# Event COT102 Cotton

# Gel-based, 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 |
| DNA | deoxyribonucleic acid |
| PCR | polymerase chain reaction |

# 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 controls against several lepidopteran pests of cotton.

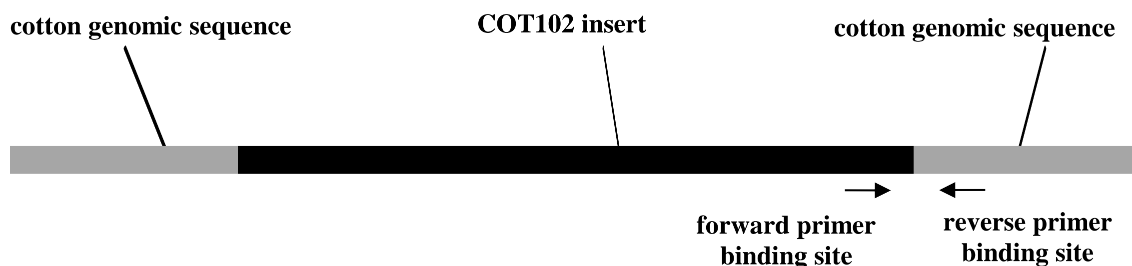
A gel-based, event-specific polymerase chain reaction (PCR) method was developed to detect and quantify COT102 deoxyribonucleic acid (DNA) extracted from seed, leaf, or other plant material in samples containing at least 0.05% (21 copies) COT102 DNA in 100 ng total DNA with 35 amplification cycles and an annealing temperature of 58°C. Cotton genome copy numbers were based on the assumption that 100 ng cotton DNA corresponds to approximately 42918 haploid genome equivalents of the cotton genome, assuming a genome weight of 2.33 pg (Arumuganathan and Earle 1991).

# 2.0 METHOD

## 2.1 Principle of the COT102 Gel-based, Event-specific Method

For specific detection of COT102 genomic DNA, a 132-bp fragment 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 within the COT102 insert, and the reverse primer binding site is located in cotton genomic sequence that flanks the 3′ end of the COT102 insert (Figure 1).

**FIGURE 1 Location of the COT102 Gel-based, Event-specific PCR Primer Binding Sites**




In addition, two oligonucleotide primers are used to amplify a 170-bp DNA fragment specific to the cotton gene for chitinase (*chi-2b*) (National Center for Biotechnology Information accession number Z68153.1) to monitor PCR performance.

## 2.2 Reagents and Equipment

All materials (e.g. vials, containers, and pipette tips) should be suitable for PCR and molecular biology applications. Table 1 contains a list of equipment and materials needed to perform the PCR method. Materials should be deoxyribonuclease-free, DNA-free, sterile, and unable to absorb protein or DNA. To avoid contamination, materials for use in this method should be stored separately from materials used in other laboratory procedures, benches and pipettes should be regularly cleaned with 70% v/v ethanol, filter tips should be

used with all pipettes, and disposable gloves should be used and changed often. The use of an electronic, repeat pipette is recommended to reduce sample to sample variability and to reduce the time needed to set up the reactions. The method should be used in conjunction with a DNA extraction method that yields DNA of sufficient purity and quantity (http://gmo-crl.jrc.ec.europa.eu/summaries/MON1445_DNAExtr_report.pdf) and quantitated using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™ catalog number P11496). Table 2 contains a list of reagents and solutions needed to perform the PCR method.

**TABLE 1 Equipment and Materials**

| **Equipment and materials** | **Specification** |
|---|---|
| GeneAmp® PCR System 9700 | Applied Biosystems® part number N8050200 |
| Veriti™ 96-well thermal cycler | Applied Biosystems® part number 4375786 |
| Thermo-Fast® 96 PCR Detection Plate MKII | 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 |

**TABLE 2 Reagents, Buffers, and Solutions**

| **Reagents, buffers, and solutions** | **Specification** |
|---|---|
| JumpStart™ REDTaq® ReadyMix™ Reaction Mix for high throughput PCR | Sigma-Aldrich® catalog number P1107 |
| Nuclease-free water | Ambion® catalog number AM9932 |
| Low DNA Mass™ ladder | Invitrogen™ catalog number 10068-013 |

## 2.3 Event-specific PCR Method for the Detection of Event COT102 DNA

For the specific detection of COT102 genomic DNA, two primers (Table 3) are used to amplify a 132-bp fragment (Figure 2) that spans the 3′ insert-to-plant genome junction. The forward primer (FE5164 forward) binding site is located within the COT102 insert, and the reverse primer (FE1117 reverse) binding site is located in cotton genomic sequence that flanks the 3′ end of the COT102 insert.

**TABLE 3 Primers Used with the COT102 Gel-based, Event-specific PCR Method**

| Primer name | Length (bp) | Primer sequence 5′ to 3′ |
|---|---|---|
| FE5164 forward | 23 | CGTGACTCCCTTAATTCTCCGCT |
| FE1117 reverse | 25 | AGGCCGGAGTCTATTACAGTAACAG |

**FIGURE 2 Sequence of the 132-bp Amplicon Generated by PCR Amplification with COT102-specific PCR Method**

```
              FE5164 forward
CGTGACTCCCTTAATTCTCCGCTCATGATCAGATTGTCGTTTCCCGCCTTCAG
TTTAAACTATCAGTGTTTAATAAATATGGGCAATCTTTCCCTACACCGACTGTA
CTGTTACTGTAATAGACTCCGGCCT
              FE1117 reverse
```

Primer binding sites are underlined.

## 2.4 The *chi-2b*-specific PCR Method

The *chi-2b*-specific PCR method serves as a reference method. For the specific detection of cotton DNA, two primers (Table 4) are used to amplify a 170-bp fragment (Figure 3).

**TABLE 4 Primers Used with the *chi-2b*-specific PCR Method**

| Primer name | Length (bp) | Primer sequence 5′ to 3′ |
|---|---|---|
| FE30049 forward | 19 | GCTGGTCGGTCCCTGGATA |
| FE30050 reverse | 23 | GGGTCTTTGGTTGTAGCAGTCAA |

**FIGURE 3 Sequence of the 170-bp Amplicon Generated by PCR Amplification with *chi-2b*-specific PCR Method**

```
       FE30049 forward
GCTGGTCGGTCCCTGGATACGGTGTCATCACCAACATCATCAATGGTGGCATCGAATGTGGCAAAGGATCCAA
CCCTCAAGTTGAGGATAGAATCGGGTTTTACAAGAGATACTGTGACATACTGAAAGTGAGCTACGGTGACAAT
CTTGACTGCTACAACCAAAGACCC
                FE30050 reverse
```

Primer binding sites are underlined.

## 2.5 Master Mix

All reagents should be thawed, as necessary, and thoroughly mixed before each use. A master mix that contains all components of the PCR reaction except DNA (Table 5) can be prepared in sufficient quantities before the reactions are performed. This assay was optimized for 100 ng DNA template in a 20 µl reaction. Use of more or less DNA may alter the results.

**TABLE 5 Master Mix Components**

| Components | Volume per reaction (µl)[a] | Final concentration |
|---|---|---|
| JumpStart™ REDTaq® ReadyMix™, 2X | 10 | 1X |
| Forward primer, 10 µM | 1 | 0.5 µM |
| Reverse primer, 10 µM | 1 | 0.5 µM |
| Nuclease-free water | 4 | N/A |
| **Total volume of master mix** | 16 | N/A |

[a]Total PCR reaction is 20 µl (16 µl master mix and 4 µl genomic DNA at 25 ng/µl concentration).
N/A = not applicable.

## 2.6 PCR Method Controls

The following controls are recommended for this method:

- negative control 1: genomic DNA from nontransgenic cotton
- negative control 2: nuclease-free water substituted for DNA
- positive control (optional): 0.1% COT102 DNA prepared from either mixed DNA consisting of 0.1% COT102 DNA and 99.9% DNA from COT102-free cotton or DNA extracted from mixed ground seed containing 0.1% COT102 cotton and 99.9% COT102-free cotton.

## 2.7 Cycling Parameters

The method should be performed with the cycling parameters shown in Table 6.

**TABLE 6 Cycling Parameters**

| Cycle | Step | Temperature (°C) | Time (seconds) | Number of cycles |
|---|---|---|---|---|
| A | 1 | 94 | 120 | 1 |
| B | 1 | 94 | 30 | 35 |
| | 2 | 58 | 30 | |
| | 3 | 72 | 30 | |
| C | 1 | 72 | 180 | 1 |
| D | 1 | 4 | Hold | N/A |

N/A = not applicable.

## 3.0 STEP-BY-STEP INSTRUCTIONS FOR PERFORMING THE METHOD

1. At room temperature, prepare a master mix of all reagents except DNA.
2. Mix the solution gently using a standard pipette.
3. Aliquot the appropriate amount of master mix into individual tubes (i.e. total volume of PCR reaction minus the DNA volume to be added [e.g. in the above example 20 µl minus 4 µl equals 16 µl master mix per well]).
4. Add DNA samples and controls in the following order:

   4 µl nuclease-free water to the negative control 2 well

   4 µl genomic DNA (25 ng/µl) from nontransgenic cotton to the negative control 1 well

   4 µl genomic DNA (25 ng/µl) extracted from the test samples

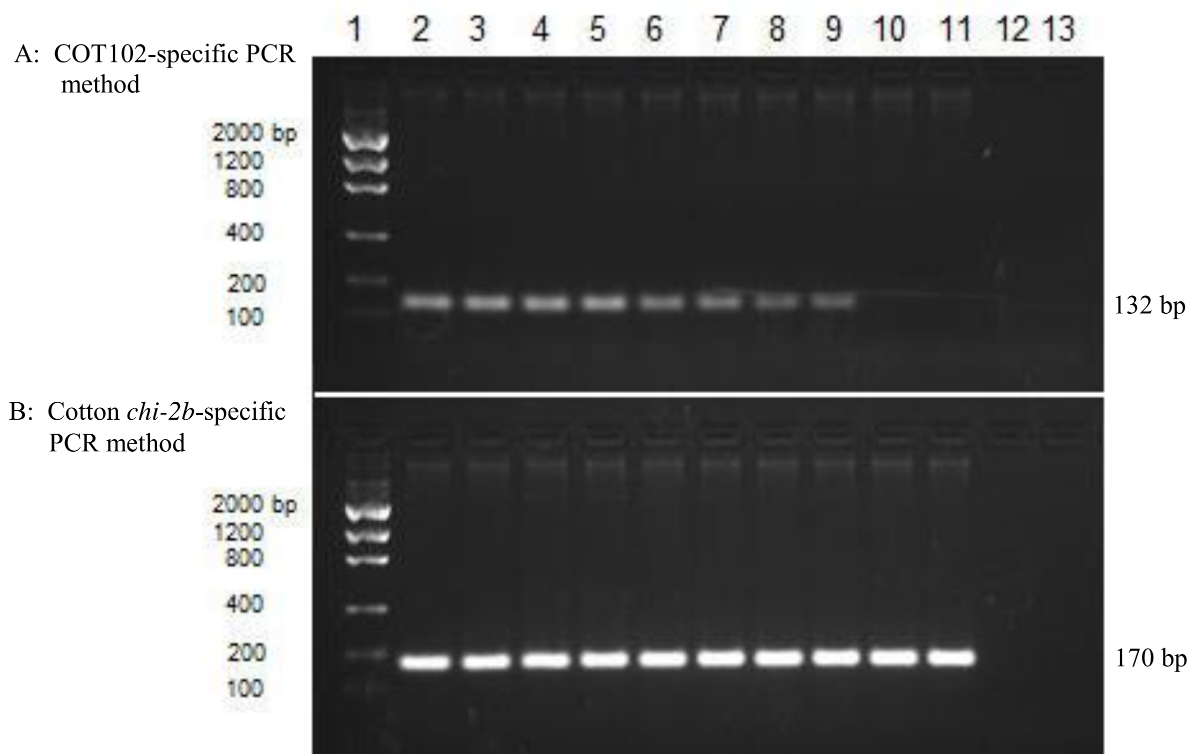
   4 µl of 0.1% COT102 DNA (25 ng/µl) to the positive control well.
5. Seal the PCR plates with adhesive PCR sealing film.
6. Centrifuge the plates at 4000 *g* for approximately 20 seconds.
7. Perform PCR using the cycling parameters in Table 6.
8. Following completion of PCR, store the PCR products at 4°C until further analysis.
9. Load the molecular weight markers and 10 to 15 µl of each PCR reaction onto a 2% agarose gel in 1X tris-acetate-EDTA or tris-borate-EDTA buffer containing 0.5 µg/ml ethidium bromide and electrophorese at 100 volts for 20 to 30 minutes. (tris = 2-amino-2-(hydroxymethyl)-1,3-propanediol and EDTA = ethylenediaminetetraacetic acid).
10. Capture the image, and score the results.

## 4.0 EXPECTED RESULTS

The agarose gel image in Figure 4 is an example of the expected results. A 132-bp PCR product is expected for all reactions containing COT102 DNA (Figure 4A, Lanes 2 through 9). The PCR product is expected to be absent in all reactions that do not contain COT102 DNA (Figure 4A, Lanes 10 through 13).

A 170-bp product is expected in all cotton DNA-containing samples using cotton *chi-2b* primers (Figure 4B, Lanes 2 through 11). No product is expected in water samples (Figure 4B, Lanes 12 and 13).

**FIGURE 4 Agarose Gel Image of the PCR Products Generated by Amplification with COT102- and *chi-2b*-specific Primers**




Lane 1: Low DNA Mass™ ladder.
Lanes 2 and 3: 1.0% COT102 DNA.
Lanes 4 and 5: 0.5% COT102 DNA.
Lanes 6 and 7: 0.1% COT102 DNA.
Lanes 8 and 9: 0.05% COT102 DNA.
Lanes 10 and 11: Negative control 1 (nontransgenic cotton).
Lanes 12 and 13: Negative control 2 (water substituted for DNA).

## 5.0 PCR INHIBITION

The absence of the expected PCR product may indicate that the DNA extract is inhibiting the PCR analysis. In this case, re-extraction of DNA and further purification may be necessary.

To determine if a DNA extract is inhibiting the PCR analysis, add 1 µl of 0.25 ng/µl 100% COT102 DNA into a well containing the reaction mixture and the DNA extract that did not show the expected PCR product. Perform PCR analysis as described in this method. If the expected PCR product is still absent, the DNA extract is inhibiting the PCR.

## 6.0 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).