ-1 at its 3'-end]

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for the soybean GMB151 and one for the *Le1* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (70 µL for the GMB151 soybean system and 70 µL for the *Le1* system). Add to each tube the correct amount

of DNA for 3.5 PCR repetitions (17.5 µL DNA). The volume for the additional 0.5 repetition will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.

6. Spin down the tubes. Aliquot 25 µL for GMB151 system and for the *Le1* reference system in each well.
7. Place an optical cover on the reaction plate and briefly centrifuge the plate.
8. Place the reaction plate in the real-time PCR apparatus (possibly apply a compression pad, depending on the model), according to the manufacturer's instructions and your Standard Operating Procedures and start the run.
9. Select FAM as reporter dye for the GMB151 and JOE for the *Le1* reference system. Define BHQ-1 or non-fluorescent as quencher dye for GMB151 specific system and BHQ or non-fluorescent for *Le1* reference system. Select ROX as the passive reference dye, according to the instructions of your instrument. Enter the correct reaction volume (25 µL).
10. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for GMB151/*Le1* assays.

Step	**Stage**		**T (°C)**	**Time (s)**	**Acquisition**	**Cycles**
1	[UNG*]		50	120	No	1
2	Initial denaturation		95	600	No	1
3	Amplification	Denaturation	95	15	No	40
		Annealing & Extension	60	60	Yes	

*UNG: Uracil-N-glycosylase

3.3 Data analysis

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

a) Set the threshold following the automatic or the manual mode. In the manual mode display the amplification curves of the event specific assay 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 Cq 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 following the automatic or the manual mode. In the manual mode: 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 Cq = 25, set the baseline crossing at Cq = 25 – 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a), b) and c) on the amplification plots of the taxon specific system.

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 Cq values for each reaction.

The standard curves are generated both for the *Le1* and for the GMB151 specific assays by plotting the Cq 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 GMB151 DNA in the unknown sample, the GMB151 copy number is divided by the copy number of the soybean endogenous gene *Le1* and multiplied by 100 (GM% = GMB151/*Le1* x 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) and appropriate analysis software
- 96-well reaction plates
- Optical caps/adhesion covers

- Microcentrifuge
- Micropipettes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex
- Racks for reaction tubes, also cooled
- 0.5, 1.5 mL and 5 or 15 mL DNAse free reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix. Applied Biosystems Part No 4304437.

4.3 Primers and Probes

Table 5. Primers and probes for the GMB151 and *Le1* methods

		DNA Sequence (5' to 3')	**Length (nt)**
GMB151			
Forward primer	PRIM1040	TCA AAT CAA CAT ggg TgA CTA gAA A	25
Reverse primer	PRIM1041	CAT TgT gCT gAA TAg gTT TAT AgC TAT gAT	30
Probe	TM1789	FAM-5'- CAg TAC Tgg gCC CTT gTg gCg CT-3'-BHQ1	23
Le1			
Forward primer	KVM164	CTT TCT CgC ACC AAT TgA CA	20
Reverse primer	KVM165	TCA AAC TCA ACA gCg ACg AC	20
Probe	TM1242	JOE-5'- CCA CAA ACA CAT gCA ggT TAT CTT gg-3'-BHQ1	26

FAM: 6-carboxyfluorescein; JOE: 4,5-dichloro-dimethoxy-fluorescein; BHQ1: black hole quencher.

5. References

1. Plant DNA C-values Database. Royal Botanic Gardens, Kew, http://data.kew.org/cvalues/