International Journal of
Basic and Applied Sciences

(A peer reviewed International Journal)

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International Journal of Basic and Applied Sciences, Vol. 1 No. 2. pp. 92-102 2277-1921. 2012

ISSN *2277 – 1921*

Article type *Full Length Research Article*

Submission date *12 January 2012*

Acceptance date *30 March 2012*

Publication date *15 April 2012*

Article URL *http://www.crdeep.org/category/ijbas*

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International Journal of Basic and Applied Sciences Vol. 1 No. 2. 2012. Pp. 92-102

Full Length Research Paper

Quantitative competitive PCR for the detection and quantification of genetically modified cotton event MON-531

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ABSTRACT

A Conventional based approach of quantitative competitive polymerase chain reaction (QC-PCR) system for the detection and quantification of the genetically modified cotton MON-531 was developed. This system was developed based on the validation of internal standards by real-time PCR. A tandem marker plasmid was constructed and used as a competitor for the detection and quantification of genetically modified cotton MON-531.The competitor plasmid contains event specific sequence of GM bt cotton MON-531and taxon-specific sequence of cotton that is fsACP (fiber specific acyl carrier protein) with distinguishable size difference with genomic sequence of respective regions. The event specific competitor has 40bp deletion in the sequence and endogenous competitor has 22bp deletion in the sequence. This plasmid DNA was used as standards in which copy number of targets was adjusted. The competitor has the same priming sites as target gene and was used as standards with the same amount of genomic DNA in quantitative-competitive PCR and co-amplified. QC-PCR amplified products were run on 3% agarose gel and equivalent point was calculated where the intensities of competitor and target gene are equal. To test the limit of detection (LOD), the DNA mixture was prepared with GM cotton MON-531 events and non GM cotton DNA. By this QC-PCR method we have detected and quantified up to 1% GM bt cotton MON-531.This QC-PCR method is simple and alternative method for real-time PCR for detection and quantification of GM foods.

Key words: *Quantitative Competitive PCR; Genetically Modified Organisms (GMOs); MON 531, event specific.*

INTRODUCTION

In 2008, the number of countries planting the biotech crops reached milestone of 25 countries in 2008 and 800 million hectarage of commercialized biotech/GM was planted, in 2008 the global hectarage of biotech crops reached 125 million hectares up from 114.3 million hectares in 2007 *(1)*.GM cotton is the first biotech crop in India, and it is mainly used for textile fiber, grown in 70 countries in the world *(2)*. GM cotton, the first transgenic crop in India, was introduced by US based company Monsanto in collaboration with the Maharashtra hybrid seed company (Mahyco) and got approval for commercialization in 2002.

In recent years, gene modification/recombinant DNA techniques have been used worldwide, and some insect-resistant and herbicide tolerant crops developed and widely cultivated, commercialization of GM crops developed by recombinant DNA technology has been increased along with the expanding population to meet the demand for food grains *(3)*.

The insect-resistant, herbicide tolerant traits incorporated into GM crops should be safe to the human health, environment *(4)*. Consumers are concerned about the use of GM crops in food and feed, they have to be labeled if the content of GM organism(GMO) in food products exceed certain level of contamination such as 0,2,3,5% *(5)*.Therefore development of GM detection methods are essential to know the percentage of contamination or percentage of presence of GM content in Gm foods and for the labeling *(6)*.The number of countries introduced their own regulation and labeling system, main benefits of labeling system is to inform the consumers of the presence of GM content in food products. Because of labeling, the development of accurate and reliable quantitative detection methods is in demand.

Qualitative and Quantitative PCR methods are mostly used for GMO identification and Quantification *(7)*. PCR based detection methods are highly specific and sensitive for DNA derived from different crops and they are qualitative *(8-10)*. PCR based quantitative methods can detect wheather the sample contains GM foods or not. Quantitative detection methods are required for accurate measurement of GM content. The best way for the quantitative analysis of GMO is by real-time PCR method due to its accuracy and specificity (ref real time PCR *(11)*.But real time PCR instrument and reagents are very expensive and can't be affordable by every laboratory. In quantitative competitive polymerase chain reaction, an internal standard (competitor) is co-amplified with the target DNA and this method has proven to be a feasible method for the detection and quantification of GMO *(12)*.

A competitive plasmid used for QC PCR contains an insertion or deletion in the sequence from the plant specific reference gene (endogenous gene) and GMO specific sequence, which is enough to allow separation by size on agarose gel. In QC PCR method, each sample is titrated with the internal standard (competitor) by increasing way of copy number is added to the constant amount of DNA and followed by PCR and electrophoresis. At the equivalent point where the density of the internal standard and target are observed, the quantity of the target and competitor are determined to be equal *(13).*

The main objective of this study is to develop and quantify the GM bt cotton MON531 by using plasmid DNA as internal standards. Competitors were developed by deleting 22bp from the endogenous sequence of *fsACP* amplified product, 40bp from the event specific MON-531 amplified products by using restriction enzymes. These two competitors were cloned into a single plasmid, with this plasmid internal standards were prepared for this study and these internal standards were calibrated by real-time PCR method. This QC PCR method is repeated two times to asses the applicability of the above method.

MATERIALS AND METHODS

Cotton seeds:

100 % GM and non GM bt cotton (MON-531 event) developed by Monsanto Company, Hyderabad and kindly provided by the developer. All of these samples were used in QC PCR.

Preparation of Sample and DNA extraction:

Dry seeds of GM and non-GM cotton were ground with mortar and pestle and fine powder was prepared from that by using liquid nitrogen.DNA extraction was performed by using DNeasy plant Mini kit (QIAGEN). The DNA concentration of the solutions were determined by Nanodrop (N1000) and on 0.8% agarose gel by comparing with lambda-Hind-III molecular marker and quality was evaluated by absorbance ratio at 260/280 was between 1.7 and 2. Different GM percentages 100%, 10%, 5%, 1% DNA samples were prepared by mixing 100ng of Non GM cotton and GM bt cotton (MON531) DNA samples.

Oligonucleotide primers and enzymes:

For the amplification of *fsACP* primer pair was designed by primer-3 (version 0.4.0) software *(26).* For ESP MON 531 *(7),* published primer was used. Oligonucleotide primers were synthesized and purified on a High Performance Salt Purification by Ocimum biosolutions Pvt Ltd, Hyderabad. Each Oligonucleotide was diluted to 100µM concentration with appropriate volume with MilliQ water. For QC PCR, 0.5µM was used. Primer sequences used in this study and enzymes used for the construction of competitor plasmid are listed in **Table. (1)**

Construction of competitor plasmid: Competitor for the QC PCR was constructed by deleting 40bp from the amplified event specific GM bt cotton MON-531 sequence using *SfaNI, Cac8I* enzymes and competitors for taxon-specific gene *fsACP* (cotton endogenous gene) was constructed by deleting 22bp from the amplified fsACP sequence using *AluI, SfaNI* enzymes. These competitors were ligated and cloned into PCR2.1 plasmid vector (Initrogen).The cells of *Escherichia coli* strain Top10 (Invitrogen) was transformed with the plasmid. The plasmid was extracted and purified by QIAGEN plasmid mini kit (QIAGEN).The competitor fragments were restricted from the TOPOTA plasmid. Endogenous competitor (*fsACP*) was cut from the TOPOTA plasmid by using *HindIII, XhoI* restriction enzymes. Event specific GM bt cotton MON-531 competitor (Esp MON-531) was cut from the TOPOTA plasmid by using *BamHI, XhoI* restriction enzymes. These two competitors were ligated with T4 ligase enzyme and amplified by PCR and PCR purified product was ligated into PCR2.1 vector (Invitrogen).The cells of E.coli strain TOPO10 (Invitrogen) was transformed with the plasmid. The plasmid was extracted and purified with the QIAGEN plasmid mini kit.

Real time PCR conditions and calculation of GM contents: 20 µl reaction solution for PCR contained 1X universal SYBR green master mix (Applied Biosystems). Plasmid DNA (1 µl) was used as a template, 0.5µm of each primer. The real- time PCR was performed with 7500 real time PCR system (Applied Biosystems), with the following step cycle programme: 90˚C for 10 min,40 cycles consisting of denaturation at 95˚C for 0.5 min and annealing and extension at 60˚C for 1 min.

For the analytical calibration, a dilution series of the plasmid (having two competitors) was done to five different concentrations, *i.e*, 203000, 20300, 2030, 203.0, 20.30, per 1 µl based on the concentration of plasmid DNA. Calibration curves were prepared for *fsACP* (cotton endogenous gene) and ESP MON-531 (GM cotton) using the plasmid DNA (containing both competitors) as a template, which contains the different concentration of reference molecules 203000, 20300, 2030, 203.0, 20.30, per 1 µl. The GM content of the samples were calculated from the proportion of the copy number of the GM-specific segment to that of the taxonomic marker for cotton by the following formula:

Cf (conversion factor) = copy number of recombinant DNA sequence in the DNA from GM sample/copy number of Taxon-specific sequence in the DNA extracted from GM seed -formula (1)

GM content (%) = Cf X100 -formula (2)

The Real time PCR for the quantification of the copy number of the GM-specific segment and that of the taxon specific segment were performed. The ratios of the GM-specific sequence to the taxon specific sequence in the DNA extracted from the GM seeds are defined as conversion factor (Cf). For comparison we analyzed the same sample (plasmid having two competitors) in real time PCR by using *fsACP*, ESP MON 531 specific primer sets.

QC- PCR: The genomic DNA from the prepared test samples (10%, 5%,1%) were co amplified with the competitor plasmid in a single PCR reaction tube with respective primer sets (*fsACP*,ESP MON-531).Each reaction mix contained 100ng of sample DNA,1µl of competitor having different copy

number,2µl reaction buffer,0.2mM dNTPs,1.5mM Mgcl2,0.5U Taq polymerase (Fermantas) and 0.5mM each primer pair for a final volume 20µl.

All PCR analyses were performed on DNA Engine, thermal cycler (Bio-Rad laboratories). Thermal cycler conditions were as follows: pre incubation at 95º for 5 min, 35 cycles consisting of ds DNA denaturation at 95º for 30s, primer annealing at 57º for 50 s, primer extension at 72º for 50s and final elongation at 72º for 5 min.

Analysis of PCR products:
After the PCR amplification products were analyzed using agarose gel electrophoresis. The gel was prepared with 3% agarose (SIGMA, Germany). In that 2% high resolution (SIGMA, Germany) and 1% normal agarose was mixed, in 1x Tris acetate EDTA (TAE) buffer with 0.5 mg/ml of ethidium bromide (Etbr). The running conditions were constant voltage 150V for 1 hour in 1x TAE buffer.

Analysis of Amplified Fragments*:* To examine the applicability of QC-PCR, analytical software such as Alpha Imager was used to determine the relative band intensities of QC-PCR products. The copy number of competitor plasmid and target should be same at the equivalence point as shown in **Figure (5-10)**. The decimal logarithm of the copy number of QC plasmid was plotted on x-axis, and that of the intensity ratio of QC plasmid to target was plotted on y-axis. The intersection between the calibration curve and the y-axis was defined to be the equivalence point, and the copy number of target was determined. The copy number of unknown sample is determined, and the Cf value was calculated by using the formula (1). The GM content in a sample was calculated using an obtained Cf value with the formula (2).

RESULTS AND DISCUSSION

In this present study, we developed a simple and efficient QC PCR method for the detection and Quantification of GM bt cotton MON531.Two primer pairs were used for the amplification of taxon specific sequence of cotton *fsACP* and ESP MON531. Competitors were prepared for taxon specific sequence (*fsACP*), and event specific sequence (MON531).These two competitors were cloned into single TOPOTA plasmid and used in QC PCR for detection of different GM percentages.

Construction of Calibrant plasmid and its performance in real time PCR:
We have developed QC-PCR method for the detection and Quantification of GM bt cotton MON 531, plasmid DNA containing tandomly ligated GM specific DNA segment (ESP MON531) and taxon-specific DNA segment was used as analytical calibrator. The constructed calibrated plasmid had two inserts having 188 bp of taxon specific DNA segment and 306 bp of event specific DNA segment.

Real- time PCR and QC-PCR was performed with the same plasmids diluted to a concentration ranging from 203000, 20300, 2030, 203.0, 20.30, per 1 µl. **Figure.1** shows the amplification plots of the real time PCR.
Amplification plots and standard curve for fsACP are shown in **figure(1-2)**. Amplification was performed using real-time PCR with a 7500 real-time PCR system.

Specificity of the primers for competitors
The primer pair (*fs ACP*) was designed by primer-3 (version 0.4.0) and primer pair (ESP 531) was taken from literature *(7).*The primer pairs used in this study were mentioned in the table.1.The efficiency of the primers in amplifing competitors were checked by conventional PCR by using plasmid DNA (having competitors) as a template.The results showed that each primer set was able to amplify respective competitors *i.e., fsACP*, ESP 531. PCR with each primer pair *fsACP*, ESP 531 produced a single specific amplicon of expected size i.e. 188 bp for *fsACP* competitor and 306 bp for ESP 531 competitor (**figure 3**).

Validation of QC-PCR for the detection of different GM percentages:
In QC-PCR competitor and genomic DNA were coamplified by using taxon specific primer (*fsACP*) and event specific primer (ESP 531).Competitor and genomic DNA had same primer binding sites, so competitor and genomic DNA could be amplified in the same reaction tube.The PCR conditions were standardised to get the amplification of both competitor and genomic DNA segments. As expected, two bands corresponding to taxon speciifc (*fsACP*) gene product and event specific gene product (ESP 531) were simultaneouly amplified for competitor and genomic DNA segments. After running the gel two bands were observed corresponding to the competitor and genomic DNA segments, based upon their size difference. These results suggest that QC-PCR is effective for the specific detection and quantification of GM bt cotton MON 531.
QC-PCR method was used for specific detection and quantification of GM cotton.To get this, we developed a simple and cost effective QC-PCR method for detection and quantification of insect resistant cotton variety MON 531. competitors for taxon specific sequence (*fsACP*) and event specific sequence MON 531 were designed and PCR conditions were standardised. The expected amplicon size for taxon specific QC-PCR is 188 bp & 210 bp and for event specific QC-PCR is 306 bp & 346 bp.(**figure5-10**)

Limit of detection:
Sensitivity assay was carried out to determine the limit of detection (LOD) of GM bt cotton MON 531 event. Different levels of DNA mixture was prepared with GM bt cotton event MON 531 and non-GM cotton using serial dilution of 100ng/µl DNA sample of GM cotton (100%) with 100ng/µl of non-GM DNA (100%) with different percentages i.e. 100%,10%,5%,1%. The GMO copy number of cotton was calculated by dividing the copy number of GM by the copy number of the taxon specific gene (*fsACP*) multiplied with 100 to get percentage value. By this assay, we could detect upto 1% of GM bt cotton MON 531.
QC-PCR for the detection of 10% GM bt MON-531 :

For 10% GM bt cotton MON 531 analysis, 150ng of genomic DNA was added to the decreasing number of copy number (203000, 20300, 2030, 203.0, 20.30) of plasmid DNA and PCR was carried out. For fsACP amplification 1µl of plasmid DNA was added to the constant amount (150ng) of genomic DNA. For event specific QC-PCR, 1µl of plasmid DNA was added to the constant amount of (150ng) genomic DNA and PCR was carried out. Ampliffication was checked on 3% agarose gel and equivalent point was calculated and expected result was observed as shown in **figure 5-6.**

QC-PCR for the detection of 5% GM bt MON-531 :

For 5% GM bt cotton MON 531 analysis 150ng of genomic DNA was added to the decreasing number of copy number (203000, 20300, 2030, 203.0, 20.30) of plasmid DNA and PCR was carried out. For *fsACP* amplification 1µl of plasmid DNA was added to the constant amount (150ng) of genomic DNA. For event specific QC-PCR, 1µl of plasmid DNA was added to the constant amount of (150ng) genomic DNA and PCR was carried out. Ampliffication was checked on 3% agarose gel and equivalent point was calculated and expected result was observed as shown in **Figure 7-8.** In event specific QC-PCR, hetero duplex formation was observed because if genomic DNA amount exceeds 100 ng, heteroduplex formation is observed during the later cycles of a 30-cycles program *(14).*

QC-PCR for the detection of 1% GM bt MON-531 :

For 1% GM bt cotton MON 531 analysis, 150ng of genomic DNA was added to the decreasing number of copy number(203000, 20300, 2030, 203.0, 20.30) of plasmid DNA and PCR was carried out. For *fsACP* amplification 2µl of plasmid DNA was added to the constant amount (150ng) of genomic DNA. For event specific QC-PCR, 1µl of plasmid DNA was added to the constant amount of (150ng) genomic DNA and PCR was carried out. Ampliffication was checked on 3% agarose gel and equivalent point was calculated and expected result was observed as shown in **figure 9-10.** *fsACP* and ESP MON 531 QC PCR was performed at a time, negative controle was not shown in **figure 9** but shown in **figure 10**.

Table 1: Details of primers used for this study

Gene	Gene bank accession No.	Primer	Primer sequence 5'-3'	Expected size (bp)	Source
fsACP	U48777.1	fsACP	F TGTGTTGGGACTTGAGGAA R GTTCACACATGATTTCCCCC	210	Our laboratory data
Event specific Mon531		ESP 531	F AAGAGAAACCCCAATCATAAAA R GAGAATGCGGTAAAGATACGTC	346	Ref no.7

Table 2: Details of Enzymes used for this study

S.No	Name of the Competitor	Enzymes used	Competitor size
1.	*fsACP* Competitor	*SfaNI,Cac8I*	188bp
2.	ESP MON531 Competitor	*SfaNI,AluI*	306bp

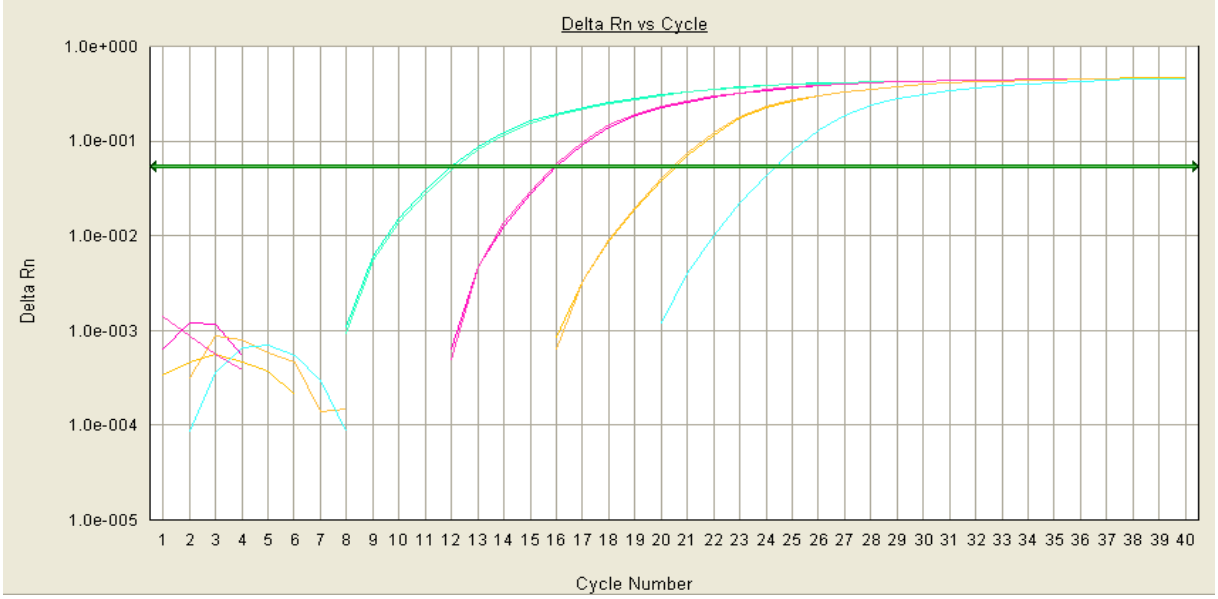

Figure 1. Amplification plot for dilution series of plasmid

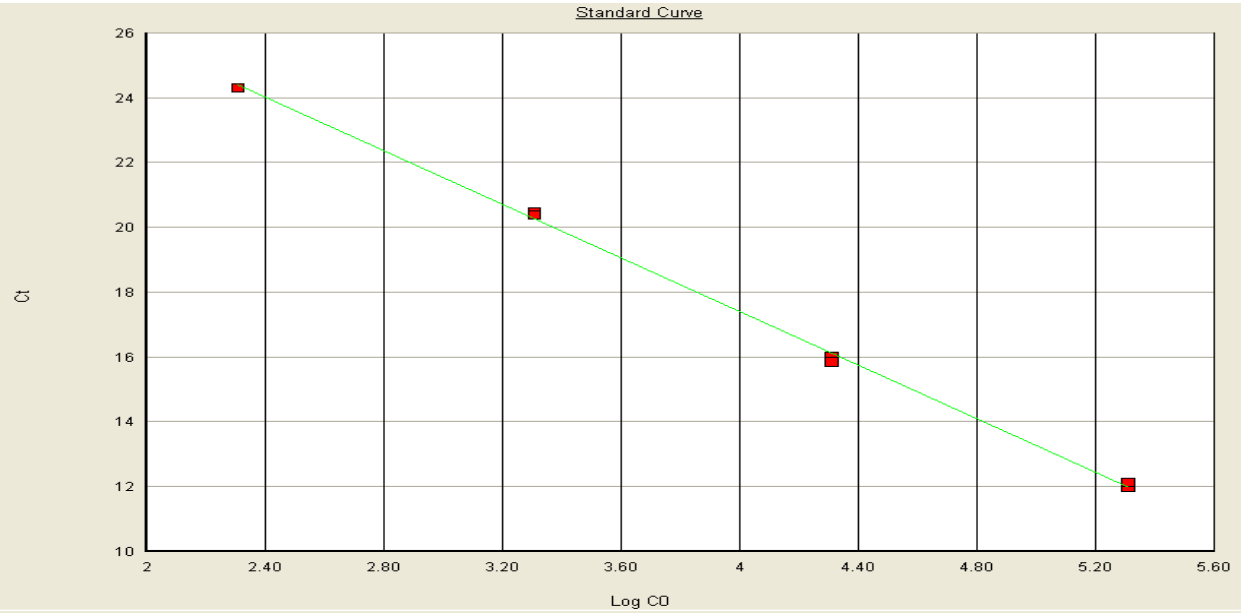

Figure 2. Standard curve for the above amplification plot

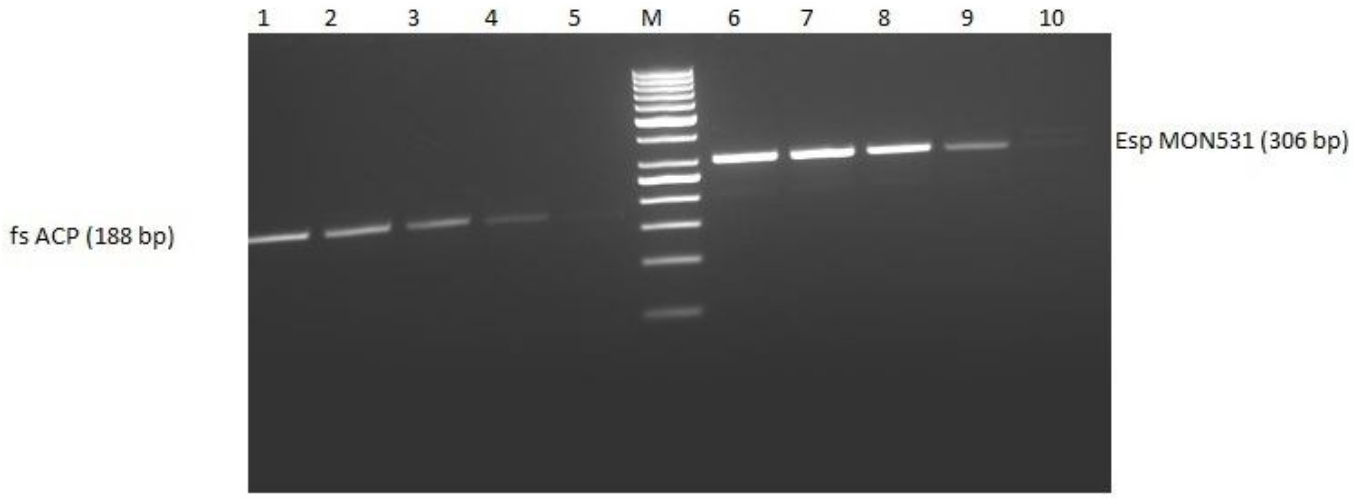

Figure 3. PCR assay for testing of primer specificity for competitors. Lane 1-5, fs ACP competitor in different dilutions, Lane M,50 bp ladder, Lane 6-10, ESP 531 competitor of different dilutions.

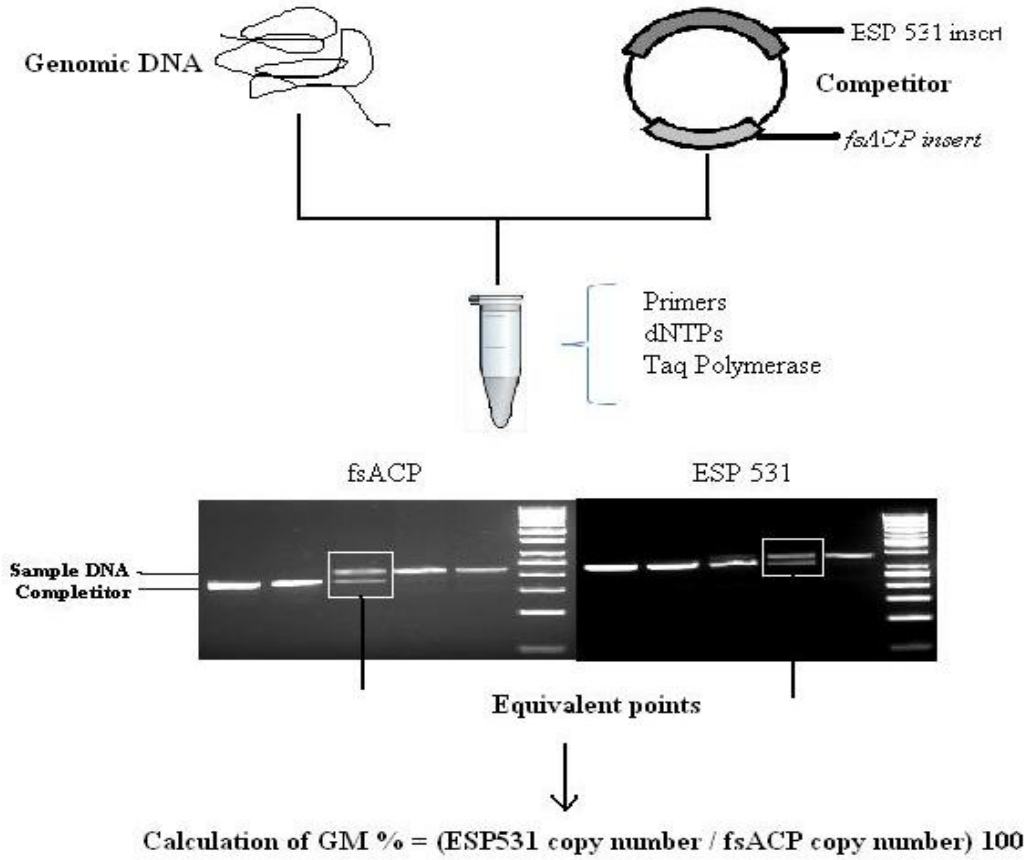

Figure 4. Principle of GMO quantificationm by QC-PCR method. Sample DNA and competitor plasmid were co-amplified in the same reaction tube. The amount of DNA was constant in all reaction tubes. After PCR, the amplification products were separated by agarose gel electrophoresis on which the amplified competitor could be distinguished from the amplified target gene by size. Equivalent point was identified and GM percentage in the sample was calculated.

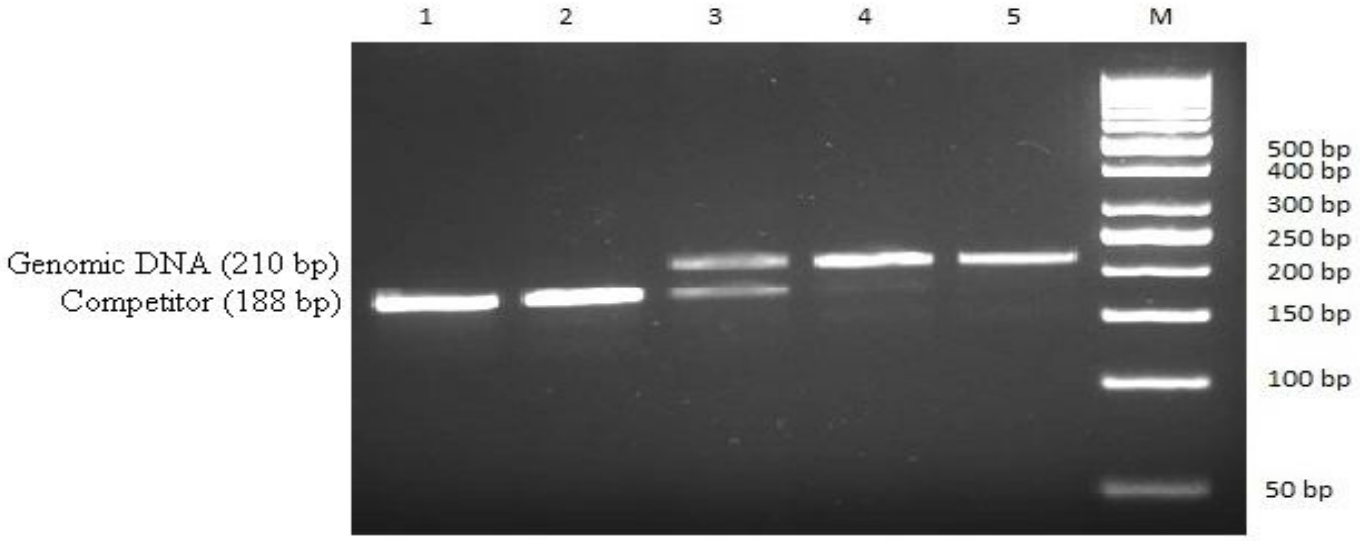

Figure 5. Detection of 10% GM bt cotton MON 531 event by QC-PCR method. Lane 1-5, QC-PCR for *fsACP* with different copy numbers of plasmid and same amount of genomic DNA, Lane 3 is the equivalence point for *fsACP* competitor and genomic DNA, Lane M, 50 bp ladder.

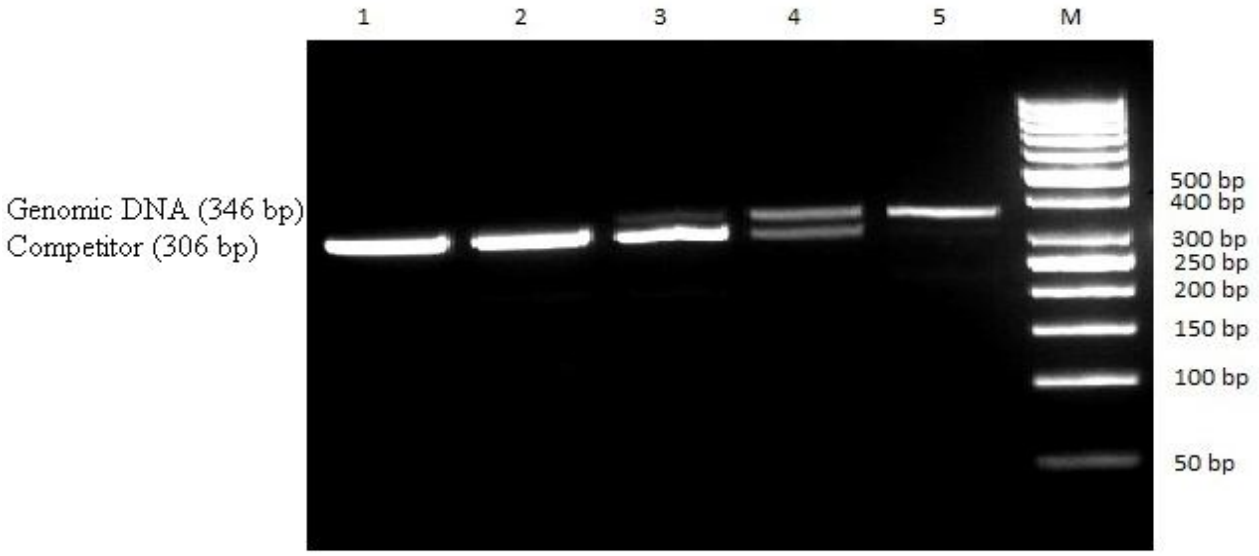

Figure 6: Detection of 10% GM bt cotton MON 531 event by QC-PCR method. Lane 1-5, QC-PCR for ESP 531 with different copy numbers of plasmid and same amount of genomic DNA, Lane 4 is the equivalence point for ESP 531 competitor and genomic DNA. Lane M, 50 bp ladder.

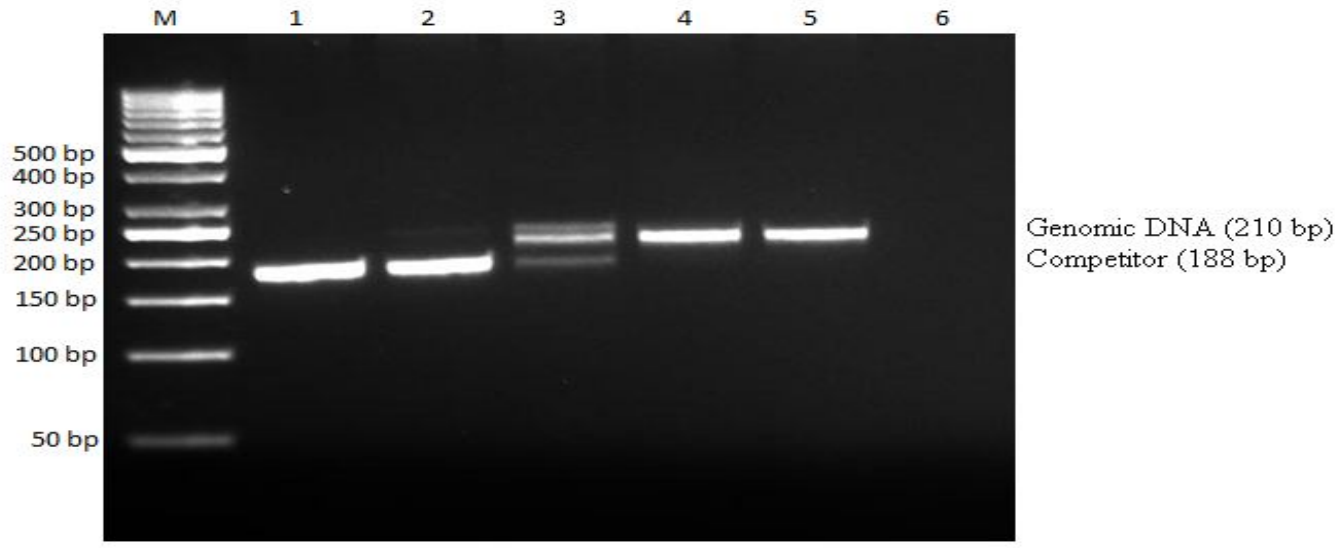

Figure 7. Detection of 5% GM bt cotton MON 531 event by QC-PCR method. Lane 1, 50 bp Ladder, Lane 1-5, QC-PCR for fs ACP with different copy numbers of plasmid and same amount of genomic DNA, Lane 6, Negetive control (without template). Lane 3 is the equivalence point for *fsACP* competitor and genomic DNA.

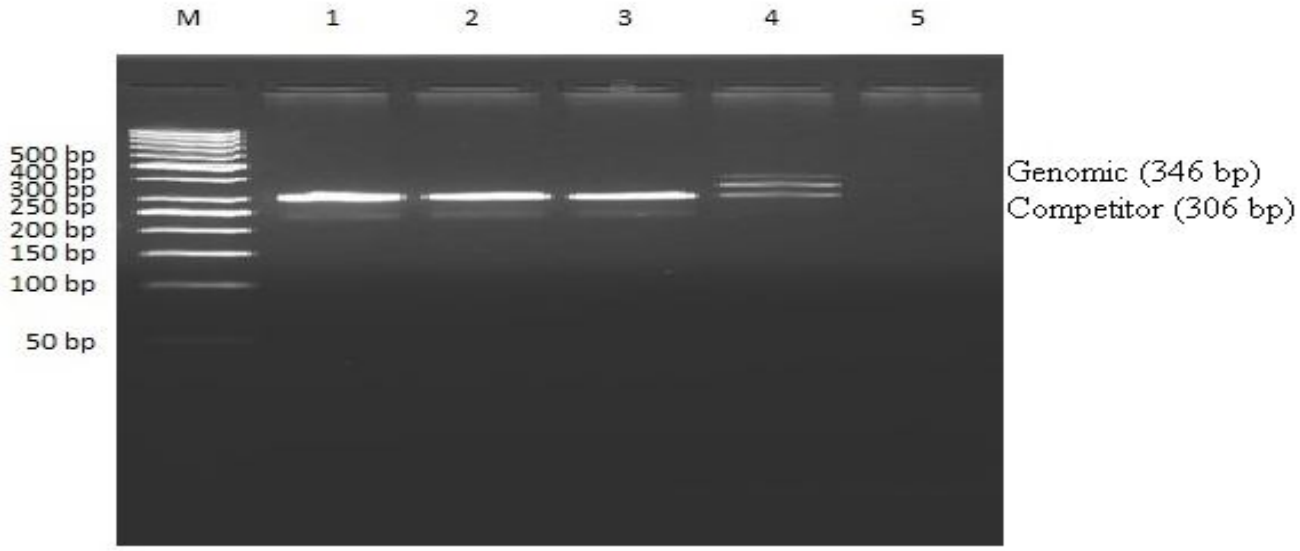

Figure 8: Detection of 5% GM bt cotton MON 531 event by QC-PCR method. Lane M, 50 bp ladder, Lane 1-4, QC-PCR for ESP 531 with different copy numbers of plasmid and same amount of genomic DNA, Lane 4 is the equivalence point for ESP 531 competitor and genomic DNA.

Lane 5, negative control.

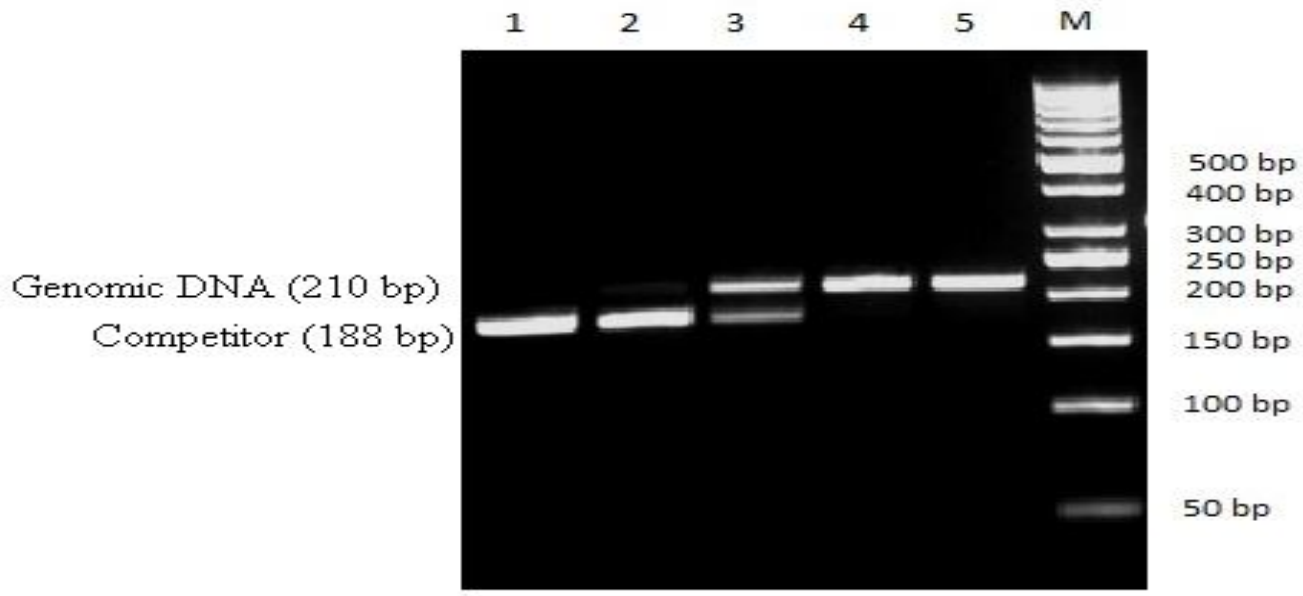

Figure 9. Detection of 1% GM bt cotton MON 531 event by QC-PCR method. Lane 1-5,QC-PCR for *fsACP* with different copy numbers of plasmid and same amount of genomic DNA, Lane 3 is the equivalence point for *fsACP* competitor and genomic DNA, Lane M 50 bp ladder.

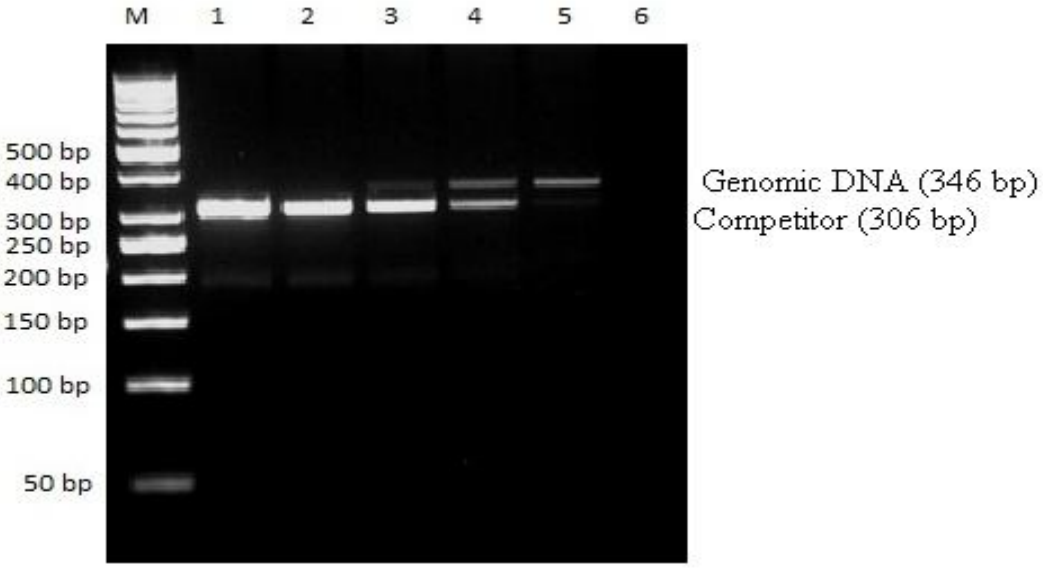

Figure 10. Detection of 1% GM bt cotton MON 531 event by QC-PCR method. Lane M, 50 bp ladder, Lane 1-5, QC-PCR for ESP 531 with different copy numbers of plasmid and same amount of genomic DNA, Lane 4 is the equivalence point for ESP 531 competitor and genomic DNA, Lane 6, negative control.

Applicability of QC-PCR PCR method for quantification of GM bt cotton MON 531:

QC-PCR method has already been developed to quantify GM bt crop soya bean *(15).* The QC-PCR is the best method of for the detection and quantification of GM food crops. In this study same set of primers are used for the amplification of both competitors and genomic DNA, to quantify GM bt cotton MON 531. This method could be used to quantify different percentage mixing of GMO products. This approach is simple and cost effective method for GMO quantification.

CONCLUSION

In the present study competitors were designed for both taxon specific sequence and event specific sequence and used in QC-PCR for the quantification of GM bt cotton MON 531.By using this method we could detect up to 1% of GM cotton MON 531.This method is simple, cost effective and time saving method. This method can be used as a alternative method for the real time PCR for quantification and certification of GMO.

Abbreviations used

GMO, genetically modified organisms; bt cotton, genetically modified cotton contains transgenes derived from *Bacillus thuringiensis*; LOD, limit of detection; *fsACP*, cotton endogenous reference gene codes for fiber specific acyl carrier protein; ESP 531, event specific primer pair, which targets the junction sequence between the transgene cassette of MON 531 event and host genome of the plant; QC-PCR, Quantitative Competitive Polymerase Chain Reaction.

ACKNOWLEDGEMENT

We thank the Director, Centre for DNA Fingerprinting and Diagnostics for the support. We are grateful to Dr.J.Nagaraju, Head of Laboratory of Molecular Genetics & co-investigator of GMO project for his encouragement and support. We also thank the Department of Biotechnology, Government of India for financial support. We are grateful to Monsanto Company, Hyderabad for providing GM cotton seeds and leaf samples.

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