



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F – Health, Consumers and Reference
Materials

Food & Feed Compliance (F.5)



Event-specific Method for the Quantification of cotton DAS-81910-7 by Real-time PCR

Validated Method

Method development:

Dow AgroSciences LLC

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan[®] PCR (polymerase chain reaction) procedure for the determination of the relative content of cotton event DAS-81910-7 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 assays. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are also recommended to ensure suitability of the extracted DNA.

For the detection of GM event DAS-81910-7, a 120 bp fragment of the region spanning the 5' plant-to-insert junction in cotton DAS-81910-7 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of GM event DAS-81910-7, a cotton taxon-specific system amplifies a fragment of a cotton *Sinapis Arabidopsis Homolog 7* (*SAH7*) endogenous gene (Accession number, GeneBank: FN610856.1), using *SAH7* gene-specific primers and an *SAH7* gene-specific probe labelled with FAM as reporter dye at its 5' end and TAMRA as quencher dye at its 3' end.

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-subgenome specific copy by several single or double nucleotide substitutions and small deletions/insertions. The 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 differences within the amplified region, the size of the amplicons resulting from the A- and D-subgenomes differs 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 "Cq" value. For quantification of the amount of DAS-81910-7 DNA in a test sample, Cq values for the DAS-81910-7 and the *SAH7* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DAS-81910-7 DNA to total cotton DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from genetically modified and conventional cotton seeds. Precision and trueness 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 (EURL GMFF). The study was undertaken with twelve participating laboratories in October-November 2018.

A detailed validation report can be found at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.035 % (copies GM/total haploid genome copies) in 150 ng of total suitable 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 0.078 % (copies GM/total haploid genome copies) in 150 ng of total suitable cotton DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1 % (mass fraction of GM-material).

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' plant-to-insert junction in cotton DAS-81910-7 and is therefore event-specific for the event DAS-81910-7.

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 should be sterilised prior to use and any residue of DNA should have been 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 regularly

- Laboratory benches and equipment should be cleaned periodically, with 10% sodium hypochlorite solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps, unless specified otherwise, should be carried out at room temperature.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of cotton event DAS-81910-7

3.2.1 General

The real-time PCR set-up for the taxon (*SAH7*) and the GMO (event DAS-81910-7) target sequences are carried out in separate vials. Multiplex qPCR (using differential fluorescent labels for the probes) has not been tested or validated by the EURL GMFF.

The method is developed for a total volume of 25 µL per reaction mixture for the GM (event DAS-81910-7) and the taxon (*SAH7*) assay with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curves are established on four calibration samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % cotton DAS-81910-7 DNA in a total of 175 ng of cotton DNA (corresponding to 75107 cotton haploid genome copies with one haploid genome assumed to correspond to 2.33 pg of cotton genomic DNA)⁽¹⁾. Standards S2 to S4 are to be prepared by serial dilutions (dilution factor 5 for samples S2-S3 and dilution factor 8 for standard S4) according to Table 1 below.

Table 1. Copy number values of the standard curve samples

| Sample code | S1 | S2 | S3 | S4 |
|----------------------------------------------|-------|-------|------|------|
| Total amount of cotton DNA in reaction (ng)* | 175 | 35 | 7 | 0.88 |
| Cotton haploid genome copies | 75107 | 15021 | 3004 | 376 |
| DAS-81910-7 copies | 7511 | 1502 | 300 | 38 |

* Total nanograms are rounded to the integral value

A calibration curve is to be produced by plotting the C_q values against the logarithm of the target 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 software.

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

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. In two tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mixes for the DAS-81910-7 cotton specific system (Table 2) and the *SAH7* reference gene system (Table 3). According to the method developer, the ROX concentration has to be adjusted depending on the real-time PCR instrument used (§ <https://biotium.com/fags/what-rox-concentration-should-i-use-in-my-qpcr-reaction/>).

The three options (No ROX, 0.1x ROX and 1x ROX) are reported as follows and are indicated in Tables 2 and 3:

- *'No ROX' applies to instruments:*
 - BioRad: iCycler™, MyiQ™, MiQ™ 2, iQ™ 5, CFX-96 Touch™, CFX-384 Touch™, Chromo4™, MiniOpticon™
 - Qiagen: Rotor-Gene® Q, Rotor-Gene® 3000, Rotor-Gene® 6000
 - Eppendorf: Mastercycler® Realplex
 - Illumina: Eco™ RealTime PCR System
 - Cepheid: SmartCycler®
 - Roche: LightCycler® 480, LightCycler® 2.0
- *'0.1x ROX' applies to instruments:*
 - ABI: 7500, 7500 Fast, ViiA 7™, QuantStudio™
 - Stratagene: MX4000P, MX3000P, MX3005P
- *'1x ROX' applies to instruments:*
 - ABI: 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne®, StepOne Plus®

Please note that additional volume is included to cover pipetting variability due to the viscosity of the solution.

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the DAS-81910-7 assay.

| Component | Final concentration | µL/reaction |
|-------------------------------|---------------------|--------------------|
| Jumpstart™ Taq ReadyMix™ (2x) | 1x | 12.5 |
| 10x ROX | No ROX / 0.1x / 1x* | 0 / 0.25 / 2.5* |
| MgCl ₂ (100 mM) | 3.5 mM | 0.88 |
| 1706-f2 (10 µM) | 300 nM | 0.75 |
| 1706-r3 (10 µM) | 300 nM | 0.75 |
| 1706-p3** (10 µM) | 150 nM | 0.38 |
| Nuclease free water | - | 4.75 / 4.5 / 2.25* |
| DNA | - | 5 |
| Total reaction volume: | | 25 µL |

* see 3.2.3 for the selection of the ROX concentration according to the instrument used

** TaqMan® probe labelled with 6-FAM at its 5'-end and TAMRA at its 3'-end

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the cotton *SAH7* assay.

| Component | Final concentration | µL/reaction |
|-------------------------------|---------------------|---------------------|
| Jumpstart™ Taq ReadyMix™ (2x) | 1x | 12.5 |
| 10x ROX | No ROX / 0.1x / 1x* | 0 / 0.25 / 2.5* |
| MgCl ₂ (100 mM) | 3.5 mM | 0.88 |
| Sah7-uni-f1 (10 µM) | 350 nM | 0.88 |
| Sah7-uni-r1 (10 µM) | 250 nM | 0.63 |
| Sah7-uni-s1** (10 µM) | 175 nM | 0.44 |
| Nuclease free water | - | 4.69 / 4.44 / 2.19* |
| DNA | - | 5 |
| Total reaction volume: | | 25 µL |

* see 3.2.3 for the selection of the final ROX concentration according to the instrument used

** TaqMan® probe is labelled with 6-FAM at its 5'-end and TAMRA at its 3'-end

- Mix well and centrifuge briefly.
- Prepare two 0.5 mL reaction tubes (one for the cotton DAS-81910-7 and one for the *SAH7* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (70 µL for the DAS-81910-7 cotton system and for the *SAH7* system). Add to each tube the correct amount of

DNA for 3.5 PCR repetitions (17.5 μ L DNA). The volume for the additional 0.5 repetition will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.

6. Spin down the tubes. Aliquot 25 μ L for the DAS-81910-7 system and for the *SAH7* reference system in each well.
7. Place an optical cover on the reaction plate and briefly centrifuge the plate.
8. Place the reaction plate in the real-time PCR apparatus (apply a compression pad, depending on the model), according to the manufacturer's instructions and your Standard Operating Procedures and start the run.
9. Select FAM as reporter dye for the DAS-81910-7 and for the *SAH7* reference system. Define TAMRA as quencher dye for the DAS-81910-7 specific system and for the *SAH7* reference system. Select ROX as the passive reference dye. Enter the correct reaction volume (25 μ L).
10. Run the PCR with the cycling program described in Table 4. Users who plan to use the second derivative maximum analysis method (an option e.g. on Roche LC480 instruments) are advised to program 45 cycles instead of 40, in order to be able to quantify down to Cq 40.

Table 4. Cycling program for DAS-81910-7/*SAH7* assays.

| Step | Stage | T (°C) | Time (s) | Acquisition | Cycles | |
|------|----------------------|-----------------------|----------|-------------|--------|-----|
| 1 | Initial denaturation | 95 | 600 | No | 1 | |
| 2 | Amplification | Denaturation | 95 | 15 | No | 40* |
| | | Annealing & Extension | 60 | 60 | Yes | |

* see comment above for users of second derivative maximum analysis method

3.3 Data analysis

After the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold following the automatic or the manual mode. In the manual mode display the amplification curves of the event specific assay 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 Cq 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 following the automatic or the manual mode. In the manual mode: 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 Cq = 25, set the baseline crossing at Cq = 25 – 3 = 22).
- c) Save the settings.
- d) Repeat the procedure described in a), b) and c) on the amplification plots of the taxon specific system.
- e) Save the settings and export all the data 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 Cq values for each reaction.

The standard curves are generated both for the *SAH7* and the DAS-81910-7 specific assays by plotting the Cq 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 number in the unknown samples.

To obtain the percentage value of event DAS-81910-7 DNA in the unknown sample, the DAS-81910-7 copy number is divided by the cotton haploid genome copy number measured with the endogenous gene *SAH7* and multiplied by 100 (GM% = DAS-81910-7/*SAH7* x 100).

4. Equipment and Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition) and appropriate analysis software
- 96-well reaction plates
- Optical caps/adhesion covers
- Microcentrifuge
- Micropipettes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex
- Racks for reaction tubes, also cooled
- 0.5, 1.5 mL and 5 or 15 mL DNase free reaction tubes

4.2 Reagents

- JumpStart™ Taq ReadyMix™ Sigma-Aldrich®, catalogue number P2893
- Nuclease-free Water (e.g. HPLC Gradient Grade) Ambion®, product number AM9937
- 1 M MgCl₂ Sigma-Aldrich®, catalogue number M1028
- ROX Reference Dye Invitrogen, catalogue number 12223012

4.3 Primers and Probes

Table 5. Primers and probes for the DAS-81910-7 and *SAH7* methods

| | Name | DNA Sequence (5' to 3') | Length (nt) |
|--------------------|-------------|-----------------------------------------------------------|-------------|
| <i>DAS-81910-7</i> | | | |
| Forward primer | 1706-f2 | AAG CTT AGG TGA TTT CGA TGA TG | 23 |
| Reverse primer | 1706-r3 | GAC CTC AAT TGC GAG CTT TC | 20 |
| Probe | 1706-p3 | FAM - CAC ACC AAA AGT TAG GCC CG - TAMRA | 20 |
| <i>SAH7</i> | | | |
| Forward primer | Sah7-uni-f1 | AGT TTG TAG GTT TTG ATG TTA CAT TGA G | 28 |
| Reverse primer | Sah7-uni-r1 | GCA TCT TTG AAC CGC CTA CTG | 21 |
| Probe | Sah7-uni-s1 | FAM - AAA CAT AAA ATA ATG GGA ACA ACC ATG ACA TGT - TAMRA | 33 |

FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.

5. References

1. Plant DNA C-values Database. Royal Botanic Gardens, Kew, <http://data.kew.org/cvalues/>