EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Cotton T304-40 using Real-time PCR

Validated Method

19 December 2012

Method development:

Bayer CropScience

Method validation:

European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF)

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.

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Content

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® polymerase chain reaction (PCR) procedure for the determination of the relative content of cotton event T304-40 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 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 T304-40, a 78-bp fragment of the region spanning the 3′ insert-to-plant junction in cotton event T304-40 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 (6-carboxytetramethylrhodamine) as a quencher dye at its 3′ end.

For the relative quantification of cotton event T304-40, a cotton-specific reference system amplifies a 73-bp fragment of the alcohol dehydrogenase 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 a reporter dye at its 5′ end and TAMRA as a 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 T304-40 DNA in a test sample, Ct values for the T304-40 and *AdhC* systems are determined for the sample. Standard curves are then used to estimate the relative amount of T304-40 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 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 (EU-RL GMFF). The study was undertaken with twelve participating laboratories in July/ August 2012.

Each participant received twenty test samples containing cotton T304-40 genomic DNA at five GM contents, ranging from 0.1% to 4.5%.
Each laboratory received each GM level of event T304-40 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

The method exploits a unique DNA sequence spanning the 3' region of recombination between the insert and the plant genome and is therefore event-specific for the event T304-40.

The specificity of the assay was assessed by the applicant in real-time PCR using genomic DNA (50 ng) extracted from event T304-40 as a positive control sample and from conventional cotton, rice LLRICE62, oilseed rape (OSR) MS1, MS8, RF1, RF2, RF3, Topas19-2, T45, OXY-235, RT73, soybean LL27, LL55, FG72, GTS 40-3-2, cotton LLCotton25, GHB614, GHB119, MON1445, and 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 T304-40 system showed no amplification signals following quantitative PCR analysis.

The specificity of the cotton 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 cotton-specific reference system did not react with any target DNA except the positive control.

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 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 T304-40

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*AdhC*) and for the GMO target sequence (event T304-40) is to be 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 1 and Table 2.

3.2.2 Calibration

The calibration curves has to be established on at lest five DNA samples. The first point of the calibration curve (S1) should be established for a sample containing 10% cotton event T304-40 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 to be prepared by serial dilutions 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 DNA in reaction (ng)	300	100	20	5	1
Target taxon *AdhC* copies	128755	42918	8584	2146	429
T304-40 Cotton GM copies	12876	4292	858	215	43

A calibration curve is to be 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 within the sequence detection system software.

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. To prepare the amplification reaction mixtures add the following components (Table 2 and 3) in two reaction tubes (one for the T304-40 assay and one for the *AdhC* assay) on ice and in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture, final volume/concentration per reaction well for the T304-40 method.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
SHA 029 (10 µM)	400 nM	1
SHA 030 (10 µM)	400 nM	1
TM 089 (10 µM)	200 nM	0.5
Nuclease free water	#	5.0
DNA	#	5.0
Total reaction volume:		25 µL

Table 3. 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)	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 T304-40 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* cotton system and 70 µL for the event T304-40 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 PCR 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 T304-40/*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. T304-40) 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 T304-40 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 samples.

To obtain the percentage value of event T304-40 DNA in the unknown sample, the T304-40 copy number is divided by the copy number of the cotton reference gene (*AdhC*) and multiplied by 100 (GM% = T304-40/*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 (catalogue n. 4318157)

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5' to 3')	Length (nt)
T304-40			
Forward primer	SHA029	5'- AGC GCG CAA ACT AGG ATA AAT T-3'	22
Reverse primer	SHA030	5'- CCT AGA TCT TGG GAT AAC TTG AAA AGA-3'	27
Probe	TM089	6-FAM 5'- TCG CGC GCG GTG TCA TCT ATC TC - TAMRA 3'	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 - TAMRA 3'	26

FAM: 6-carboxyfluorescein, TAMRA: 6-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.