DNA Extraction from Bt-cotton and its counterpart

One critical point in the development of quantitative PCR methods is, DNA extraction method and different sources may require different extraction protocols. DNA extracted from different sample matrixes was evaluated for suitability in quantitative analysis. In order to select for the best applicable DNA isolation procedure we tried two methods Bangalore GeneiPure Column kit and CTAB. They are mentioned as follows.

**Bangalore GeneiPure Column kit**

DNA isolated from Bt-cotton and its counterpart by Bangalore GeneiPure Column kit, showed clear bands of genomic DNA with no contamination of RNA and other impurities, as can be seen Figures. It clarifies that quality and quantity of DNA was good. Isolated DNA was checked on 0.8% agarose gel electrophoresis, Figure 22 shows clear bands genomic DNA.

**CTAB method**

It was found that DNA isolated from CTAB method was of high quality and high yield, but was applicable only to seeds and leaf. On running the DNA samples on 0.8% agarose gel, a single clear band of genomic DNA was observed in all Bt-cotton and its counterpart. The table 10, below mentions the quantity and the quality of DNA extracted from CTAB protocol. It was observed we get DNA when the CTAB methods was applied on Bt-cotton and its counterpart but we could not get good quality of DNA. Further the DNA easy kit from Bangalore GeneiPure Column was applied to extract DNA for getting sharp bands & high yield.
PCR for different transgenes used in the study

The primers to detect 26S r-RNA for cotton as an internal control already published primers were adapted to compare the two for suitability.

Likewise respective transgenes i.e. Cry1A(c), Cry2Ab, 35S Promoter, Nos terminator and npt-II as a marker gene were also detected by PCR based strategies adopted in the present study. DNA from all and then to perform PCR to detect respective internal control i.e. 26Sr-RNA as an internal control for cotton samples, so as to make sure that attempts to detect respective transgenes in authentic way was possible. These specific PCR products are depicted in figure 24 and figure 25 showing 516bp amplicon for 26S r-RNA and 230 bp amplicon, for Cry1A(c) 326bp in figure 27 and figure 29 Cry2Ab and 453bp from Bt-Cotton (BG-II).

Detection of Internal Control and transgene

Using single primer pairs up to 286ng DNA was easily detectable which comes around to be of internal control of 26S r-RNA ,Cry1A(c) and Cry2Ab presence of the transgenes in Bt-Cotton was characterized by the PCR amplicons of 35S promoter (195bp), and Nos terminator (180bp)and npt-II gene(215bp). In addition to above transgene 230 bp for Cry1A(c), 326bp and 453bp in Cry2Ab in figure 28.

The presence of the transgenes in Mon15985 was characterized by the PCR amplicon of 35S promoter (195bp) and MON15985 construct specific Cry1A(c) transgene (230bp), another transgene Cry2Ab(326 bp)and 453 bp. Nos terminator be detected in DNA isolated from MON 15985 standards, as it was reported in literature that this is already present. In addition to above transgenes, 156bp 26S r-RNA as an internal control was detected as positive control for cotton specific gene.
26S r-RNA and Cry1A(c) gene

Duplex PCR for Internal control 26S r-RNA and transgene Cry1A(c) in Bt-cotton, Figure 26 showed that the transgenes were detectable. It was calculated that 20ng of DNA was used for this PCR of internal control and transgene with this duplex.

26S r-RNA and Cry2A (b) gene

Duplex PCR for internal control 26Sr-RNA and transgene Cry2Ab in MON 15985 Figure 29 was detectable.

35S and Nos Terminator

The duplex PCR for 35S, Nos terminator and npt-II gene which are commonly used promoter and terminator sequences present in many GM Crops / foods is a good choice. This duplex can easily used as a simple tool for initial screening of a big lot of suspected feed products, which if found positive can be further processed for the detection of specific transgenes namely 26S r-RNA and Cry1A(c) in the case of MON 15985 in present scenario & 26S r-RNA and Cry2A(b). Duplex PCR for transgenes 35S, NOS terminator and npt-II gene (Figure 28) showed that the positive result gets for their amplification.

Isolation of DNA using Bangalore GeneiPure Column Kit Method

DNA from Bt and non Bt cotton was isolated using GeneiPure Column as per manufacturer instructions. The isolated DNA was checked on 0.8% agarose gel by electrophoresis. Very sharp bands were seen in the gel that clarifies that the DNA quantity and quality was very good. (Figure 22)
Isolation of DNA using CTAB-Method

DNA from Bt and non- Bt cotton was isolated using CTAB method as per protocol instructions. The isolated DNA was checked on 0.8% agarose gel by electrophoresis. Very sharp bands were seen lane 2, 3, 5, and 6 the gel that clarifies that while in lane 1 faint band has been seen.
Figure 23: Isolation of DNA by CTAB method

Lane 1- Gujarat Cotton Hybrid-6 (Non- Bt)
Lane 2- Gujarat Cotton Hybrid-6 (BG-II) Bt
Lane 3- Gujarat Cotton Hybrid-8 (Non- Bt)
Lane 4- Gujarat Cotton Hybrid-8 (BG-II) Bt
Lane 5- Gujarat Cotton 10 (Bt)
Lane 6- Gujarat Cotton 10 (Non- Bt)

Purity of DNA at 260/280 Isolation of DNA using Bangalore GeneiPure Column Kit Method

<table>
<thead>
<tr>
<th>Bt-cotton &amp; its counterpart</th>
<th>OD 260/280</th>
<th>Quantity ng/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Gujarat Cotton Hybrid-6 (Non Bt)</td>
<td>1.79</td>
<td>286.5</td>
</tr>
<tr>
<td>2- Gujarat Cotton Hybrid-6 (BG-II)  Bt</td>
<td>1.75</td>
<td>155.5</td>
</tr>
<tr>
<td>3- Gujarat Cotton Hybrid-8 (Non Bt)</td>
<td>1.85</td>
<td>353</td>
</tr>
<tr>
<td>4- Gujarat Cotton Hybrid-8 (BG-II)  Bt</td>
<td>1.85</td>
<td>318.5</td>
</tr>
<tr>
<td>5- Gujarat Cotton 10 (Non Bt)</td>
<td>1.78</td>
<td>371.5</td>
</tr>
<tr>
<td>6- Gujarat Cotton 10 (BG-II) (Bt)</td>
<td>1.78</td>
<td>371.5</td>
</tr>
</tbody>
</table>

Table 11: Quantity of DNA by using Bangalore GeneiPure Column kit
Purity of DNA at 260/280 Isolation of DNA using CTAB- Kit Method

<table>
<thead>
<tr>
<th>Bt-cotton &amp; its counterpart</th>
<th>OD 260/280</th>
<th>Quantity ng/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Gujrat Cotton Hybrid-6(Non Bt)</td>
<td>1.79</td>
<td>37.51</td>
</tr>
<tr>
<td>2-Gujrat Cotton Hybrid-6(BG-II) Bt</td>
<td>1.77</td>
<td>55.84</td>
</tr>
<tr>
<td>3-Gujrat Cotton Hybrid-8(Non Bt)</td>
<td>2.08</td>
<td>54.45</td>
</tr>
<tr>
<td>4-Gujrat Cotton Hybrid-8(BG-II) Bt</td>
<td>1.31</td>
<td>36.49</td>
</tr>
<tr>
<td>5-Gujrat Cotton 10(Non Bt)</td>
<td>1.32</td>
<td>65.21</td>
</tr>
<tr>
<td>6-Gujrat Cotton 10 (BG-II) (Bt)</td>
<td>1.46</td>
<td>65.56</td>
</tr>
</tbody>
</table>

Table 12: Quantity of DNA from Cotton samples by CTAB-Method

Figure 24: PCR showing amplified product of 516 bp in all cotton samples

Lane 1-Gujarat Cotton Hybrid-6(Non- Bt)
Lane 2-Gujarat Cotton Hybrid-6(BG-II) Bt
Lane 3-Gujarat Cotton Hybrid-8(Non- Bt)
Lane 4-Gujarat Cotton Hybrid-8(BG-II) Bt
Lane 5-Gujarat Cotton 10(Bt)
Lane 6-Gujarat Cotton 10(Non- Bt)
Detection of Internal Control 26S r-RNA in cotton and Bt-cotton seeds samples.

After isolation and quantification of DNA from different Bt and non Bt cotton seed samples (I, II, III, IV, V, VI) they were put to PCR amplification using specific set of primers of 26Sr-RNA (Sequence \( F \) - CAC AAT GAT AGG AAG AGC CGA \( R \) - CAA GGG CTT GGC AGA ATC). PCR product so obtained was checked on 2% Agarose gel electrophoresis, with a 100bp ran parallel to check the size of amplicon. A product of 516 bp was observed which confirms the presence of 26S r-RNA in all samples as shown in figure 24.

![PCR Product](image)

Figure 25: Transgene Cry1A(c) in Bt – Cotton Samples

M-100bp Marker

Lane 1-Gujarat Cotton Hybrid-6(Non Bt) no amplified product
Lane 2-Gujarat Cotton Hybrid-6(BG-II) Bt 230 bp amplified product
Lane 3-Gujarat Cotton Hybrid-8(Non Bt) no amplified product
Lane 4-Gujarat Cotton Hybrid-8(BG-II) Bt 230 bp amplified product
Lane 5- Gujarat Cotton 10(Non Bt) no amplified product
Lane 6- Gujarat Cotton 10(Bt) 230 bp amplified product
Detection of Transgenes Cry1A(c) in Bt-cotton (Bollgard-II)

In Bt-cotton i.e. Bollgard II there are two types of transgenes has been detected i.e. Cry1A(c) having 230 bp. By using specific set of primers of Cry1A(c) having 230bp (Sequence \( F - TGACCGCTTACAAGGAGGGATACG \) \( R - CACGGAGGCATAGTTCAGCAGGAAC \)). PCR products so obtained was checked on 2% Agarose gel Electrophoresis, with a 100 bp ladder ran parallel to check the size of amplicon. A product of 230 bp was observed only in Bt-cotton samples but not present in non-Bt cotton samples as shown in figure 25.

Figure 26: Duplex PCR having internal control in all cotton samples and Transgenes in Bt-cotton samples

M-100 bp Marker
Lane 1-Gujarat Cotton Hybrid-6(Non Bt) showing 516 bp amplified product
Lane 2-Gujarat Cotton Hybrid-6(BG-II) showing 516 bp and Bt 230 bp amplified product
Lane 3-Gujarat Cotton Hybrid-8(Non Bt) showing 516bp amplified product
Lane 4-Gujarat Cotton Hybrid-8(BG-II) Bt showing 516bp and 230 bp amplified product
Lane 5-Gujarat Cotton 10(Non- Bt) showing 516 bp amplified product
Lane 6-Gujarat Cotton 10(Bt) showing 516 bpand 230 bp amplified product

Duplex PCR of Internal control and transgene (26Sr-RNAand Cry1A (c))

In Duplex PCR we have to add 2 set of primers i.e.(internal control of 26S r-RNA and transgene of Cry1A(c). PCR product was checked on 2% agarose gel electrophoresis, with a 100 bp ladder
ran parallel to check the size of amplicon. A product of 516bp was observed in all the samples of Bt-cotton & cotton seed samples while product of 230 bp i.e Cry1A(c) was observed only in Bt-cotton samples not in cotton samples as shown in figure 26.

**Detection of Transgenes Cry2Ab in Bt-cotton (Bollgard-II)**

PCR of another transgene i.e. Cry2A(b) having 326 bp by using specific set of primers(F-
\[CGCGACTACCTCAAGA\]ACTACACC  R- GAGAGCGAGCACC\[ACT\]GAAC). PCR product so obtained was checked on 2% agarose gel electrophoresis, with a 100 bp ladder ran parallel to check the size of amplicon. A product of 326 bp was observed in Bt-cotton samples having 326 bp i.e. Cry2A(b) was observed as shown in figure 27.

![Image of gel electrophoresis](image)

**Figure 27:** Transgene Cry2Ab only in Bt-cotton samples having 326 bp product

M-100 bp Marker
Lane 1-Gujarat Cotton Hybrid-6(Non Bt) showing no amplified product
Lane 2-Gujarat Cotton Hybrid-6(BG-II) Bt showing 326 bp amplified product
Lane 3-Gujarat Cotton Hybrid-8(Non Bt) showing no amplified product
Lane 4-Gujarat Cotton Hybrid-8(BG-II) Bt showing 326bp amplified product
Lane 5-Gujarat Cotton 10( Non- Bt) showing no amplified product
Lane 6-Gujarat Cotton 10(Bt) showing 326bp amplified product
A Standard PCR and Multiplex PCR analysis of Cry2A(b) (MON15985)

326 bp Cry2Ab added in 1 PCR, 195bp(P-35S) Promoter in another tube, npt-II gene  215 in another tube & Duplex PCR with Cry2Ab326 bp with 195, 326bp with 180 bp and 326 bp with 215bp as shown in figure 28.

Figure 28: Transgene, 35S promoter, NOS terminator, npt-II and duplex PCR

M- 100 bp DNA Ladder
Lane 1- Water control
Lane 2- Transgene Cry2Ab having 326bp
Lane 3- 35 S Promoter having 195 bp
Lane 4- NOS terminator having 180bp
Lane 5- npt-II gene having 215 bp
Lane 6- Duplex PCR with Cry2Ab and 35S Promoter
Lane 7- Duplex PCR with Cry2Ab and NOS Terminator
Lane 8- Duplex PCR with Cry2Ab and npt-II gene
Duplex PCR with another Cry2Ab having 453 bp with 26Sr-RNA as an internal control

Cry2Ab

\[ F - GGACCTACCGCGACTACCTGAAGA \]

\[ R - TGAACGGCCGATGCACCAATGTC \]

Figure 29: Duplex PCR with Cry2Ab and Internal control 26S r-RNA in Cotton samples

M-100 bp Marker
Lane 1-Gujarat Cotton Hybrid-6(Non Bt) 516bp amplified products
Lane 2-Gujarat Cotton Hybrid-6(BG-II) Bt 516 and 453 bp amplified product
Lane 3-Gujarat Cotton Hybrid-8(Non Bt) 516bp amplified product
Lane 4-Gujarat Cotton Hybrid-8(BG-II) Bt 516bpand 453bp amplified product
Lane 5-Gujarat Cotton 10(Non- Bt) 516bp amplified product
Lane 6-Gujarat Cotton 10(Bt) 516bpand 453 bp amplified product

Elisa TEST- For the Transgene Cry2Ab
<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Samples</th>
<th>O.D. at 450 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+ve Control Standard</td>
<td>2.427</td>
</tr>
<tr>
<td>2</td>
<td>+ve Control Standard</td>
<td>1.893</td>
</tr>
<tr>
<td>3</td>
<td>-ve Control Standard</td>
<td>0.822</td>
</tr>
<tr>
<td>4</td>
<td>-ve Control Standard</td>
<td>0.569</td>
</tr>
<tr>
<td>5</td>
<td>Extraction Buffer</td>
<td>0.154</td>
</tr>
<tr>
<td>6</td>
<td>Extraction Buffer</td>
<td>0.175</td>
</tr>
<tr>
<td>7</td>
<td>Gujarat Cotton Hybrid-6(Non Bt)</td>
<td>1.009</td>
</tr>
<tr>
<td>8</td>
<td>Gujarat Cotton Hybrid-6(BG-II) Bt</td>
<td>2.019</td>
</tr>
<tr>
<td>9</td>
<td>Gujarat Cotton Hybrid-8(Non Bt)</td>
<td>0.893</td>
</tr>
<tr>
<td>10</td>
<td>Gujarat Cotton Hybrid-8(BG-II) Bt</td>
<td>2.028</td>
</tr>
<tr>
<td>11</td>
<td>Gujarat Cotton 10( Non- Bt)</td>
<td>0.488</td>
</tr>
<tr>
<td>12</td>
<td>Gujarat Cotton 10(BG-II) Bt</td>
<td>2.002</td>
</tr>
</tbody>
</table>

Table 13: O.D. at 450nm of Transgene Cry2Ab in Cotton samples by ELISA test

Figure 30: Elisa TEST for Cry2Ab
Table 14: O.D. at 450nm of Transgene Cry1A(c) in Cotton samples by ELISA test

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Samples</th>
<th>O.D. at 450 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+ve Control Standard</td>
<td>2.486</td>
</tr>
<tr>
<td>2</td>
<td>+ve Control Standard</td>
<td>2.015</td>
</tr>
<tr>
<td>3</td>
<td>-ve Control Standard</td>
<td>0.137</td>
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<tr>
<td>4</td>
<td>-ve Control Standard</td>
<td>0.131</td>
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<td>5</td>
<td>Extraction Buffer</td>
<td>0.118</td>
</tr>
<tr>
<td>6</td>
<td>Extraction Buffer</td>
<td>0.109</td>
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<tr>
<td>7</td>
<td>Gujarat Cotton Hybrid-6(Non Bt)</td>
<td>0.0894</td>
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<tr>
<td>8</td>
<td>Gujarat Cotton Hybrid-6(BG-II) Bt</td>
<td>2.055</td>
</tr>
<tr>
<td>9</td>
<td>Gujarat Cotton Hybrid-8(Non Bt)</td>
<td>0.062</td>
</tr>
<tr>
<td>10</td>
<td>Gujarat Cotton Hybrid-8(BG-II) Bt</td>
<td>2.015</td>
</tr>
<tr>
<td>11</td>
<td>Gujarat Cotton 10( Non- Bt)</td>
<td>0.071</td>
</tr>
<tr>
<td>12</td>
<td>Gujarat Cotton (BG-II) 10 (Bt)</td>
<td>1.878</td>
</tr>
</tbody>
</table>
Figure 32: ELISA TEST – For Cry1A(c)

Figure 33: ELISA TEST – For Cry1A(c)
Cloning of Bt-Cotton by TOPOTA Cloning Kit

Figure 34: Transgene Cry 1A(c) and Cry2Ab in MON 15985

- M: shows 100 bp Ladder
- L1, L2, L3 shows 230 bp product of Cry1A(c) and L4, L5, L6 shows 453 bp product of Cry2Ab

On 2% Agarose Gel Electrophoresis showing Cry 1A(c) and Cry2Ab in MON 15985 gene in Bt-Cotton before cloning

Figure 35: Control 1 (1C) Masterplate

In control 1(1C) master plate bacteria are grown in LB Agar medium without antibiotics ampicillin for 12 hours in incubator at 37°C. It shows numerous non-recombinant blue coloured bacterial colonies as well as fungal contamination.
In experimental 1 (1E) master plate recombinant white colonies were seen in LB Agar medium with antibiotics ampicillin for 12 hours in incubator at 37°C. It shows approximately 20 recombinant white colonies as well as one non-recombinant type blue coloured colony.

In control 2 (2C) master plate bacteria are grown in LB Agar medium without antibiotics ampicillin for 12 hours in incubator. It shows numerous non-recombinant blue coloured bacterial colonies as well as fungal contamination.
Figure 38: Experimental 2E Masterplate

In experimental 2 (2E) master plates recombinant white colonies were seen in LB Agar medium with antibiotic ampicillin for 12 hours in incubator at 37°C. It shows approximately 10 recombinant white colonies.

Figure 39: Subculture Streaked Plate

Eight recombinant white colonies from each experimental master plate (1E and 2E) are picked up & streaked on to a fresh LB Agar medium plate with antibiotics ampicillin & grown for 12 hours in incubator at 37°C.
Eight colonies of experimental 1(1E) are streaked on the upper part of plate and eight colonies of experimental 2 (2E) are streaked on lower part of plate. The eight recombinant colonies of plate 1 shows growth. There is growth in all colonies of plate 2 except colony 3.

On 2% Agarose Gel Electrophoresis showing Cry 1A(c) and Cry2Ab in MON 15985 after cloning

![Electrophoresis Image]

Figure 40: Transgene Cry1A(c) and Cry2Ab in MON-15985 after cloning

M shows 100 bp Ladder
L1, L2, L3, shows 230 bp amplified product of CRY1A(c) in Bt-Cotton
L4, L5, L6 shows 453 bp amplified product of transgene Cry2Ab in in Bt-Cotton.