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DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Event-specific method for the quantitation of cotton 3006-210-23 using real-time PCR

Protocol

Method development:
Dow AgroSciences LLC

Method validation:
Joint Research Centre – European Commission
Biotechnology & GMOs Unit
Community Reference Laboratory for GM Food and Feed

Contents

Document Approval		
Name / *Function*	**Date**	**Signature**
Marco Mazzara *Sector Head*	**21/04/2006**	**Signed**
Stephane Cordeil *Quality Manager*	**21/04/2006**	**Signed**
Guy Van den Eede *B&GMOs Unit Head*	**21/04/2006**	**Signed**

Address of contact laboratory:

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory
Via Fermi 1, 21020 Ispra (VA) - Italy

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 3006-210-23 DNA to total cotton DNA in a sample.

The PCR assay has been 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 the use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run, use of DNA spikes) are recommended.

For specific detection of event cotton 3006-210-23 genomic DNA, a 90-bp fragment of the recombination area between the transgenic insert and the plant genome (located at the 5′ border junction) 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 relative quantitation of event 3006-210-23 DNA, a cotton-specific reference system amplifies part of the intron sequences of the putative SAH7 protein gene of *Gossypium hirsutum*. The SAH7 gene is present not only in the A-subgenome but also in the D-subgenome of *Gossypium hirsutum*. The A-subgenome specific copy differs from the D-sub-genome specific copy by several single or double nucleotide substitutions and small deletions/insertions. Primers and probe of the cotton-specific reference PCR system match perfectly without any single mismatch to both subgenome gene copies. However, due to sequence deviations within the amplified region, the size of the amplicons resulting from A- and D- subgenome differ slightly, being respectively 115 bp and 123 bp of length.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantitation of the amount of event 3006-210-23 DNA in a test sample, event 3006-210-23 and SAH7 Ct values are determined for the sample. Standard curves are then used to calculate the relative content of cotton 3006-210-23 DNA to total cotton DNA.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for DNA extracted from cotton seeds, containing mixtures of genetically modified and conventional cotton.

The reproducibility and trueness of the method was tested through collaborative trial using samples at different GMO contents.

2.2 Collaborative trial

The method was validated in a collaborative trial by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 12 laboratories.

Each participant received twenty unknown samples containing cotton genomic DNA at five concentration levels, between 0.10 % and 5.5 %.

Each test sample was analyzed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM 281-24-236 in four unknown samples. Two replicates of each GM level were analyzed 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

According to the method developer, the relative LOD of the method is at least 0.04%. The relative LOD was not assessed in a collaborative trial. The lowest relative concentration of the target sequence included in collaborative trial was 0.10%.

2.4 Limit of quantitation

According to the method developer, the relative LOQ of the method is 0.09%. The lowest relative concentration of the target sequence included in collaborative trial was 0.10%.

2.5 Molecular specificity

The method utilizes a unique DNA sequence of the recombination region between the transgenic insert and the plant genome. This sequence is specific to 3006-210-23 cotton and thus imparts event-specificity to the detection method.

The specificity of the 3006-210-23 system was assessed by Blast search (19 October 2004) on the amplicon resulting from the event-specific amplification of the transition region of the cotton genomic DNA into the specific event.
No 100% match with other sequences was found.

The specificity was experimentally tested against DNA extracted from plant materials containing the specific targets (at least 1000 genomic copies/reaction) of soybean, rapeseed, rice, wheat, maize, cotton (PH98M.3199, PSC355.112, PH00A.303), Bt176 maize, Bt11 maize, GA21 maize, DAS-59122-7 maize, RR soybean, RR rapeseed, NewLeaf potato, MON 810 maize, Cry1Ac 'sister event', and Cry1F 'sister event'.

None of the materials yielded detectable amplification. Only the event 3006-210-23 and the 281-24-236/3006-210-23 gave a positive signal.

The specificity of the cotton specific reference system (SAH7) was assessed by Blast search (19 October 2004) on the amplicon resulting from the amplification of the species specific sequence.
No 100% match with other sequences except for *Gossypium* was found.

The specificity of the cotton reference PCR system was experimentally tested against DNA extracted from plant materials (at least 1000 genomic copies/reaction) of soybean, rapeseed, rice, wheat, potato, sugar beet, tomato, maize, *Malva sylvestris*, *Althaea offcinalis*, *Alcea rosea* and cotton (PSC355).

None of the non-*Gossypium* species yielded detectable amplification.

3. Procedures

3.1 General instructions and precautions

- All handling of reagents and controls should occur in an ISO 9001:2000 or ISO 17025 environment or equivalent.

- The procedures require experience of working under sterile conditions.

- Laboratory organization, e.g. "flow direction" during PCR-setup, should follow the guidelines given by relevant authorities like e.g. ISO, CEN, Codex alimentarius commission.

- PCR-reagents shall be stored and handled in a separate room and freezer and in equipment 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 must be sterilized 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 shall not adsorb protein or DNA.

- In order to avoid contamination, filter pipette tips protected against aerosol should be used.

- Use only powder-free gloves and change them frequently.

- Clean lab-benches and equipment periodically with 10% sodium hypochloride solution (bleach).

- Pipettes should be checked regularly for precision and calibrated, if necessary.

- All handling steps - unless specified otherwise - shall 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 3006-210-23 cotton

3.2.1 General

The PCR set-up for the taxon specific target sequence (SAH7) and for the GMO (3006-210-23) 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 of 100 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

Separate calibration curves with each primer/probe system are generated in the same analytical amplification run.

The calibration curves consist of four samples. The first point of the calibration curves is a 10% 3006-210-23 in non-GM cotton DNA for a total of 100 ng of DNA (corresponding to approximately 42918 haploid genome copies with one genome assumed to correlate to 2.33 pg of haploid cotton genomic DNA) (Arumuganathan & Earle, 1991).
A series of six-fold dilutions down to 0.5 ng of total cotton DNA/sample (S4) starting from S1 may be used.

A calibration curve is produced by plotting Ct-values against the logarithm of the target copy number for the calibration points. This can be done e.g. by use of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

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

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 the 3006-210-23 system and one for the SAH7 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 SAH7 reference specific system.

Component	Final concentration	µl/reaction
PCR buffer II 10x	1x	2.5
Rox Reference Dye (50x)	0.7x	0.35
Tween-20 1%	0.01%	0.25
Glycerol 20%	0.8%	1
dATPs (10 mM)	200 µM	0.5
dCTPs (10 mM)	200 µM	0.5
dGTPs (10 mM)	200 µM	0.5
dUTPs (20 mM)	400 µM	0.5
$MgCl_2$ (100 mM)	6.0 mM	1.5
Sah7-uni-f1 primer (10 µM)	350 nM	0.875
Sah7-uni-r1 primer (10 µM)	250 nM	0.625
Sah7-uni-s1 probe (10 µM)	175 nM	0.438
Ampli Taq Gold (5U/µl)	1 U/rxn	0.2
Nuclease free water	#	10.263
Template DNA (see 3.2.1 and 3.2.2)		(5)
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for 3006-210-23 specific system.

Component	Final concentration	µl/reaction
PCR buffer II 10x	1x	2.5
Rox Reference Dye (50x)	0.7x	0.35
Tween-20 1%	0.01%	0.25
Glycerol 20%	0.8%	1
dATPs (10 mM)	200 µM	0.5
dCTPs (10 mM)	200 µM	0.5
dGTPs (10 mM)	200 µM	0.5
dUTPs (20 mM)	400 µM	0.5
$MgCl_2$ (100 mM)	6.0 mM	1.5
3006-f3 primer (10 µM)	400 nM	1.0
3006-r2 primer (10 µM)	400 nM	1.0
3006-s2 (10 µM)	150 nM	0.38
Ampli Taq Gold (5U/µl)	1 U/rnx	0.2
Nuclease free water	#	9.83
Template DNA (see 3.2.1 and 3.2.2)		(5)
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the 3006-210-23 and one for the SAH7 master mix) for each DNA sample to be tested (reference 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). Low-speed vortex each tubes at least three times for approx 30 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
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 cycling conditions described in Table 3:

Table 3. Reaction conditions.

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	Initial denaturation		95 °C	600″	No	1x
2a	Amplification	Denaturation	95 °C	15″	No	45x
2b		Annealing & Extension	60 °C	60″	Measure	

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. 3006-210-23) 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. SAH7 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 instruments software calculated the Ct-values for each reaction.

The standard curves are generated both for the SAH7 and 3006-210-23 specific systems 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 copy numbers in the unknown sample DNA by interpolation from the standard curves.

For the determination of the amount of 3006-210-23 DNA in the unknown sample, the 3006-210-23 copy number is divided by the copy number of the maize reference gene (SAH7) and multiplied by 100 to obtain the percentage value (GM% = 3006-210-23/SAH7 * 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 evaluating data after standard curve method (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes with adjustable volume
- Filter tips
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml tubes

4.2 Reagents

(equivalents may be substituted)

- PCR buffer II 10x (Applied Biosystems Part No. N8080241)

- $MgCl_2$ for molecular biology (Applied Biosystems Part No. N8080241)
- AmpliTaq Gold polymerase (Applied Biosystems Part No N8080241)
- Rox Reference Dye (Invitrogen Part No 12223-012)
- Tween20 for molecular biology (SIGMA Part No P9416-50 ML)
- Glycerol for molecular biology (minimum 99%) (SIGMA Part No G5516-100 ML)
- dATP (Amersham-Pharmacia Part No 27-2050-02)
- dCTP (Amersham-Pharmacia Part No 27-2060-02)
- dGTP (Amersham-Pharmacia Part No 27-2070-02)
- dUTP (Amersham-Pharmacia Part No 27-2040-01)
- 1 x TE-Buffer pH=8.0 (10/1 mM) (Applichem Part No A2575,1000)
- Water (Roth Part No 3255.1

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
3006-210-23 target sequence	
3006-f3	5'- AAA TAT TAA CAA TGC ATT GAG TAT GAT G -3'
3006-r2	5'- ACT CTT TCT TTT TCT CCA TAT TGA CC -3'
3006-s2	6-FAM– TAC TCA TTG CTG ATC CAT GTA GAT TTC CCG–TAMRA-3'
Reference gene SAH7 target sequence	
Sah7-uni-f1	5'- AGT TTG TAG GTT TTG ATG TTA CAT TGA G -3'
Sah7-uni-r1	5'- GCA TCT TTG AAC CGC CTA CTG -3'
Sah7-uni-s1	6-FAM - AAA CAT AAA ATA ATG GGA ACA ACC ATG ACA TGT – TAMRA-3'

5. References

Arumuganathan, K., Earle, E.D. (1991). Nuclear content of some important plant species. Plant Mol Biol Reporter 9, 208-218.