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Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Soybean Event FG72 Using Real-time PCR

Protocol

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

Method development:

Bayer CropScience

Method validation:

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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 FG72 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 FG72, a 70-bp fragment of the region spanning the 3′ insert-to-plant junction in soybean FG72 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 MGB-NFQ (minor groove binding non-fluorescent quencher) at its 3′ end.

For the relative quantification of soybean event FG72 DNA, a soybean-specific reference system amplifies a 102-bp fragment of *lectin1 (le1)*, a soybean endogenous gene (Accession number, GeneBank: K00821) using *le1* gene-specific primers and a *le1* gene-specific probe labelled with VIC 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 FG72 DNA in a test sample, Ct values for the FG72 and *le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of FG72 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. 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 EU- RL GMFF. The study was undertaken with twelve participating laboratories in August-September 2011.

Each participant received twenty blind samples containing soybean FG72 genomic DNA at five GM contents, ranging from 0.1% to 8%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each GM level of event FG72 in four replicates. 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 0.023% in 200 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 0.08% in 200 ng of total soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1%.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region spanning the 3' insert-to-plant junction in soybean FG72; the sequence is specific to event FG72 and thus imparts event-specificity to the method.

The specificity of the soybean taxon-specific assay was assessed by the method developer in real-time PCR using 200 ng of conventional genomic DNA extracted from soybean, rice, cotton, oilseed rape and maize. According to the method developer the soybean-specific reference system did not react with any target DNA except the positive control.

The specificity of the event-specific assay was assessed by the applicant in real-time PCR using genomic DNA (50 ng) extracted from conventional soybean and soybean FG72 event as positive control sample and, rice LLRICE62, oilseed rape (OSR) MS1, MS8, RF1, RF2, RF3, Topas19-2, T45, OXY-235, RT73; soybean A2704-12, A5547-127, GTS40-3-2; Cotton LLCotton25, GHB614, T304-40, GHB119, MON1445; maize MON810, BT11, GA21, NK603, T25.
According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the Taqman probe of the FG72 event showed no amplification signals following quantitative PCR analysis (45 cycles).

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 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 FG72

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*le1*) and for the GMO (event FG72) target sequence is carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.
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 five samples. The first point of the calibration curves is a sample containing 10% soybean FG72 DNA in a total of 150 ng of soybean DNA (corresponding to approximately 132743 soybean genome copies with one genome assumed to correspond to 1.13 pg of haploid soybean genomic DNA) [(1)]. The other four standards are prepared by serial 4-fold dilution of the 10% standard.

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 each unknown sample DNA is obtained by interpolation from the standard curves.

3.2.2 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 1 and 2) in two reaction tubes (one for the FG72 assay and one for the *le1* assay) on ice in the order mentioned below (except DNA).

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the FG72 assay.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x) with UNG	1x	12.5
MAE071 (10 µM)	400 nM	1.0
SHA097 (10 µM)	400 nM	1.0
TM325 (10 µM)	200 nM	0.5
Nuclease free water	#	5.0
Template DNA (100 ng)	#	5.0
Total reaction volume:		25 µL

Table 2. 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) with UNG	1x	12.5
KVM164 (10 µM)	200 nM	0.5
KVM165 (10 µM)	200 nM	0.5
TM021 (10 µM)	200 nM	0.5
Nuclease free water	#	6.0
Template DNA (100 ng)	#	5.0
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.

4. Prepare two reaction tubes (one for the soybean FG72 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 FG72 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 room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 3.

Table 3. Cycling program for FG72/*le1* assays.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	UNG*		50	120	No	1X
2	Initial denaturation		95	600	No	1X
3	Amplification	Denaturation	95	15	No	45X
		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: display the amplification curves of one assay (e.g. FG72) 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 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 FG72 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 numbers in the unknown sample.

To obtain the percentage value of event FG72 DNA in the unknown sample, the FG72 copy number is divided by the copy number of the soybean reference gene (*le1*) and multiplied by 100 (GM% = FG72/*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 (2X). Applied Biosystems Part No 4326708.

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5' to 3')	Length (nt)
FG72			
Forward primer	MAE071	5' AgA TTT gAT Cgg gCT gCA gg 3'	20
Reverse primer	SHA097	5' gCA CgT ATT gAT gAC CgC ATT A 3'	22
Probe	TM325	6-FAM-5' AAT gTg gTT CAT CCg TCT T-MGBNFQ-3'	19
le1			
Forward primer	KVM164*	5' CTT TCT CgC ACC AAT TgA CA 3'	20
Reverse primer	KVM165	5' TCA AAC TCA ACA gCg ACg AC 3'	20
Probe	TM021	6-VIC®-5' CCA CAA ACA CAT gCA ggT TAT CTT gg-TAMRA-3'	26

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

NOTE. The same primer name KVM164 is attributed by Bayer CropScience to two different primer sequences used as forward primer in the reference system for relative quantification of soybean events A2704-12, A5547-127 and FG72.
The difference lies in an additional triplet 'CAC' at the 5' end of the KVM164 applied for the relative quantification of soybean event A2704-12 (KVM164: CAC CTT TCT CgC ACC AAT TgA CA, 23bp long, http://gmo-crl.jrc.ec.europa.eu/summaries/A2704-12_soybean_validated_Method.pdf). The CAC triplet is not present in the KVM164 primer used in the reference system for relative quantification of soybean events A5547-127 (http://gmo-crl.jrc.ec.europa.eu/summaries/A5547_validated%20Method.pdf) and FG72

5. References

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