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# Decaplex and Real-Time PCR Based Detection of MON531 and MON15985 *Bt* Cotton Events

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The genetically modified (GM) *Bt* crops expressing delta-endotoxins from *Bacillus thuringiensis* provide protection against a wide range of lepidopteron insect pests throughout the growing season of the plant. *Bt* cotton is the only commercialized crop in India that is planted on an area of 7.6 million hectares. With the increase in development and commercialization of transgenic crops, it is necessary to develop appropriate qualitative and quantitative methods for detection of different transgenic events. The present study reports on the development of a decaplex polymerase chain reaction (PCR) assay for simultaneous detection of transgene sequences, specific transgene constructs, and endogenous stearoyl acyl desaturase (*Sad1*) gene in two events of *Bt* cotton, i.e., MON531 and MON15985. The decaplex PCR assay is an efficient tool to identify and discriminate the two major commercialized events of *Bt* cotton, i.e., MON531 and MON15985, in India. Real-time PCR assays were also developed for quantification of *cry1Ac* and *cry2Ab* genes being employed in these two events. The quantitative method was developed using seven serial dilutions containing different levels of *Bt* cotton DNA mixed with a non-*Bt* counterpart ranging from 0.01 to 100%. The results revealed that the biases from the true value and the relative standard deviations were all within the range of ±20%. The limit of quantification (LOQ) of the developed real-time PCR method has also been established up to 0.01%.

**KEYWORDS: *Bt* cotton; construct-specific; *cry1Ac* gene; *cry2Ab* gene; decaplex PCR; limit of quantification; real-time PCR**

## INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is an important fiber crop, which is being cultivated in the area of 9.5 million hectares in India, the largest cotton growing country in the world. The first and only genetically modified (GM) crop approved in 2002 for commercial cultivation in India is "*Bt* cotton", conferring resistance to a lepidopteran insect pest, the bollworm. To date, 618 hybrids and 1 variety of six events of *Bt* cotton, i.e., MON531 with *cry1Ac* gene, MON15985 with *cry1Ac* and *cry2Ab* genes, Event1 with *cry1Ac* gene, GFM-*cry1A* with fused *cry1Ab* and *cry1Ac* genes, 9124 *Bt* cotton with synthetic *cry1C* gene in hybrids, and Dharwad Event (*Bt Bikaneri Nerma*) in a variety with truncated *cry1Ac* gene, have been commercialized and planted in an area of 7.6 million hectares (*1*). Out of commercialized 618 hybrids and 1 variety, 514 hybrids (83.03%) belong to two major events, i.e., 205 hybrids of MON531 (BollgardI) and 309 hybrids of MON15985 (BollgardII), which are being grown in farmer's fields in the North, Central, and South zones of India. The MON531 event of *Bt* cotton, BollgardI (BGI), expressing the single gene *cry1Ac*, and the MON15985 event, BollgardII (BGII), expressing two genes, *cry1Ac* and *cry2Ab*, were commercialized by Maharashtra Hybrid Seeds Company Ltd. (Mahyco). Based on the performance, farmers prefer multiple genes over *Bt* cotton hybrids with single gene, as *Bt* cotton hybrids with multiple genes provide additional protection to *Spodopetra* (a leaf eating tobacco caterpillar) while it also increases efficacy of protection to American bollworm, Pink bollworm, and Spotted bollworm. Farmers adopting *Bt* cotton with multiple genes earn higher profits through cost savings associated with fewer sprays for *Spodopetra* control as well as increased yield by 8−10% over *Bt* cotton hybrids with single gene (*2*).

The market price of BGII is comparatively higher as compared to BGI. It is difficult to differentiate the seeds of two events, so there are chances of adulteration or mixing of the events by the traders to earn profits. Hence, distinguishing these two events of *Bt* cotton has become an important concern so that the farmers may get the authentic seed. Hence, development of efficient and precise detection assays is required on priority for identification and discriminating the widely cultivated events of *Bt* cotton.

In India, the regulation of all activities related to genetically modified organisms (GMOs) and products derived from GMOs is governed by "*Rules for the Manufacture/Use/Import/Export and Storage of Hazardous Microorganisms, Genetically Engineered Organisms or Cells, 1989*" (commonly referred to as *Rules, 1989*) under the provisions of the Environment Protection Act, 1986, through the Ministry of Environment and Forests (MoEF). The *Rules, 1989*, primarily implemented by MoEF and the Department of Biotechnology (DBT), Ministry of Science and Technology, essentially take care of all activities, products, and processes related to or derived from biotechnology, including foods derived from biotechnology,

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**Table 1.** Details of Primer Pairs and Probes Employed for the Study

| gene | primer/probe | primer sequence (5′−3′) | expected amplicon size (bp) | reference |
|---|---|---|---|---|
| | | For Simplex and Decaplex PCR | | |
| *cry1Ac* | Cry1Ac-F/R | F-GACCGCTTACAAGGAGGGATACG<br>R-ACGGAGGCATAGTCAGCAGGACC | 228 | present study |
| *cry2Ab* | Cry2Ab-F/R | F-CAGCGGCGCCAACCTCTACG<br>R-TGAACGGCGATGCACCAATGTC | 260 | present study |
| *aadA* | AadA-1-F/R | F-TCCGCGCTGTAGAAGTCACCATTG<br>R-CCGGCAGGCGCTCCATTG | 406 | *11* |
| *nptII* | APH2 short/APH2 reverse | F-CTCACCTTGCTCCTGCCCGAGA<br>R-CGCCTTGAGCCTGGCGAACAG | 215 | *22* |
| *uidA* | Gus F/R | F-TTTCTTTAACTATGCCGGAATCCATC<br>R-CACCACGGTGATATCGTCCAC | 82 | *23* |
| *CaMV* 35S promoter | SP1 F/R | F-TTGCTTTGAAGACGTGGTTG<br>R-ATTCCATTGCCCAGCTATCT | 196 | *7* |
| *nos* terminator | NOS1/NOS3 | F-GAATCCTGTTGCCGGTCTTG<br>R-TTATCCTAGTTTGCGCGCTA | 180 | *24* |
| *Sad1* | S3 F/S4 R | F-CCAAAGGAGGTGCCTGTTCA<br>R-TTGAGGTGAGTCAGAATGTTGTTC | 107 | *25* |
| *Cry1Ac* transgene construct | Cry1Ac-35SF/R | F-CTTCGCAAGACCCTTCCTCTAT<br>R-GAACTCTTCGATCCTCTGGTTG | 326 bp region between *CaMV* 35S promoter and *cry1Ac* gene | present study |
| *Cry2Ab* transgene construct | CTCR-F/CTCR-2R | F-ATT GAA GAA GAG TGG GAT GAC GTT A<br>R-GAC CAG AGT TCA GGA CGG AGT T | 116 bp junction region between *CTP* and *cry2Ab* genes | *16* |
| | | For Real-Time PCR | | |
| *cry1Ac* | Cry1Ac-192-f/r | F-TTGCCTGAGTTGTCCGTGATC<br>R-GACGGAACGCTGATTGTTCTGT | | present study |
| | Cry1Ac-TM (probe) | FAM-ACGGTGACTTCAACAATGGCCTCA-TMR | | present study |
| *cry2Ab* | Cry2Ab-f/r | F-GAGTCCTTCGAGACCACCC<br>R-TCCTCTCGTCGTCCGCAA | | present study |
| | Cry2Ab-TM (probe) | FAM-TCCGCCGTCCACTGCACTACAAC-TMR | | present study |

thereby making the Genetic Engineering Approval Committee (GEAC) as the competent authority to approve or disapprove the release of GM foods in the marketplace (*3*). So far, no labeling threshold has been implemented in India. However, to meet the regulatory obligations, as per the Hon'ble Supreme Court of India's instructions for conducting field trials of GM crops, a protocol for testing presence of transgenes up to 0.01% has to be established (http://www.envfor.nic.in/divisions/csurv/geac/decision-jul-95.pdf).

Multiplex and real-time polymerase chain reaction (PCR) based detection assays have higher specificity for detecting target nucleotide sequences than the simplex PCR. Multiplex PCR, a variant of conventional PCR, is the reliable, efficient, and cost-effective qualitative assay and, hence, has been employed successfully for detection and identification of GM crops, which are under different stages of testing in containment or field trials in India, such as *Bt* cauliflower with insect resistant *cry1Ac* gene (*4*), *Bt* cotton with vegetative insecticidal protein (*vip*) 3A-type gene (*5*), GM tomato with salinity and drought tolerant *osmotin* gene (*6*), *Bt* potato with insect resistant *cry1Ab* gene (*7*, *8*), and GM potato with *AmA1* gene for better protein quality (*8*, *9*). A multiplex PCR assay has been developed for the detection and characterization of a *cry1Ac* transgene construct in *Bt* cotton (*10*). A hexaplex PCR assay for simultaneous amplification of commonly used marker genes has also been developed, which can be used as an efficient tool for initial screening of GM planting materials, irrespective of the crop and GM trait (*11*). Among the PCR-based quantitative assays, real-time PCR is considered to be an easy, useful, and accurate quantification method (*12*, *13*). Real-time PCR assays have been reported as the robust and reliable methodology for quantitative detection of various GM crops such as Widestrike cotton event expressing *cry1F* and *cry1Ac* genes (*14*) and also in other GM insect resistant cotton lines expressing *cry1Ac* gene (*15*, *16*), MON863 maize event expressing *cry3Bb1* gene (*17*), *Bt* rice with fused *cry1A*(*b*) and *cry1A*(*c*) gene (*18*), GT73 rapeseed event with *gox* gene (*19*), and three *Bt* potato lines containing *cry3A* gene (*20*).

In the present study, the decaplex PCR method simultaneously detecting the transgenes (i.e., *cry1Ac* and *cry2Ab*), marker genes (i.e., *nptII*, *aadA*, and *uidA* encoding, respectively, for neomycin phosphotransferase, aminoglycoside-3′-adenyltransferase, and β-D-glucuronidase), control elements (i.e., *Cauliflower Mosaic Virus* (*CaMV*) 35S promoter and nopaline synthase (*nos*) terminator), two construct-specific sequences (i.e., *cry1Ac* transgene construct and *cry2Ab* transgene construct), and endogenous *Sad1* gene, was developed for identification and differentiation of MON531 and MON15985 *Bt* cotton events. Real-time PCR assays have also been developed for quantification of *cry1Ac* and *cry2Ab* genes in these events.

## MATERIALS AND METHODS

**Planting Material.** Seeds of commercialized events of *Bt* cotton, i.e., MON531 (BollgardI) with *cry1Ac* gene and MON15985 (BollgardII) with *cry1Ac* and *cry2Ab* genes, were procured from M/s Maharashtra Hybrid Seeds Company Ltd. (Mahyco), Jalna. The seeds of both the events along with non-GM cotton seeds were grown in the National Containment Facility, National Bureau of Plant Genetic Resources, New Delhi, under optimum conditions.

**Genomic DNA Isolation and Quantification.** The isolation and purification of genomic DNA from fresh leaves of 5−6 weeks old seedlings of MON531 and MON15985 events of *Bt* cotton and non-GM cotton was carried out using a modified CTAB extraction method (*21*). The DNA samples were quantified by measuring UV absorption at 260 nm, while DNA purity was evaluated on the basis of the UV absorption ratio at 260 nm/280 nm ranging from 1.6 to 1.9 using a DU 640 UV spectrophotometer

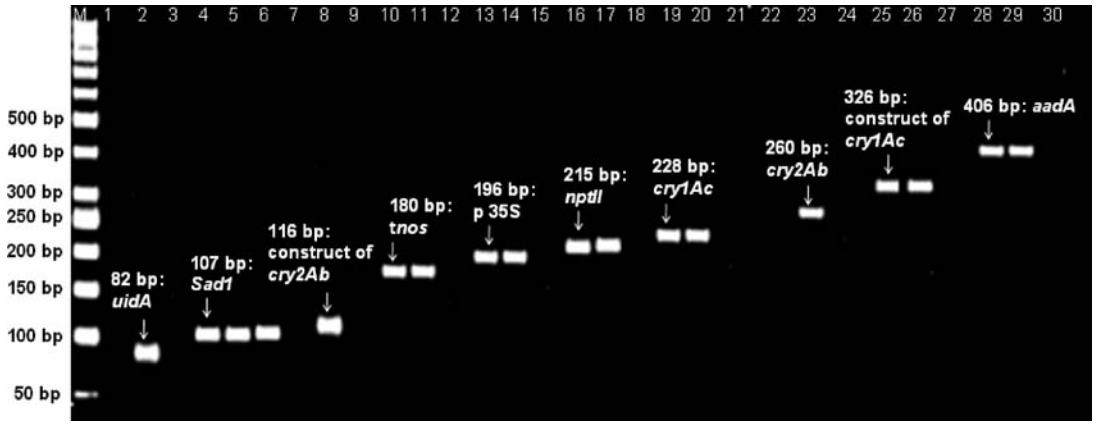



**Figure 1.** Simplex PCR for amplification of inserted genes, construct-specific sequences, and endogenous gene in two *Bt* cotton events, i.e., MON531 and MON15985 using primer pairs for *cry1Ac* and *cry2Ab* transgenes, *nptII*, *aadA*, and *uidA* marker genes, *CaMV* 35S promoter, *nos* terminator, endogenous *Sad1* gene, and specific gene constructs in MON531/MON15895 and MON15985: (lane M) 50 bp ladder; (lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28) samples of MON531 cotton; (lanes 2, 5, 8, 11, 14, 17, 20, 23, 24, 29) samples of MON15985 cotton; (lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30) samples of non-GM cotton.

(Beckman, USA). The final concentration of extracted DNA was made to 20 ng/$\mu$L for both simplex and real-time PCR and 35 ng/$\mu$L for the multiplex PCR assays.

**Primer and Probe Designing.** Sequences of primers and TaqMan probes are listed in **Table 1**. The primer pairs for the amplification of *cry1Ac* gene, *cry2Ab* gene, and *cry1Ac* transgene construct of MON531/MON15985 were designed using "Primer3 Online" primer designing software. Published primers for the amplification of *aadA* (*11*), *nptII* (*22*) selectable marker genes and *uidA* reporter gene (*23*), *CaMV* 35S promoter (*7*), *nos* terminator (*24*), *cry2Ab* transgene construct of MON15985 (*16*), and *Sad1* (*25*) endogenous reference gene were employed. All primers were synthesized by M/s Pivotal Marketing Ltd. The designed TaqMan probes and primer pairs employed in real-time PCR for *cry1Ac* and *cry2Ab* genes were synthesized by Roche Applied Sciences, Germany. The dilutions were made to have a final concentration of 10 $\mu$M with Milli-Q water to carry out simplex PCR, and a 4× concentration of primer mix was made by mixing 1.6 $\mu$M of each primer pair for performing decaplex PCR.

**Simplex and Decaplex PCR for Detection of MON531 and MON15985 Events.** Simplex and decaplex PCR assays were carried out on a PTC-200 Programmable Thermal Cycler (MJ Research Inc., USA). The specificity of the primer pairs to amplify the target gene sequences was checked by simplex PCR using both the *Bt* cotton events. Simplex PCR assays were performed in a final volume of 25 $\mu$L with the following reagent concentrations: 100 ng of template DNA, 1× Taq PCR buffer, 1.5 mM $MgCl_2$, 200 $\mu$M dNTP mix, 0.4 $\mu$M each primer and 0.5 U of Taq DNA polymerase (MBI Fermentas Inc., USA). The following program was used for the qualitative simplex PCR: one cycle of initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s; annealing at 59 °C for 1 min and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 8 min.

For decaplex (10-plex) PCR designed to amplify nine different elements of the inserted gene constructs, i.e., *cry1Ac*, *cry2Ab* gene, *CaMV* 35 promoter, *nos* terminator, *nptII*, *aadA*, *uidA*, *cry1Ac* transgene construct, MON15985 transgene construct, and an endogenous gene *Sad1*, simultaneously, a reaction mixture (25 $\mu$L) containing 175 ng of template DNA, 1× Hot Taq PCR buffer, 3.2 mM of $MgCl_2$, 600 $\mu$M of dNTP mix, 1× primer mix, and 0.2 U/$\mu$L of Hot Start Taq DNA polymerase (MBI Fermentas Inc., USA) was used. The amplification conditions were as follows: initial denaturation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 50 s, primer annealing at 59 °C for 50 s, primer extension at 72 °C for 50 s, and final extension at 72 °C for 5 min.

The PCR amplicons were analyzed by horizontal gel electrophoresis using 4.0% Metaphor agarose (Cambrex Bioscience Rockland, Inc., Rockland, ME) and were further visualized under UV light using a Gel Documentation System (Alpha Innotech, USA).

**Reference Molecule for Real-Time PCR.** A standard plasmid was constructed on the basis of a pCR2.1-TOPO vector (Invitrogen Life Technologies Inc.), in which the real-time PCR product amplified with the designed primer pair specific for *cry1Ac* gene was integrated using the TOPO TA cloning kit (Invitrogen Life Technologies Inc.). This recombinant plasmid was used to transform *Escherichia coli* strain TOP10 cell (Invitrogen Life Technologies Inc.). The cloned plasmid was selected by restriction digestion with *Eco*RI (Roche Applied Sciences, Germany). The cloned plasmid's DNA was extracted by the Qiagen Plasmid Midi kit (Qiagen, Germany), which was digested with *Hind*III restriction endonuclease. The linearized plasmid DNA was purified from 2% agarose gel by the QIA Quick Gel Extraction kit (Qiagen, Germany). For the generation of a standard curve, eight serial diluted concentrations (10, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, and $10^8$ copies per reaction) of standard plasmid DNA were used as reference molecules. The repeatability of the standard plasmid's copy numbers was estimated from the data of triplicate reactions. The accuracy and precision of the developed assays were determined by calculating the standard deviation and relative standard deviation (RSD) values.

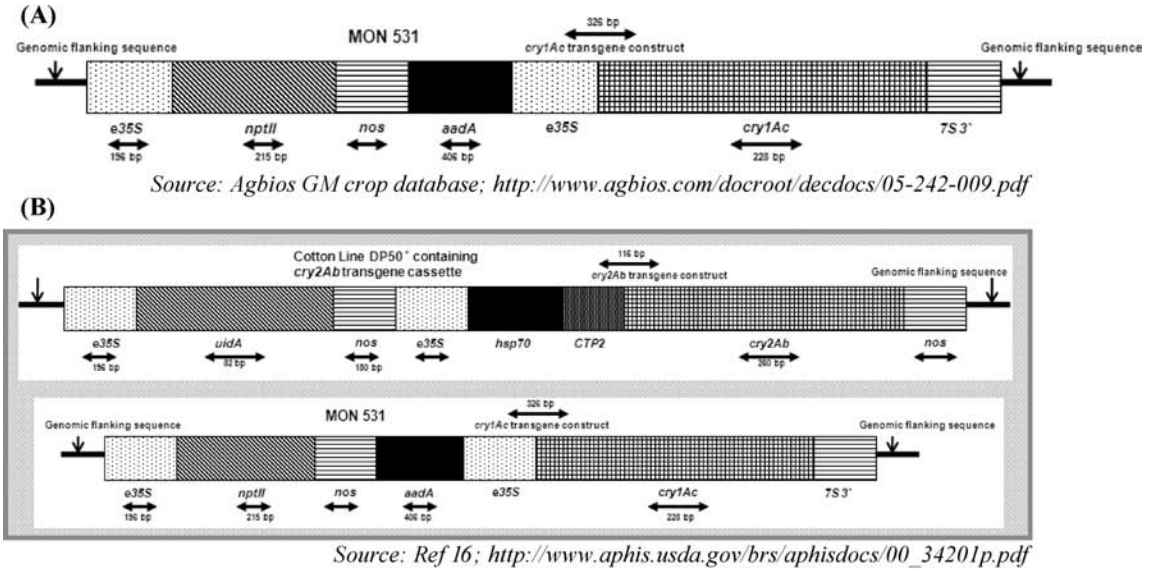
**Real-Time PCR for Quantitative Analysis.** For quantification of both the *Bt* cotton events, designed primer pairs and Taqman probes for *cry1Ac* and *cry2Ab* genes were standardized for real-time PCR assay in triplicate for each DNA sample, using a Light cycler480 system (Roche Applied Sciences, Germany). In each well, a 20 $\mu$L volume of reaction mixture was composed of 100 ng of template DNA, 0.4 $\mu$M of primer pair, 0.1 $\mu$M Taqman probe, and 10.0 $\mu$L of universal master mix (Roche Applied Science, Germany). The PCR conditions were as follows: initial denaturation at 95 °C for 7 min, 55 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 s.

The sensitivity of developed real-time PCR assays was also tested using the serial dilutions of 20 ng/$\mu$L of both the *Bt* cotton events DNA with non-GM to have varying concentrations of transgene: i.e., 100, 50, 10, 1.0, 0.1, 0.05, and 0.01 ng per reaction. The sensitivity of the method was evaluated by comparing the experimental mean value with the theoretical value of the GM content.

## RESULTS AND DISCUSSION

In India, among the six commercialized events of *Bt* cotton, the MON531 (BollgardI) with *cry1Ac* gene and MON15985 (BollgardII) with *cry1Ac* and *cry2Ab* genes are being widely cultivated in the North, Central, and South zones. To develop a reliable and efficient diagnostic method for detection of these two major events of *Bt* cotton is of utmost importance to ensure the seed quality, to meet the regulatory obligations, and to solve legal disputes, if they arise. Hence, the present study aimed at detecting efficiently MON531 and MON15985 events of *Bt* cotton using transgene- and construct-specific decaplex PCR and quantitative real-time PCR methods.

**Qualitative PCR Analysis of MON531 and MON15985 *Bt* Cotton Events.** In attempting to set up the combination of a transgene-and construct-specific decaplex PCR system for



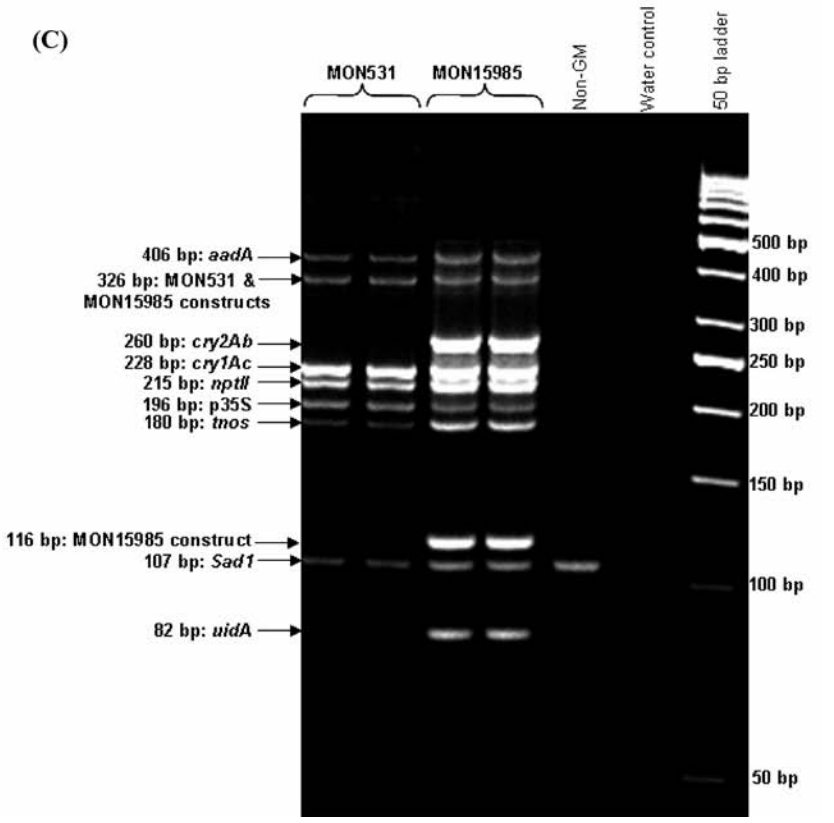




**Figure 2.** Transgene- and construct-specific multiplex PCR for discrimination of two *Bt* cotton events, i.e., MON531 and MON15985 using primer pairs for *cry1Ac* and *cry2Ab* transgenes, *nptII*, *aadA*, and *uidA* marker genes, *CaMV* 35S promoter, *nos* terminator, endogenous *Sad1* gene, and specific gene constructs in MON531/MON15895 and MON15985. (**A**) Linear transgene construct of MON 531 and the primer's target positions are shown by double-headed arrows along with the amplified base pairs. (**B**) Linear transgene construct of MON 15985 (cotton line DP50 containing *cry2Ab* transgene cassette[16] × MON 531) and the target positions of decaplex PCR. Primer's target positions are shown by double-headed arrows along with the amplified base pairs. (**C**) Amplification of targeted regions using decaplex PCR.

detection of the two *Bt* cotton events, simplex PCR assays were performed to assess the specificities of all the ten primer pairs employed in the study. The specific amplicons of desired size for all the ten target sequences, i.e., 228 bp for *cry1Ac* gene, 260 bp for *cry2Ab* gene, 196 bp for *CaMV* 35 promoter, 180 bp for *nos* terminator, 215 bp for *nptII*, 406 bp for *aadA*, 82 bp for *uidA*, 326 bp for *cry1Ac* construct-specific (targeting the desired region between *CaMV* 35S promoter and *cry1Ac* gene), 116 bp for MON15985 construct-specific (targeting the junction region between chloroplast transit peptide *CTP* and *cry2Ab* genes), and 107 bp for *Sad1*, were detected in the sample of MON15985 cotton, whereas only seven of the target sequences, i.e., 228 bp for *cry1Ac* gene, 196 bp for *CaMV* 35 promoter, 180 bp for *nos* terminator, 215 bp for *nptII*, 406 bp for *aadA*, 326 bp for *cry1Ac* construct specific, and 107 bp for *Sad1*, were detected in the sample of MON531 event of cotton (**Figure 1**). Amplicon of 107 bp for *Sad1* gene was also detected in the non-*Bt* cotton sample.

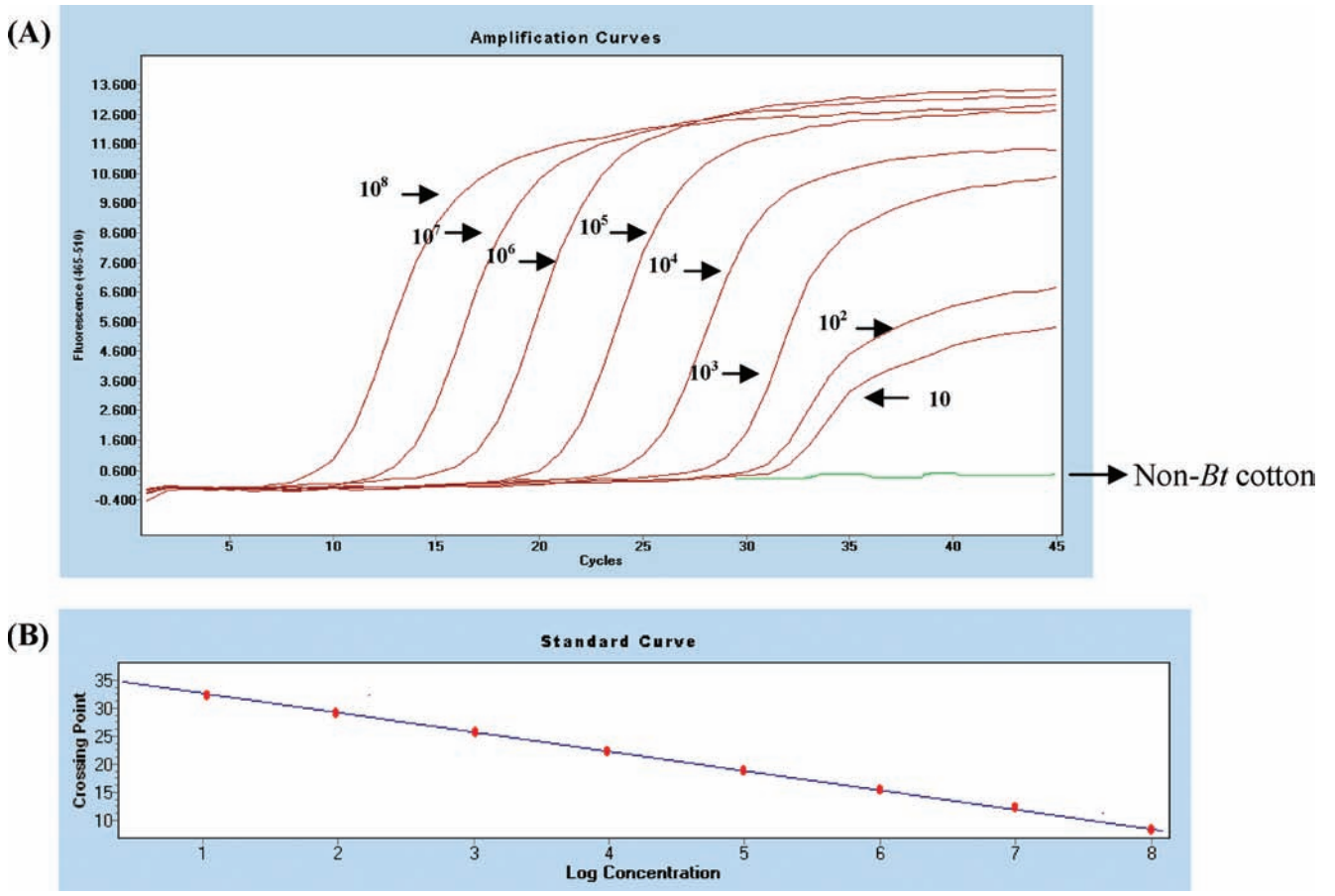



**Figure 3.** Amplification plots and standard curves using real-time PCR for *cry1Ac* gene. (**A**) Amplification curves generated for eight serial dilutions of standard plasmid with 10 to $10^8$ copies of *cry1Ac* gene, respectively. (**B**) Standard curve generated from the amplification data for standards shown in part **A**.

To carry out decaplex PCR, ten primer pairs listed in **Table 1** were employed to amplify ten target elements in a single run. Using a Hot Start PCR kit, the first focus was on the thermal cycler program and primer concentration for the optimization of PCR parameters. A primer concentration of 0.4 $\mu$M for each primer pair yielded intense bands for their target elements. MON531 and MON15985 *Bt* cotton events have most of the common inserted gene sequences, i.e., *cry1Ac* gene, *CaMV* 35S promoter, *nos* terminator, *nptII* and *aadA* selectable marker genes (**Figures 2**A and B). The MON15985 event has additional *cry2Ab* gene and *uidA* reporter gene transformed in it. In decaplex PCR, for the MON 15985 event, all the seven inserted gene sequences, i.e., *cry1Ac*, *cry2Ab*, *CaMV* 35S promoter, *nos* terminator, *uidA*, *nptII*, and *aadA,* two transgene constructs, i.e., *cry1Ac* gene construct and *cry2Ab* gene construct, along with an endogenous reference gene were amplified with the desired band size of 228 bp, 260 bp, 196 bp, 180 bp, 82 bp, 215 bp, 406 bp, and 107 bp, respectively. In the samples of MON531 cotton event, five inserted genes, i.e., *cry1Ac*, *CaMV* 35S promoter, *nos* terminator, *nptII*, and *aadA* and *cry1Ac* gene construct, along with an endogenous reference gene were amplified with the desired amplicons of 228 bp, 196 bp, 180 bp, 215 bp, 406 bp, and 107 bp, respectively (**Figure 2C**). In the non-GM cotton sample, only endogenous reference gene was amplified and in the water sample taken as negative control, no gene was amplified, showing specificity of the developed PCR protocol. Since no amplification was detected for *cry2Ab* gene, *uidA* reporter gene, and *cry2Ab* transgene construct in MON531 event of *Bt* cotton, hence, the developed decaplex method can be used as an efficient tool for differentiating the MON531 and MON15985 events of *Bt* cotton.

**Standard Plasmid as a Reference Molecule Used in the Quantitative Method.** As a reference molecule, eight levels of standard plasmids for *cry1Ac* and *cry2Ab* genes were set to $10^8$, $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, and 10 copies per reaction for the quantitative real-time PCR. This was sufficient to quantify as low as 0.01% of transgenes in *Bt* cotton events using 100 ng of genomic DNA template per reaction. The linearity of the standard curves for MON531 and MON15985 cotton was confirmed in the quantitative PCR using the designed primer pair, the Taqman probe, and the standard plasmid. Good linearity between copy number and fluorescence values (Ct), as visualized in the calibration curves for *cry1Ac* and *cry2Ab* genes (**Figures 3** and **4**), indicated that the developed real-time PCR assays combined with the reference molecule established in this study are well-suited for further quantitative measurements. The repeatability of the copy number for all the eight levels of standard plasmid was confirmed from the data of triplicate reactions. The values of relative standard deviation (RSD) of the triplicate reactions ranged from 1.71 to 12.2% (**Table 2**). All of the RSD values were found below 20%, revealing that the variation within this range was not significant, so the standard plasmid was confirmed to be a stable and reliable reference molecule.

**Quantitative Real-Time PCR for Detection of *cry1Ac* and *cry2Ab* genes.** The precision of the method was evaluated as the bias (percent) of the experimental mean value from the theoretical value. The accuracy was evaluated by RSD values. For the validation of the quantitative PCR method, the test DNA samples were prepared by mixing the GM with non-GM DNA at seven levels: 50, 10, 1, 0.5, 0.1, 0.05, and 0.01%. For *cry1Ac* gene, at low mixing levels, i.e., 1.0, 0.5, 0.05, and 0.01%, the biases

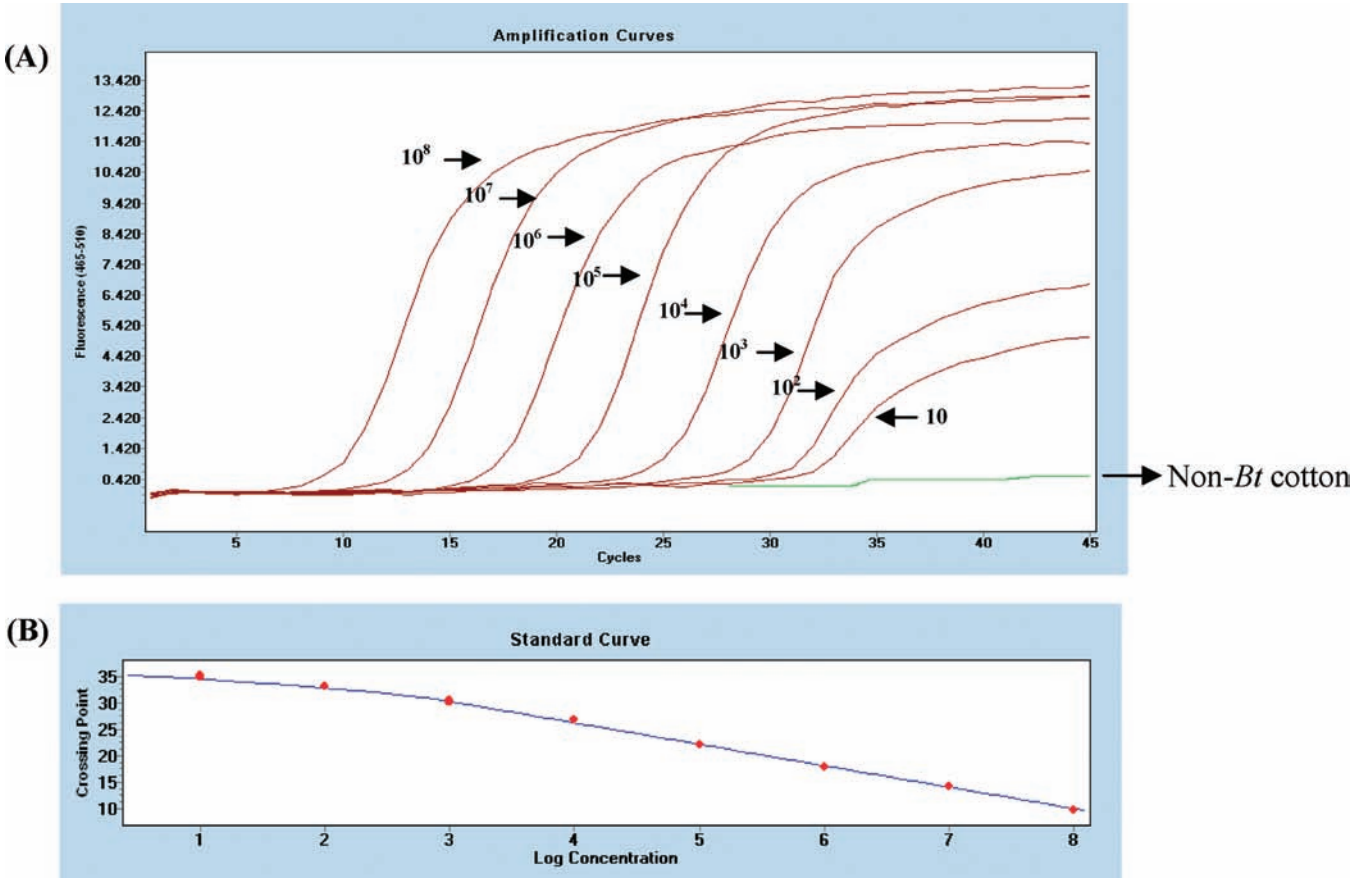



**Figure 4.** Amplification plots and standard curves using real-time PCR for *cry2Ab* gene. (**A**) Amplification curves generated for eight serial dilutions of standard plasmid with 10 to $10^8$ copies of *cry2Ab* gene, respectively. (**B**) Standard curve generated from the amplification data for standards shown in part **A**.

**Table 2.** Repeatability of the Copy Numbers of Standard Plasmids

| | copy no. | | |
|---|---|---|---|
| target | true value | mean value | relative standard deviation |
| *cry1Ac* gene | 10 | 12.1 | 12.2 |
| | 100 | 487 | 9.7 |
| | 1000 | 1520 | 1.71 |
| | 10000 | 13000 | 4.07 |
| | 100000 | 159000 | 3.78 |
| | 1000000 | 1450000 | 6.54 |
| | 10000000 | 12700000 | 3.18 |
| | 100000000 | 103000000 | 2.54 |
| *cry2Ab* gene | 10 | 14 | 10.68 |
| | 100 | 110 | 10.18 |
| | 1000 | 1000 | 0.21 |
| | 10000 | 9700 | 1.89 |
| | 100000 | 102000 | 4.76 |
| | 1000000 | 1010000 | 0.569 |
| | 10000000 | 10200000 | 6.84 |
| | 100000000 | 101500000 | 4.1 |

**Table 3.** Accuracy and Precision Statistics for Quantitative Real-Time Assays

| | | accuracy | | precision | |
|---|---|---|---|---|---|
| transgene | true value of transgene content (%) | mean transgene content (%) | bias true value (%) | standard deviation | relative standard deviation |
| *cry1Ac* | 0.01 | 0.011 | 10.0 | 0.002 | 18.2 |
| | 0.05 | 0.049 | −2.0 | 0.0021 | 4.2 |
| | 0.5 | 0.461 | −7.8 | 0.067 | 14.5 |
| | 1.0 | 1.07 | 7.0 | 0.055 | 5.1 |
| | 10 | 9.93 | −0.7 | 0.25 | 2.5 |
| | 50 | 50.01 | 0.02 | 0.48 | 0.96 |
| | 100 | 99.7 | −0.3 | 0.33 | 0.33 |
| *cry2Ab* | 0.01 | 0.011 | 10.0 | 0.002 | 18.18 |
| | 0.05 | 0.051 | 10.0 | 0.002 | 4.07 |
| | 0.5 | 0.54 | 8.0 | 0.03 | 5.6 |
| | 1.0 | 0.986 | −1.4 | 0.11 | 11.1 |
| | 10 | 9.93 | −0.7 | 0.71 | 7.15 |
| | 50 | 46.93 | −6.14 | 1.72 | 3.66 |
| | 100 | 95.7 | 4.3 | 1.95 | 2.03 |

were 7.0, −7.8, −2.0, and 10%, respectively, and their RSDs were 5.1, 14.5, 4.2, and 18.2%, respectively. In the case of *cry2Ab* gene, the biases at low mixing levels, i.e., 1.0, 0.5, 0.05, and 0.01%, were 10, 10, 8, and −1.4%, respectively, and their RSDs were 11.1, 5.6, 4.07, and 18.18%, respectively. Overall, the values of the RSDs of the three time repeated tests ranged from 0.33 to 18.2% for *cry1Ac* gene and 2.03 to 18.18% for *cry2Ab* gene.

Linearity was assessed for both the genomic DNA samples and the plasmid standard curve. The range of plasmid DNA samples was defined between 10 and $10^8$ copies, whereas the range of genomic DNA was defined between 0.01 and 100%. The correlation coefficient ($r^2$) of the regression line was >0.99 with the $Y = -3.435x + 38.12$ and $Y = -3.44x + 37.15$ regression equations for *cry1Ac* and *cry2Ab* assays, respectively. The repeatability, accuracy, and precision of this quantification system were also tested (**Tables 2** and **3**). According to the approach suggested by Codex, the limit of detection (LOD) should correspond to the lowest level of analyte, for which the RSD for reproducibility is 33% or less, and the limit of quantification (LOQ) should correspond to the lowest level of analyte, for which the RSD is 25% or less (*26*). In this study, the RSD values of the lowest concentration level (0.01%) for both the genes, i.e., *cry1Ac* and

*cry2Ab*, were below the 25% criteria (18.2 and 18.18%, respectively). In conclusion, according to the Codex Alimentarius guidelines, both the LOD and LOQ of this method were 0.01%, which is a feasible level for detection of a particular GM crop.

In the present study, qualitative and quantitative PCR methods for detection of MON531 and MON15985 events of *Bt* cotton were developed. The decaplex PCR detection method so developed would assist to effectively identify and differentiate two widely cultivated *Bt* cotton events, MON531 and MON15985, in India to meet the regulatory obligations for GM labeling and identification of genetic traits and to check the adulteration or mixing of these events by the traders, which would ensure the supply of authentic seeds to the farmers and solve legal disputes, if they arise. The reported real-time PCR assays will have also immense use in estimating the copies of inserted *cry1Ac* and *cry2Ab* genes and in quantitative analysis of *Bt* cotton to meet the threshold level.

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This paper published ASAP August 5, 2010 with several reference citation errors in the fourth paragraph of the Introduction section. The correct version published on August 19, 2010.

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