EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Cotton GHB119 Using Real-time PCR

Protocol

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Joint Research Centre
Institute for Health and Consumer Protection
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Method development:

Bayer CropScience

Method validation:

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

Quality assurance

The EU-RL GMFF is ISO 9001:2008 certified (certificate number: CH-32232) and 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.

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, 21027 Ispra (VA) - Italy

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 cotton event GHB119 DNA to total cotton DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in polymerase chain reaction (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 cotton event GHB119, a 90-bp fragment of the region spanning the 3′ insert-to-plant junction in cotton event GHB119 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, TAMRA (carboxytetramethylrhodamine) as a quencher dye at its 3′ end.

For the relative quantification of cotton event GHB119, a cotton-specific reference system amplifies a 73-bp fragment of the alcoholdehydrogenase C (AdhC) gene, a cotton endogenous gene (Accession number, GeneBank: AF036569 and AF403330), using *AdhC* gene-specific primers and a *AdhC* gene-specific probe labelled with VIC as reporter dye at its 5′ end, and TAMRA (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 GHB119 DNA in a test sample, Ct values for the GHB119 and *AdhC* systems are determined for the sample. Standard curves are then used to estimate the relative amount of GHB119 DNA to total cotton DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional cotton seeds, grain and leaves. The trueness and precision 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 European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF). The study was undertaken with twelve participating laboratories in June-July 2012.

Each participant received twenty test samples containing cotton GHB119 genomic DNA at five GM contents, ranging from 0.1% to 4.5%.
Each laboratory received each GM level of event GHB119 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.

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 cotton 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 cotton 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 of recombination between the insert and the plant genome; the sequence is specific to event GHB119 and thus imparts event-specificity to the method.

The specificity of the cotton taxon-specific assay was assessed by the method developer in real-time PCR using 200 ng conventional genomic DNA extracted from Soybean, Rice, Cotton, Oil Seed Rape and Corn. According to the method developer the cotton-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 DNA (50 ng) extracted from GHB119 event as positive control sample for the GHB119 system reaction and conventional Cotton, Rice LLRICE62, Oil Seed Rape (OSR) MS1, MS8, RF1, RF2, RF3, Topas19-2, T45, OXY-235, RT73, Soybean LL27, LL55, FG72, Round-up Ready Soybean GTS40-3-2, Cotton LLCotton25, GHB614, T304-40, MON1445, Cotton MON810, BT11, GA21, NK603, Corn T25.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the TaqMan® probe of the GHB119 event showed no amplification signals following quantitative PCR analysis.

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 cotton event GHB119

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*AdhC*) and for the GMO (event GHB119) 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 DNA samples. The first point of the calibration curve (S1) is a sample containing 10% cotton GHB119 DNA in a total of 300 ng of cotton DNA (corresponding to approximately 128755 cotton genome copies with one genome assumed to correspond to 2.33 pg of haploid cotton genomic DNA) [(1)]. Standards S2 to S4 are prepared by serial dilutions of the 10% standard. S2 contains 4292 cotton GM copies (3-fold dilution), S3 contains 858 GM copies (five-fold dilution), S4 contains 215 GM copies (four-fold dilution) and S5 contains 43 GM copies (five-fold dilution).

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	300	100	20	5	1
Target taxon *AdhC* copies	128755	42918	8584	2146	429
GHB119 Cotton GM copies	12876	4292	858	215	43

A calibration curve is produced by plotting the Ct values against the logarithm of the target genome 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 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 GHB119 assay and one for the *AdhC* assay) on ice in the order mentioned below (except DNA).

Table 1. Amplification reaction mixture, final volume/concentration per reaction well for the GHB119 method.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x) with UNG	1x	12.5
SHA021 (10 µM)	400 nM	1.0
NEL109 (10 µM)	400 nM	1.0
TM082 (10 µM)	200 nM	0.5
Nuclease free water	#	5.0
Template DNA	#	5.0
Total reaction volume:		25 µL

Table 2. Amplification reaction mixture, final volume/concentration per reaction well for the cotton *AdhC* assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x) with UNG	1x	12.5
KVM157 (10 µM)	200 nM	0.5
KVM158 (10 µM)	200 nM	0.5
TM012 (10 µM)	200 nM	0.5
Nuclease free water	#	6.0
Template DNA	#	5.0
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.
4. Prepare two reaction tubes (one for the GHB119 and one for the *AdhC* assay) 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 *AdhC* reference system and 70 µL for the GHB119 cotton system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (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 GHB119/*AdhC* methods.

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG*		50°C	120	No	1x
2	Initial denaturation		95°C	600	No	1x
3	Amplification	Denaturation	95°C	15	No	45x
		Annealing & Extension	60°C	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. GHB119) 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. *AdhC*).

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 *AdhC* and the GHB119 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 GHB119 DNA in the unknown sample, the GHB119 copy number is divided by the copy number of the cotton reference gene (*AdhC*) and multiplied by 100 (GM% = GHB119/*AdhC* 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 with UNG. Applied Biosystems

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5' to 3')	Length (nt)
GHB119			
Forward primer	SHA021	5'- CCA.gTA.CTA.AAA.TCC.AgA.TCA.TgC.A-3'	25
Reverse primer	NEL109	5'- gAA.ATT.gCg.TgA.CTC.AAA.TTC.C-3'	22
Probe	TM082	6-FAM-5'- CCT.gCA.ggT.CgA.Cgg.CCg.AgT.AC-3'-TAMRA	23
AdhC			
Forward primer	KVM157	5'-CAC.ATg.ACT.TAg.CCC.ATC.TTT.gC-3'	23
Reverse primer	KVM158	5'-CCC.ACC.CTT.TTT.Tgg.TTT.AgC-3'	21
Probe	TM012	VIC -5'-TgC.Agg.TTT.Tgg.TgC.CAC.TgT.gAA.Tg-3'-TAMRA	26

FAM: 6-carboxyfluorescein, TAMRA: carboxytetramethylrhodamine

5. References

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