Event COT102 Cotton

Real-time, Event-specific Polymerase Chain Reaction Method

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LIST OF ACRONYMS AND ABBREVIATIONS

Definitions of International System of Units (SI) base units and derived units may be found in NIST (2011).

bp	base pair
CT	cycle threshold
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
PCR	polymerase chain reaction
sah	sinapis arabidopsis homolog
TE	tris (2-amino-2(hydroxymethyl)-propane-1,3-diol)/EDTA (ethylenediaminetetraacetic acid)

1.0 INTRODUCTION

Using the techniques of modern molecular biology, Syngenta has transformed cotton (*Gossypium hirsutum* L.) to produce Event COT102 cotton, a new cultivar that has controls against several lepidopteran pests of cotton.

A real-time, event-specific polymerase chain reaction (PCR) method was developed to detect and quantify COT102 deoxyribonucleic acid (DNA) extracted from seed and other plant material samples. The method consists of a cotton-specific PCR method as a reference, and an event-specific PCR method for detection of COT102 cotton.

2.0 METHOD

2.1 Principle of the COT102 Real-time, Event-specific Method

The COT102 real-time, event-specific PCR method is optimized for use on an Applied Biosystems® 7500 Fast Real-Time PCR System using the standard mode. The amount of PCR product is determined during each cycle (real-time) by measuring the fluorescence produced by a target-specific oligonucleotide probe labeled with two fluorescent dyes.

- COT102-specific PCR method: oligonucleotide probe with 6-FAM™ as a reporter dye at its 5′ end and TAMRA™ as a quencher dye at its 3′ end
- Cotton-specific PCR method: oligonucleotide probe with VIC® as a reporter dye at its 5′ end and TAMRA™ as a quencher dye at its 3′ end

The 5′-nuclease activity of *Taq* DNA polymerase cleaves the probe and liberates the fluorescent moiety from proximity to the quenching moiety during the amplification process. The resulting increase in fluorescence during amplification is monitored and recorded.

A cotton-specific PCR method previously validated within the context of the validation of the event-specific quantitative method for Event 3006-210-23/281-23-235 cotton (http://gmo-crl.jrc.ec.europa.eu/summaries/281-24-36_cotton_Protocol.pdf, http://gmo-crl.jrc.ec.europa.eu/summaries/3006-210-23_cotton_Protocol.pdf) is used as a reference method.

For specific detection of COT102 genomic DNA, a 101-base pair (bp) fragment of the region that spans the 3′ insert-to-plant genome junction in COT102 cotton is amplified using two specific primers. The forward primer binding site is located in the COT102 insert, the reverse primer binding site is located in the cotton genomic sequence, and the probe binding site spans the junction between the COT102 insert and the adjacent cotton genomic DNA.

2.2 Reagents and Equipment

All materials (e.g., vials, containers, pipette tips) should be suitable for PCR and molecular biology applications (Table 1). Materials should be deoxyribonuclease (DNase)-free, DNA-free, sterile, and unable to absorb protein or DNA. Table 2 contains a list of reagents, buffers, and solutions needed to perform the PCR method.

TABLE 1 Equipment and Materials

Equipment and materials	Specification
Applied Biosystems® 7500 Fast Real-Time PCR System	Applied Biosystems® part number 4351107 or equivalent
Vortex	NeoLab VM-300 or equivalent
Thermo-Fast® 96-well PCR plate	Abgene® catalog number AB-1400 or equivalent
Clear seal diamond heat sealing film	Abgene® catalog number AB-0812 or equivalent
Thermo-sealer	Abgene® catalog number AB-0384/240 or equivalent
Pipettes with adjustable volume	Eppendorf Research®, 2 – 20 µl, 20 – 200 µl, 100 – 1000 µl or equivalent
Filter tips	Fitting the pipette models used
Microcentrifuge tubes 1.5 ml	Roth catalog number 4182.1 or equivalent
Microcentrifuge tubes 1.5 ml, screw lids	Sarstedt part number 72.692.005 or equivalent

TABLE 2 Reagents, Buffers, and Solutions

Reagents, buffers, and solutions	Specification
TaqMan® Universal PCR Master Mix	Applied Biosystems® catalog number 4326708
1X TE buffer pH 8.0	Applichem part number A2575, 1000
Water (HPLC Gradient Grade)	Rotisolv® product number A511.1 or equivalent
Salmon sperm DNA	Sigma-Aldrich® catalog number D7656 or equivalent

TE = tris (2-amino-2(hydroxymethyl)-1,3-propanediol)/EDTA (ethylenediaminetetraacetic acid).
HPLC = high performance liquid chromatography.

The handling of all reagents and controls should be carried out under sterile conditions and in a manner that precludes contamination of reagents or controls with exogenous DNA or undesired enzymatic activities (e.g., DNase).

The dilution buffer is 0.1X TE containing salmon sperm DNA. Resuspend 1 mg of salmon sperm DNA in 100 ml of 0.1 X TE (pH 8.0) to make a 10 ng DNA/µl 0.1X TE solution. Solution should be vortexed and stored at −20°C.

2.3 DNA Concentration of Samples

For the unknown samples, the use of 100 ng of template DNA per reaction is recommended. This corresponds to approximately 42918 haploid copies of the cotton genome, assuming a genome weight of 2.33 pg (Arumuganathan and Earle 1991).

2.4 Calibration Standards

The method format uses five calibration standards containing different amounts of COT102 genomic DNA (Standard 1 through Standard 5); each calibration standard is analyzed in both the COT102-specific and Sinapis Arabidopsis Homolog (*sah*) *7*-specific PCR methods. The calibration standards are produced by preparing solutions of 20 ng/µl (100 ng/reaction) of total genomic DNA with 10%, 5%, 1%, 0.5%, and 0.07% copy/copy COT102 DNA mixed with nontransgenic cotton genomic DNA. Table 3 lists the total DNA content, as well as the total copy numbers of COT102, for each calibration standard. COT102 copy numbers are based on the fact that the COT102 seeds are homozygous.

TABLE 3 Dilution Scheme of the Calibration Standards

Sample	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
Total DNA content	100 ng	100 ng	100 ng	100 ng	100 ng
COT102 DNA copies	4292	2146	429	215	30

2.5 The *sah7*-specific PCR Method

For relative quantification of COT102 DNA, two specific primers (Table 4) are used to amplify a fragment of the cotton endogenous gene *sah7*. This cotton-specific PCR method has been validated by the JRC (http://gmo-crl.jrc.ec.europa.eu/summaries/281-24-36_cotton_Protocol.pdf, http://gmo-crl.jrc.ec.europa.eu/summaries/3006-210-23_cotton_Protocol.pdf). Whereas primer and probe sequences and concentrations were taken from the original protocol, the buffer system was modified and validated. The gene *sah7* is present in both the A- and the D-subgenomes of *G. hirsutum*. The A-subgenome specific copy differs from the D-subgenome specific copy by several single or double nucleotide substitutions and small deletions or insertions. Primers and probe sequences of the cotton-specific reference PCR system match perfectly without any single mismatch to both subgenomes' gene copies. However, due to sequence deviations within the amplified region, the sizes of the amplicons resulting from the A- and D- subgenomes differ slightly, being 115 bp and 123 bp in length (Figures 1 and 2), respectively. The amount of PCR product is determined during each cycle in real-time by measuring

fluorescence produced by a *sah7*-specific oligonucleotide probe (Table 4) labeled with VIC® as a reporter dye at its 5′ end and TAMRA™ as a quencher at its 3′ end.

TABLE 4 The *sah7*-specific PCR Primers and Probe Sequences

Primer/probe name	Primer sequence 5′ to 3′
SAH7-uni-f1 primer	AGTTTGTAGGTTTTGATGTTACATTGAG
SAH7-uni-r1 primer	GCATCTTTGAACCGCCTACTG
SAH7-uni-s1 probe	VIC®-AAACATAAAATAATGGGAACAACCATGACATGT-TAMRA™

FIGURE 1 Sequence of the 115-bp Amplicon Generated by PCR Amplification with the *sah7*-specific PCR Method from the A-subgenome of Cotton

SAH7-uni-f1 primer SAH7-uni-s1 probe
AGTTTGTAGGTTTTGATGTTACATTGAGTGACAGTGAATGAAAGGGTGTGTAAACATAAAATAATGGGAA

CAACCATGACATGTTGGACTGGATCAGTAGGCGGTTCAAAGATGC
SAH7-uni-r1 primer

Primer and probe binding sites are underlined.

FIGURE 2 Sequence of the 123-bp Amplicon Generated by PCR Amplification with the *sah7*-specific PCR Method from the D-subgenome of Cotton

SAH7-uni-f1 primer SAH7-uni-s1 probe
AGTTTGTAGGTTTTGATGTTACATTGAGTGAAAGCGAATGATAGGGTGTCTAAACATAAAATAATGGGAA

CAACCATGACATGTTGGACTGGAGGTCAAAATCAGTAGGCGGTTCAAAGATGC
SAH7-uni-r1 primer

Primer and probe binding sites are underlined.

All reagents should be thawed, as necessary, and thoroughly mixed before each use. The master mix for the *sah7*-specific PCR method that contains all components of the PCR method except template DNA (Table 5) can be prepared in sufficient quantities before reactions are performed.

TABLE 5 Master Mix for the *sah7*-specific PCR Method

Components	Initial concentration	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix	2X	1X	12.50
SAH7-uni-f1 primer	10 µM	350 nM	0.875
SAH7-uni-r1 primer	10 µM	250 nM	0.625
SAH7-uni-p1 probe	10 µM	175 nM	0.438
Nuclease-free water	N/A	N/A	5.562
Total volume[a]	N/A	N/A	**20.00**

[a]Total PCR reaction is 25 µl (20 µl master mix and 5 µl genomic DNA template).
N/A = not applicable.

2.6 Event-specific PCR Method for the Detection and Quantification of COT102 DNA

For specific detection of COT102 genomic DNA, two specific primers (Table 6) are used to amplify a 101-bp fragment of the region that spans the 3' insert-to-plant genome junction (Figure 3). The amount of PCR product is determined during each cycle in real-time by measuring the fluorescence produced by a COT102-specific oligonucleotide probe labeled with two fluorescent dyes: 6-FAM™ as a reporter dye at its 5' end and TAMRA™ as a quencher dye at its 3' end (Table 6).

TABLE 6 COT102 Real-time, Event-specific PCR Primers and Probe Sequences

Primer/probe name	Primer sequence 5′ to 3′
COT102_3_89F primer	TCTCCGCTCATGATCAGATTGTC
COT102_3_181R primer	CAGTAACAGTACAGTCGGTGTAGGG
COT102_3_115T probe	6-FAM™–TCCCGCCTTCAGTTTAAACTATCAGTGTTTAAT-TAMRA™

FIGURE 3 Sequence of the 101-bp Amplicon Generated by PCR Amplification with the COT102 Real-time, Event-specific PCR Method

COT102_3_89F primer COT102_3_115T probe
TCTCCGCTCATGATCAGATTGTCGTTTCCCGCCTTCAGTTTAAACTATCAGTGTTTAATAAATATGGGC

COT102_3_181R primer
AATCTTTCCCTACACCGACTGTACTGTTACTG

Primer and probe binding sites are underlined.

All reagents should be thawed, as necessary, and thoroughly mixed before each use. The master mix for the COT102-specific PCR method that contains all components of the PCR method except template DNA (Table 7) can be prepared in sufficient quantities before reactions are performed.

TABLE 7 Master Mix for the COT102-specific PCR Method

Components	Initial concentration	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix	2X	1X	12.50
COT102_3_89F primer	10 µM	600 nM	1.50
COT102_3_181R primer	10 µM	600 nM	1.50
COT102_3_115T probe	10 µM	150 nM	0.375
Nuclease-free water	N/A	N/A	4.125
Total volume[a]	N/A	N/A	**20.00**

[a]Total PCR reaction is 25 µl (20 µl master mix and 5 µl genomic DNA template).
N/A = not applicable.

2.7 Cycling Parameters

An Applied Biosystems® 7500 Fast Real-Time PCR System using the standard mode in the instrument set-up is used with the COT102 real-time, event-specific PCR method and the *sah7*-specific PCR method. Table 8 shows the PCR cycling conditions.

TABLE 8 Cycling Parameters

Cycle	Step	Temperature (°C)	Time (seconds)	Data acquisition	Number of cycles
A	1	50	120	No	1
B	1	95	600	No	1
C	1	95	15	No	45
	2	60	60	Yes	

2.8 PCR Plate Layout

The calibration of the COT102 real-time, event-specific method includes five calibration standards, each containing different percentages of COT102 cotton genomic DNA, with a total amount of 100 ng cotton DNA. The COT102 DNA content of the standard samples ranges from 0.07% to 10% copy/copy. Each calibration standard is analyzed in duplicate with the COT102-specific PCR method, as well as with the *sah7*-specific PCR method. Duplicate negative control samples (1X TE buffer) containing no template DNA verify the purity of the reagents. Unknown samples are analyzed in triplicate for both the COT102-specific and *sah7*-specific PCR methods. Figure 4 summarizes the PCR plate layout.

FIGURE 4 PCR Plate Layout

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><th>A</th><td colspan="2">Standard 1</td><td colspan="2">Standard 1</td><td colspan="2">Standard 2</td><td colspan="2">Standard 2</td><td colspan="2">Standard 3</td><td colspan="2">Standard 3</td></tr>
<tr><th>B</th><td colspan="2">Standard 4</td><td colspan="2">Standard 4</td><td colspan="2">Standard 5</td><td colspan="2">Standard 5</td><td colspan="2">Negative control</td><td colspan="2">Negative control</td></tr>
<tr><th>C</th><td colspan="3">Unknown 1</td><td colspan="3">Unknown 1</td><td colspan="3">Unknown 2</td><td colspan="3">Unknown 2</td></tr>
<tr><th>D</th><td colspan="3">Unknown 3</td><td colspan="3">Unknown 3</td><td colspan="3">Unknown 4</td><td colspan="3">Unknown 4</td></tr>
<tr><th>E</th><td colspan="3">Unknown 5</td><td colspan="3">Unknown 5</td><td colspan="3">Unknown 6</td><td colspan="3">Unknown 6</td></tr>
<tr><th>F</th><td colspan="3">Unknown 7</td><td colspan="3">Unknown 7</td><td colspan="3">Unknown 8</td><td colspan="3">Unknown 8</td></tr>
<tr><th>G</th><td colspan="3">Unknown 9</td><td colspan="3">Unknown 9</td><td colspan="3">Unknown 10</td><td colspan="3">Unknown 10</td></tr>
<tr><th>H</th><td colspan="3">Unknown 11</td><td colspan="3">Unknown 11</td><td colspan="3">Unknown 12</td><td colspan="3">Unknown 12</td></tr>
</table>

Standard 1 to 5: calibration standards.
Unknown 1 to 12: unknown samples.
Negative control: samples containing no template DNA (1X TE buffer).
White: *sah7*-specific PCR method.
Gray: COT102-specific PCR method.

2.9 Data Analysis and Evaluation of Results

Analyze the results according to the following procedure:

a) Place the threshold in the region of exponential amplification across all of the amplification plots. This region is recognized in the logarithmic view of the amplification plots as the portion of the plot that is linear. Do not place the threshold line in the plateau phase or in the initial linear phase of amplification. Place the threshold line clearly above the background fluorescence and above the level where splitting or fork effects between replicates can be observed.

b) 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., if the earliest cycle threshold [CT] = 24, set the baseline crossing at 21, or CT of 24 − 3).

c) Save the settings.

d) Repeat the procedure described in a) and b) on the amplification plots of the other method.

e) Save the settings and export all the data into a Microsoft® Office Excel® file for further calculations.

A calibration curve can be produced by plotting mean delta CT (ΔCT) values of calibration samples against the logarithm of the respective percent COT102 DNA contents; the slope (a) and the intercept (b) of the calibration curve ($y = ax + b$) can then be used to calculate the percent COT102 DNA content of the reference samples based on their normalized mean ΔCT values.

3.0 REFERENCES

Arumuganathan K, Earle ED. 1991. Nuclear DNA content of some important plant species. *Plant Mol Biol Rep* 9:208–218.

NIST. 2011. *The NIST Reference on Constants, Units, and Uncertainty.* Gaithersburg, MD: National Institute of Standards and Technology. http://physics.nist.gov/cuu (updated June 2, 2011).