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GM Food and Feed

Event-specific Method for the Quantification of Cotton Line 'LLCotton25' Using Real-time PCR

Protocol

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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 event LLCotton25 DNA to total cotton DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in this method.

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 event LLCotton25 DNA, a 79-bp fragment of the recombination region between the insert and the plant genome (located at the 5′ plant DNA region) is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For the relative quantification of event LLCotton25 DNA, a cotton-specific reference system amplifies a 73-bp fragment of the cotton endogenous *Alcohol dehydrogenase C* gene (*AdhC*), using a pair of specific primers and an *AdhC* 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 LLCotton25 DNA in a test sample, the normalised ΔCt values of the calibration samples are used to calculate by linear regression a reference curve ΔCt-formula. The normalised ΔCt values of the unknown samples are measured and, by means of the reference ΔCt-formula, the relative amount of LLCotton25 event DNA is estimated.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for suitable DNA extracted from cotton seeds and grains.

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 a collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with eleven participating laboratories in December 2005.

Each participant received twenty blind samples containing LLCotton25 genomic DNA at five GM contents, ranging from 0.15 % to 3.3 %.

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 LLCotton25 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found under http://gmo-crl.jrc.it/statusofdoss.htm

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.045% in 200 ng of total cotton 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 0.09% in 200 ng of total cotton DNA.

The lowest relative GM content of the target sequence included in collaborative trial was 0.15 %.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to LLCotton25 event and thus imparts event-specificity to the method.

The specificity of the forward and reverse oligonucleotide primers was experimentally tested in end-point PCRs against DNA extracted from plant materials containing the specific targets of maize T25, MON810, Bt11, Bt176, GA21, NK603, CBH351, Roundup Ready soybean, soybean A2704-12, oilseed rape Ms1, Ms8, Rf1, Rf2, Rf3, Topas19/2, T45, LLRice62 and LLCotton25.
None of the GM-lines tested, except the positive control LLCotton25, yielded detectable amplicons in two replicate experiments.

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 the guidelines given by relevant authorities, e.g. ISO, CEN, Codex Alimentarius Commission.
- 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.
- In order to avoid contamination, 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 LLCotton25

3.2.1 General

The PCR set-up for the taxon specific target sequence (*AdhC*) and for the GMO (LLCotton25) 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 200 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 curve consists of five samples containing different amounts of LLCotton25 DNA in a total amount of 200 ng cotton DNA. The GM content of the standard samples ranges from 3.6% to 0.09%.

A calibration curve is produced by plotting the ∆Ct-values of calibration samples against the logarithm of the respective GM % contents; the slope (a) and the intercept (b) of the calibration curve (y = ax + b) are then used to calculate the mean % GM content of the blind samples based on their normalised ΔCt values.

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 LLCotton25 system and one for the *AdhC* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the cotton *AdhC* reference system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM157 primer (10 µM)	200 nM	0.5
KVM158 primer (10 µM)	200 nM	0.5
TM012 probe (10 µM)	200 nM	0.5
Nuclease free water	#	6
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the LLCotton25 specific system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM155 primer forward (10 µM)	400 nM	1
KVM156 primer reverse (10 µM)	400 nM	1
TM018 Probe (10 µM)	200 nM	0.5
Nuclease free water	#	5
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the LLCotton25 and one for the *AdhC* master 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 master mix (e.g. 20 x 3 = 60 µl master 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 to a minimum.
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 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 cycling conditions described in Table 3:

Table 3. Cycling program for LLCotton25/*AdhC* systems

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG		50 °C	120	No	1
2	Initial denaturation		95 °C	600	No	1
3	Amplification	Denaturation	95 °C	15	No	45
		Annealing & Extension	60 °C	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. LLCotton25) 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. *AdhC* system).

e) Save the settings and export all the data into an Excel 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 reference ΔCt-curve is generated by plotting the ΔCt-values measured for the calibration points against the logarithm of the GM% content, and by fitting a linear regression line into these data.

Thereafter, the reference ΔCt-curve formula is used to estimate the relative amount (%) of LLCotton25 event in the unknown samples of DNA.

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
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
LLCotton25 target sequence	
KVM155	5' –CAG ATT TTT GTG GGA TTG GAA TTC-3'
KVM156	5' –CAA GGA ACT ATT CAA CTG AG-3'
TM018 (Probe)	FAM 5'- CTT AAC AGT ACT CGG CCG TCG ACC GC -3' TAMRA
Reference gene AdhC target sequence	
KVM157	5' – CAC ATG ACT TAG CCC ATC TTT GC -3'
KVM158	5' – CCC ACC CTT TTT TGG TTT AGC -3'
TM012 (Probe)	FAM 5' – TGC AGG TTT TGG TGC CAC TGT GAA TG -3' TAMRA