Event-specific Method for the Quantification of Cotton Line MON 531 Using Real-time PCR

Protocol

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Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit

Method development:

Monsanto Company

Collaborative trial:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
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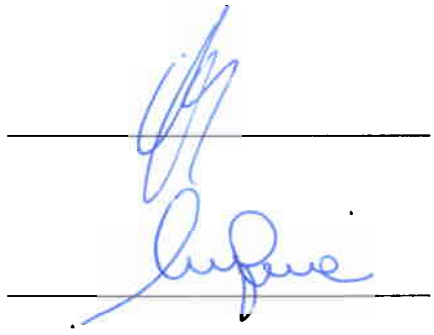

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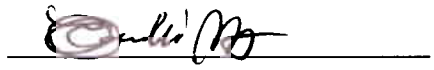

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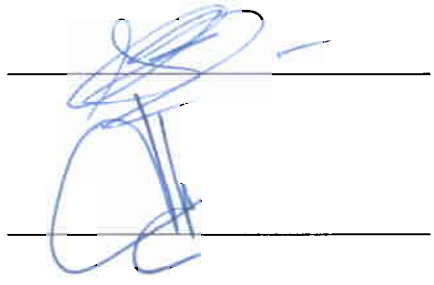

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Content

1. General information and summary of the methodology

This protocol describes a real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event MON 531 DNA to total cotton DNA in a sample.

The PCR assay was optimised for the use in real-time PCR instruments for plastic reaction vessels.

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 of diluted series, use of DNA spikes) are recommended.

For the specific detection of event MON 531 genomic DNA, a 72-bp fragment of the integration region of the construct inserted into 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 the fluorescent dye FAM as a reporter at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For relative quantification of event MON 531 DNA, a cotton-specific reference system amplifies a 76-bp fragment of a cotton fibre-specific *acy1* carrier protein gene (*acp1*), using two specific primers and one 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 MON 531 DNA in a test sample, MON 531 and *acp1* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of event MON 531 DNA to total cotton DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for DNA extracted from cotton seeds or grains containing mixtures of genetically modified and conventional cotton.

The reproducibility and trueness of the method was tested through an international collaborative trial using 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 twelve laboratories in July-August 2006.

Each participant received twenty blind samples containing cotton MON 531 genomic DNA at five GM contents, ranging from 0.1% to 6.0%.

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 cotton MON 531 in four unknown samples. Four replicates of each GM level were analysed on the same plate.

A detailed validation report can be found at 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 0.05% in 200 ng of total cotton DNA. The relative LOD was not assessed in the collaborative trial.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.1% in 200 ng of total cotton DNA. The lowest relative GM content included in the collaborative trial was 0.10%.

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 cotton event MON 531 and thus imparts event-specificity to the method.

The specificity of the assay was experimentally tested by the applicant in real-time PCR against DNA extracted from plant materials containing the specific targets of MON 531, Roundup Ready® soybean 40-3-2, Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), Roundup Ready® corn (GA21), Roundup Ready® corn (NK603), YieldGard® corn borer corn (MON 810), YieldGard® rootworm corn (MON 863), conventional corn, Bollgard® II cotton (MON 15985), conventional cotton, conventional Soybean, Roundup Ready® wheat (MON 71800), conventional wheat, Assoria rice, barley, basmati rice, lentil, quinoa, sunflower, oat, buckwheat, pinenuts, rye berries, millet, Teosinte, hard wheat.

According to the applicant, none of the plant materials tested, except the positive control cotton line MON 531, Assoria rice and cotton MON 15985 gave detectable amplifications; MON 15985 however contains event MON 531 and was expected to be positive in PCR.

Assoria rice reacted unexpectedly with the event-specific detection assay of MON 531, but this positive result is considered by the applicant to be an artefact. Bioinformatics analyses conducted by the CRL-GMFF confirmed the absence of relevant matches between the primers for MON 531 and the rice genome; this was also supported by additional tests conducted by the CRL-GMFF.

The specificity of the cotton reference assay *acp1* was experimentally tested by the applicant against DNA extracted from plant materials containing Roundup Ready® soybean 40-3-2, Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® corn (GA21), Roundup Ready® corn (NK603), YieldGard® corn borer corn (MON 810), YieldGard® rootworm corn (MON 863), conventional corn, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard II® cotton (MON 15985), conventional cotton, conventional soybean, Roundup Ready® wheat (MON 71800), conventional wheat, Assoria rice, barley, basmati rice, Teosinte, lentil, quinoa, sunflower, oat, buckwheat, pinenuts, rye berries, millet, peanut.

According to the applicant, none of the plant materials tested, except cotton MON 531, cotton MON 15985, cotton MON 1445, conventional cotton and Assoria rice yielded detectable amplifications. Assoria rice reacted unexpectedly with the *acp1* assay, but this positive result is considered by the applicant to be an artefact. Bioinformatics analyses conducted by the CRL-GMFF confirmed the absence of relevant matches between the primers for *acp1* and the rice genome; this was also supported by additional tests conducted by the CRL-GMFF.

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 24276:2005.
- 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 MON 531

3.2.1 General

The PCR set-up for the taxon specific target sequence (*acp1*) and for the GMO (MON 531) 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 50 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 10% MON 531 in non-GM cotton DNA for a total of 200 ng of DNA (corresponding to approximately 85830 cotton genome copies with one genome assumed to correspond to 2.33 pg of haploid cotton genomic DNA) [(1)].

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

The ratio of GM copy number and reference gene copy number multiplied by 100 gives the % GM contents of the samples.

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 MON 531 system and one for the *acp1* 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 MON 531 specific system.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
MON 531 primer forward (10 µM)	150 nM	0.75
MON 531 primer reverse (10 µM)	150 nM	0.75
MON 531 probe (5 µM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the cotton *acp1* reference system.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
acp 1 primer forward (10 µM)	150 nM	0.75
acp 1 primer reverse (10 µM)	150 nM	0.75
acp 1 probe (5 µM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

3. Mix gently and centrifuge briefly.

4. Prepare two reaction tubes (one for the MON 531 and one for the *acp 1* 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. 46 x 3 = 138 µL master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 µL DNA for three PCR repetitions). Vortex each tubes for approx. 10 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 microcentrifuge. Aliquot 50 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 Tables 3 and 4:

Table 3. Cycling program for the MON 531 system

Step	**Stage**		**T°C**	**Time (sec)**	**Acquisition**	**Cycles**
	UNG		50 °C	120	No	1
1	Initial denaturation		95 °C	600	No	1
2	Amplification	Denaturation	95 °C	15	No	45
		Annealing & Extension	**55** °C	60	Yes	

Table 4. Cycling program for the reference *acp1* system.

Step	**Stage**		**T°C**	**Time (sec)**	**Acquisition**	**Cycles**
	UNG		50 °C	120	No	1
1	Initial denaturation		95 °C	600	No	1
2	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 curve 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 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 *acp 1* and the MON 531 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.

For the determination of the amount of event MON 531 DNA in the unknown sample, the MON 531 copy number is divided by the copy number of the cotton reference gene (*acp 1*) and multiplied by 100 to obtain the percentage value (GM% = MON 531/*acp 1* x 100).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels
- 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')
MON 531 target sequence	
531-F forward primer	5' - TCC CAT TCG AGT TTC TCA CGT -3'
531-R reverse primer	5' - AAC CAA TGC CAC CCC ACT GA -3'
531-P probe	FAM 5' - TTG TCC CTC CAC TTC TTC TC -3' TAMRA
Reference gene acp1 target sequence	
acp 1 forward primer	5' - ATT GTG ATG GGA CTT GAG GAA GA -3'
acp 1 reverse primer	5' - CTT GAA CAG TTG TGA TGG ATT GTG -3'
acp 1 probe	FAM 5' - ATT GTC CTC TTC CAC CGT GAT TCC GAA -3' TAMRA

5. References

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