



## **Event-specific Method for the Quantification of cotton COT102 by Real-time PCR**

### **Validated Method**

#### **Method development:**

Syngenta Crop Protection NV/SA

# 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 COT102 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 COT102, a 101 bp fragment of the region spanning the 3' insert-to-plant genome junction in cotton COT102 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™ (carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantitation of event COT102, a cotton-specific reference system amplifies part of the intron sequences of the gene for the putative SAH7 protein of *Gossypium hirsutum*. The *SAH7* gene is present not only in the A-sub-genome but also in the D-sub-genome of *G. hirsutum*. The A-sub-genome 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 (no mismatch) to both sub-genomes gene copies. However, due to sequence differences within the amplified region, the size of the amplicons resulting from the A- and D- sub-genomes differ slightly, being respectively 115 bp and 123 bp long (CRLVL14/05VP, [http://gmo-crl.jrc.ec.europa.eu/summaries/281-24-36\\_cotton\\_Protocol.pdf](http://gmo-crl.jrc.ec.europa.eu/summaries/281-24-36_cotton_Protocol.pdf)); (Accession number, GeneBank: X14555). Amplification of the target *SAH7* sequence is achieved using *SAH7* gene-specific primers and a *SAH7* gene-specific probe labelled with VIC® as reporter dye at its 5' end and TAMRA™ as quencher dye at its 3' end.

The 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 COT102 DNA in a test sample, Cq values for the COT102 and the *SAH7* systems are determined for the sample. Standard curves are then used to estimate the relative amount of COT102 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 June 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 0.015 % (related to copy number ratio calculated in terms of haploid genomes) 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 % (related to copy number ratio calculated in terms of haploid genomes) in 150 ng of total suitable cotton DNA, corresponding to 50 copies of COT102 event. 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 3' insert-to-plant genome junction in cotton COT102 and is therefore event-specific for the event COT102. This was confirmed in the validation study.

# 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 COT102

### 3.2.1 General

The real-time PCR set-up for the taxon (*SAH7*) and the GMO (event COT102) 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 COT102) and the taxon (*SAH7*) assay with the reagents as listed in Table 2 and Table 3.

### 3.2.2 Calibration

The calibration curves have to be established on at least four samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % cotton COT102 DNA in a total of 150 ng of cotton DNA (corresponding to 64378 cotton haploid genome copies with one haploid genome assumed to correspond to 2.33 pg of cotton genomic DNA) <sup>(1)</sup>. Standards S2 to S4 are prepared by serial dilutions (dilution factor 8 for standard S2, dilution factor 4 for standard S3, dilution factor 5 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)	150	18.8	4.7	0.94
cotton haploid genome copies	64378	8047	2012	402
COT102 copies	6438	805	201	40

A calibration curve is to be produced by plotting the C<sub>q</sub> 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 COT102 cotton specific system (Table 2) and the *SAH7* reference gene system (Table 3). Please note that additional volume is included in the total 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 COT102 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
COT102_3_89F (10 µM)	600 nM	1.500
COT102_3_181R (10 µM)	600 nM	1.500
COT102_3_115T (10 µM)	150 nM	0.375
Nuclease free water	-	4.125
DNA	-	5
Total reaction volume:		25 µL

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
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
SAH7-uni-f1 (10 µM)	350 nM	0.875
SAH7-uni-r1 (10 µM)]	250 nM	0.625
SAH7-uni-s1 (10 µM)	175 nM	0.438
Nuclease free water	-	5.562
DNA	-	5
Total reaction volume:		25 µL

TaqMan® probe is labelled with VIC® at its 5'-end and TAMRA at its 3'-end

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for the cotton COT102 and one for the *SAH7* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (70  $\mu$ L for the COT102 cotton system and 70  $\mu$ L for the *SAH7* system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (17.5  $\mu$ 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  $\mu$ L for COT102 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 (possibly 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 COT102 and VIC® for the *SAH7* reference system. Define TAMRA as quencher dye for COT102 specific system and for *SAH7* reference system. Select ROX (if necessary according to the instructions of the real-time PCR manufacturer) as the passive reference dye. Enter the correct reaction volume (25  $\mu$ L).
10. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for COT102/*SAH7* assays.

Step	Stage	T (°C)	Time (s)	Acquisition	Cycles	
1	UNG*	50	120	No	1	
2	Initial denaturation	95	600	No	1	
3	Amplification	Denaturation	95	15	No	40*
		Annealing & Extension	60	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 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 C<sub>q</sub> 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 C<sub>q</sub> = 25, set the baseline crossing at C<sub>q</sub> = 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 C<sub>q</sub> values for each reaction.

The standard curves are generated both for the *SAH7* and for the COT102 specific assays by plotting the C<sub>q</sub> 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 COT102 DNA in the unknown sample, the COT102 copy number is divided by the copy number of the cotton endogenous gene *SAH7* and multiplied by 100 (GM% = COT102/*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

- TaqMan® Universal PCR Master Mix. ThermoFisher Scientific™ Catalogue Number 4318157

## 4.3 Primers and Probes

Table 5. Primers and probes for the COT102 and *SAH7* methods

	ID	DNA Sequence (5' to 3')	Length (nt)
COT102			
Forward primer	COT102_3_89F	TCT CCG CTC ATG ATC AGA TTG TC	23
Reverse primer	COT102_3_181R	CAG TAA CAG TAC AGT CGG TGT AGG G	25
Probe	COT102_3_115T	6-FAM™– TCC CGC CTT CAG TTT AAA CTA TCA GTG TTT AAT-TAMRA	33
<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	VIC®-AAA CAT AAA ATA ATG GGA ACA ACC ATG ACA TGT- TAMRA™	33

FAM™: 6-carboxyfluorescein; VIC®; TAMRA™: carboxytetramethylrhodamine.

## 5. References

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