

## Construction of a Standard Reference Plasmid for Detecting GM Cottonseed Meal

Qingfeng Guan · Xiumin Wang · Da Teng · Yalin Yang ·  
Fang Tian · Qingqiang Yin · Jianhua Wang

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**Abstract** A plasmid was constructed for quantification of genetically modified (GM) cottonseed meal in the gene-specific level. The *Cry1Ab/c* gene was connected with the *Sad1* gene by fusion PCR. The fusion gene was cloned into the pMD®19-T Simple Vector. The plasmid DNA was then digested with a restriction endonuclease *Sma*I to reduce the characteristic differences between the plasmid DNA and genomic DNA. For a rough quantitative analysis of GM cotton meal contents, a rapid method for measurement of the copy numbers of the transgenic *Cry* and cotton endogenous *Sad1* gene using a real-time PCR system with the plasmid DNA as a calibrator was established. The inter-run and intra-run coefficients of variation were less than 1.48% and 2.36%, respectively. The limits of detection and quantitation of the *Cry* and *Sad1* genes were 9 and 91 copies of pMDCS, respectively. These results prove that the standard plasmid represents a valuable alternative to genomic DNA as a certified reference material for the quantification of GM cotton and is a useful tool to establish a feasible identification management for GM cottonseed meal content in the feed industry.

**Keywords** Bt cottonseed meal · Standard plasmid · Construction · Real-time PCR

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Qingfeng Guan and Xiumin Wang contributed equally to this paper.

Q. Guan · X. Wang · D. Teng · Y. Yang · F. Tian · J. Wang  
Key Laboratory of Feed Biotechnology, Ministry of Agriculture, Beijing 100081,  
People's Republic of China

Q. Guan · Q. Yin  
College of Animal Science and Veterinary Medicine, Henan Agricultural University,  
Zhengzhou 450002, People's Republic of China

X. Wang · D. Teng · Y. Yang · J. Wang (✉)  
Gene Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences,  
12 Zhongguancun Nandajie St. Haidian District, Beijing 100081, People's Republic of China  
e-mail: jhwangfribio@yahoo.com.cn

## Abbreviations

CRMs	Certified reference materials
CV	Coefficients of variation
GMO	Genetically modified organism
LODs	Limits of detection
LOQs	Limits of quantitation

## Introduction

The cultivation areas of genetically modified (GM) crops, especially cotton, have increased in China over the past several years. Sixty-eight percent of China's cotton growing area was planted with Bt cotton in 2009 [1]. A total of 716 strains of Bt cotton, containing the *CryIAc* (GenBank accession no. Y09787) gene or fusion *CryIAb/c* (GenBank accession no. EU816953.1) gene of *CryIAb* (GenBank accession no. X54939) and *CryIAc*, were authorized in China during 2007–2009. Cottonseed meal is a high-protein by-product from the extraction of oil from whole cottonseed and usually used as a protein source for animal feed instead of soybean meal. Silva [2] reported that the replacement of soybean meal by cottonseed meal does not alter nutrient intake, nutrient digestibility, milk production, or milk composition in Girolando cows. The use of cottonseed meal in China is concentrated in feed for livestock, and the content of cottonseed meal in feed ranged from 10% to 50% [3].

Compared with traditional natural feed, GM feed needs more rigorous supervision and management. At present, the labeling policies regarding GM food and feed vary from country to country. Except the USA, Canada, Argentina, and Hong Kong [4], other countries or regions largely adopt a mandatory labeling policy and set up a corresponding identity threshold in GM food and feed. In order to guarantee the right to know of the consumers, it is necessary to detect and quantify the GM content of feed.

DNA-based PCR analysis is still the most widely used approach for GM organism (GMO) identification and quantification, and different types of external calibrants can be used for GMO quantification. A standard curve can be built on a basis of DNA extracted from certified reference materials (CRMs) with different GMO contents [5–8], called genomic DNA calibrants. These calibrants are made from homozygous GM seed milled into powder and mixed with non-GM seed powder. With this technique, the percentage of GMO content is generally calculated as a mass fraction. However, the production of CRMs is an extremely complex, time-consuming, and expensive process [9–12]. In October 2004, the European Commission recommended that the content of GM food and feed could be expressed as the percentage of GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes [13]. Malcolm Burns [14] compared plasmid and genomic DNA calibrants for the quantification of GM ingredients and found that plasmid calibrants gave equal or better performance characteristics in precision and closeness to the expected value than their genomic equivalents. In this way, the use of linearized plasmid DNA as a standard provides a cheaper and more flexible alternative to conventional reference material.

The current calibrants, including genomic DNA calibrants and plasmid DNA calibrants, are mainly based on an event-specific method. However, the method has a shortcoming in multispecies GMO detection. Due to hundreds of Bt cotton planted in China, it is hardly possible to quantify this cotton in the event-specific level in a short time. However, there

are only two exogenous genes said in Bt cotton, so the gene-specific method is applied as a simple way in Bt cotton detection.

In this paper, the tandem-marker plasmid containing the endogenous and the transgenic target which are present in a 1:1 ratio was constructed and amplified in GMO quantification.

## Materials and Methods

### Materials and DNA Extraction

Cottonseed meal samples were collected from Zhumadian Henan, Tacheng Xinjiang, Weixian, and Sanhe in Hebei, Qingdao and Weifang in Shandong, and Beijing. Wild cottonseed was provided by the Biotechnology Research Institute of the Chinese Academy of Agricultural Sciences. Non-transgenic maize was supplied by the Chinese Academy of Inspection and Quarantine.

The DNA was extracted from 0.1 g grinded cottonseed meal using a SDS method with some modifications [15–17]. The procedure is as follows: a 0.1-g sample was added into 1 mL 65°C preheated SDS lysis buffer (100 mmol L<sup>-1</sup> pH 4.8 NaAc, 50 mmol L<sup>-1</sup> pH 8.0 EDTANa<sub>2</sub>, 500 mmol L<sup>-1</sup> NaCl, 2% PVP, 1.4% SDS, pH 5.5) and incubated for 1 h at 65°C with occasional stirring. The suspension was added with 1 mL chloroform/isoamyl alcohol solution (24:1) and incubated for 10 min at room temperature, and was then centrifuged (10 min; 12,000×g). The upper phase was transferred to a new tube, mixed with one-third volume of KAc (2.5 mol L<sup>-1</sup>, pH 4.8) and incubated for 10 min at room temperature. After centrifugation (10 min; 12,000×g), the upper phase was mixed with 0.6 volume of -20°C pre-cooling isopropyl alcohol and incubated for 20 min at room temperature. The mixture was centrifuged (12,000×g, 10 min), and the pellet was washed with 2 mL of ethanol solution (70%, v/v) two times. The pellet was dried and dissolved in 100 µL ddH<sub>2</sub>O. The quality and quantity of the extracted DNA were evaluated using absorbance measurements at 260- and 280-nm wavelengths and further checked by 1% agarose gel electrophoresis. The plasmid DNA samples were isolated and purified using Plasmid Mini Extraction Kit (TianGen Co., Beijing).

### Primer Design

Primers of *Cry1Ab/c* and *Sad1* genes of *Gossypium hirsutum* were designed using Primer 5.0 software. The primer set of *Cry1Ab/c* F and *Sad1-Cry1Ab/c* 2R the *Cry1Ab/c* gene, and the other set of *Cry1A-Sad1* 2F and *Sad1* 2R for the *Sad1* gene. The primer pair of *Sad1-Cry1Ab/c* 2R and *Cry1Ab/c-Sad1* 2F is complementary and is called overlap primer. The restriction enzyme site of *Sma*I was added into the mid-site of the overlapping sequence. The fusion gene is expected to yield a product of 1,656 bp. Primers and TaqMan probes in real-time PCR were designed and synthesized by TaKaRa Biotechnology (Dalian, China). The sequences of all the primers and probes are listed in Table 1.

### Fusion PCR

The separate PCR amplification of the *Cry1Ab/c* and *Sad1* genes was carried out in a final volume of 25 µL, which contains 1× EasyTaq buffer, 200 µM dNTPs, 200 nM forward and

**Table 1** Nucleotide sequences of the primers and probes used in fusion PCR and real-time PCR

Gene name	Primer/probe name	Sequence (5'–3')	Product length (bp)
<i>Cry1Ab/c</i>	Cry1Ab/c F	GGCGAACGCATTGAAACCG	
	Cry1Ab/c 1R	AGATGATGTCAACTAGTCCGAGAAC	118
	Cry1Ab/cP	(FAM) CACCTGGCACGAACTCGCTG AGCA (Eclipse)	
	Sad1-Cry1Ab/c2R	TCAAGCCAAGAACATAGTCGTATCCC CCGGGGGATGTTGTGGAGCGGCGTTT	999
<i>Sad1</i>	Sad11F	GGTAGATCGAAGTGCTACAAAGAC	
	Sad11R	GATTTAAGTGTCCAAGTTCATCAC	161
	Sad1P	(HEX) CCCACGAAGCCCAATACACAAC CCA (Eclipse)	
	Sad12R	ACTTATGTCTCTACCGTCGTCTA	709
	Cry1Ab/c-Sad1 2F	AAACGCCGCTCCACAACATCCCCCGGGG GATACGACTATGTTCTTGGCTTGA	

reverse primers each, and 1  $\mu\text{L}$  extracted DNA. The PCR consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s; 60°C for 40 s; and 72°C for 1 min, with the final extension of 10 min. PCR products were excised from agarose gels and purified using TIANGel Midi Purification Kit (Beijing). In the second PCR, 1  $\mu\text{L}$  of each purified product from the above PCR was mixed in a final volume of 25  $\mu\text{L}$  PCR system without primers. The PCR included an initial denaturation at 94°C for 1 min, followed by 20 cycles of 94°C for 30 s, 60°C for 40 s, and 72°C for 2 min, with a final 10-min extension. In the third PCR, the forward primer of the *Cry1Ab/c* gene, the reverse primer of the *Sad1* gene, and the 1- $\mu\text{L}$  product from step 2 were added into the reaction system; PCR was implemented as follows: 94°C for 30 s, then 35 cycles of 94°C for 30 s, 58°C for 40 s, and 72°C for 2 min, followed by 72°C for 10 min. The PCR products were determined by gel electrophoresis and purified by TIANGel Midi Purification Kit.

### Construction of a Reference Molecule

The purified products were inserted into pMD®19-T Simple Vector and transformed into *Escherichia coli* Top10. Positive clones were confirmed by PCR. The recombinant plasmid was sequenced by Shanghai Sangon Biotech and then digested with *Sma*I. Ratio of OD 260:280 nm and concentration of total linearized plasmid DNA were determined. This concentration was used to calculate the number of copies of plasmid per microliter using the following formula when the size of the plasmid and the average molar mass ( $M$ ) of a base were given:

$$\text{Copy number concentration (cp } \mu\text{L}^{-1}) = (\text{DNA mass concentration (g } \mu\text{L}^{-1}) \times \text{NA (bp/mol)}) / (M (\text{g mol}^{-1}) \times 2 \times S (\text{bp cp}^{-1})).$$

Here, NA=Avogadro number,  $M$ =average molar mass of a nucleotide (expressed in grams per mole),  $S$ =size of the plasmid molecule (expressed in number of base pairs)

### Standard Curve

Five serially diluted plasmid DNAs were used as a calibrator for the preparation of standard curves. The standard curve was plotted for Ct values as the ordinate against the logarithm of DNA copy numbers.

### Qualitative PCR

To confirm the specific amplification of real-time primers, eight samples were analyzed by PCR amplification with the primers for real-time PCR.

### Real-time Quantitative PCR

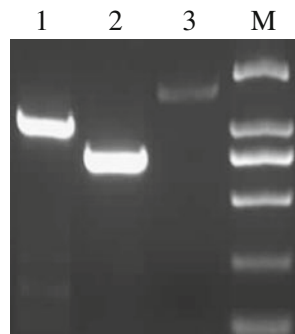
Real-time PCR was performed in a final volume of 15  $\mu\text{L}$  with 7.5  $\mu\text{L}$  of 2 $\times$  Master Mix (TaKaRa, Dalian, China), 250 nM of probe, 200 nM of forward primer and reverse primer of *Cry1Ab/c* or *Sad1* gene, and 75 ng of DNA template. PCRs were carried out as follows: 50°C, 2 min; 95°C, 10 min; followed by 40 cycles of 95°C, 15 s; 60°C, 1 min; fluorescence was collected at the annealing and extension step (60°C). All real-time PCRs were repeated three times and each time with triple replications. PCR was run on an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems), and the data were analyzed by 7500 software v 2.03. The copy numbers of *Cry1Ab/c* and *Sad1* in each sample were calculated from the Ct value of the sample using the standard curve. GMO content (percent) was measured using the ratio of the copy numbers of *Cry1Ab/c* and *Sad1*.

## Results and Discussion

### DNA Extraction

The total DNA was extracted successfully from cottonseed meal without serious degradation using the SDS-based method. The absorbance ratio of A260/280 was determined on a UV759 spectrophotometer (Shanghai Precision & Science Instrument Co. Ltd.) and ranged from 1.7 to 2.0, showing a high purity of DNA. Moreover, the total DNA yield was from 1.57 to 3.5  $\mu\text{g } \mu\text{L}^{-1}$ .

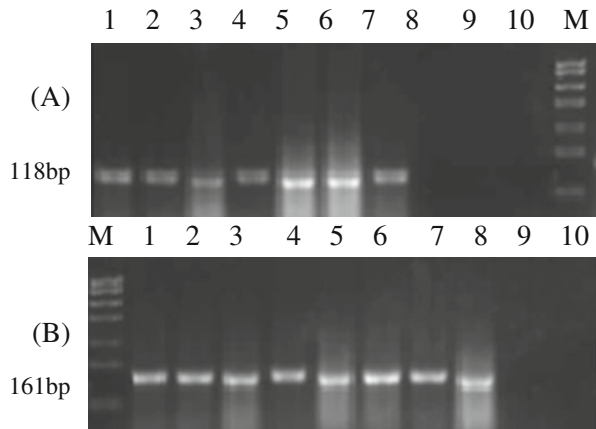
**Fig. 1** The fusion product of the *Cry1Ab/c-Sad1* gene. 1 *Cry1Ab/c* gene, 2 *Sad1* gene, 3 *Cry1Ab/c-Sad1* gene, M trans 2K marker (2,000; 1,000; 750; 500; 250; and 100 bp)



GTGCATCGGCAGTGATTAGAAGCTCGGTACGCGCGGATCTTCCAGAGATTGGCGAACGCA  
 TTGAAACCGGTTACACTCCCATCGACATCTCCTTGTCTTGACACAGTTTCTGCTCAGCG  
 Cry1Ab/c F  
 AGTTCGTGCCAGGTGCTGGGTTCGTTCTCGGACTAGTTGACATCATCTGGGGTATCTTTGG  
 Cry1Ab/c probe  
 TCCATCTCAATGGGATGCATTCTGGTGCAAATTGAGCAGTTGATCAACCAGAGGATCG  
 AAGAGTTTCGCCAGGAACCAGGCCATCTCTAGGTCGGAAGGATTGAGCAATCTCTACCA  
 AATCTATGCAGAGAGCTTCAGAGAGTGGGAAGCCGATCTACTAACCAGCTCTCCGC  
 GAGGAAATGCGTATTCAATTCAACGACATGAACAGCGCCTTGACCACAGCTATCCCATT  
 GTTCGCAGTCCAGAACTACCAAGTTCCTCTCTTGTCCGTGTACGTTCAAGCAGCTAATC  
 TTCACCTCAGCGTGCTTCGAGACGTTAGCGTGTTTGGGCAAAGGTGGGGATTTCGATGC  
 TGCAACCATCAATAGCCGTTACAACGACCTTACTAGGCTGATTGGAACTACACCGACC  
 ACGTGTTCGTTGGTACAACACTGGCTTGGAGCGTGTCTGGGGTCTGATTCTAGAGA  
 TTGGATTAGATACAACCAGTTCAGGAGAGAATTGACCCTCACAGTTTTGGACATTGTGT  
 CTCTCTTCCCGAACTATGACTCCAGAACCTACCCTATCCGTACAGTGTCCCAACTTACC  
 AGAGAAATCTATACTAACCAGTTCTTGAGAACTTCGACGGTAGCTTCCGTGGTTCTGC  
 CCAAGGTATCGAAGGCTCCATCAGGAGCCCACACTTGATGGACATCTTGAACAGCATA  
 ACTATCTACACCGATGCTCACAGAGGAGAGTATTACTGGTCTGGACACCAGATCATGGC  
 CTCTCCAGTTGGATTACGCGGGCCCGAGTTTACCTTCCTCTCTATGAAGTATGAAAACGC  
 CGCTCCACAACATCCCCCGGGGATACGACTATGTTCTTGGCTTGAATATCCTTGGCAAGA  
 Cry1Ab/c 2R  
 TTAATGCTCTATTGGTTCCTTTTGCTGGTGGCATATGTATCTTGGACACTTGTTAACGAC  
 AATGTGTTGTGCTGGTGGATCATGATGAGAAATGAGGAGCCAAGGTATTGTGACAAAT  
 CAACTAGCTAAAGATGTTTATTTTGGATAAAAACATTGACTTGGTAGATCGAAGTGCTAC  
 Sad1 2F  
 AAAGACCCCTTAGAAATGCTAGAGGTGAAAAAACCAATATAAAGCTTGTGTGAGTTAT  
 CAGTGAGGTTACCACTTATGAGAGAGGTGGGTTGTGTATTGGGCTTCGTGGGAAAAGTG  
 Sad1 probe  
 GTGATGCAAGTTGGACAGTTAAATCAAGTAAATGTTACAAGCAAGGTACATCTCAAACAC  
 Sad1 1R  
 TTTCTTAGTGTTCTACATTATGACTTACTGTTGTCTTGGAAAAACACATGAACCCCTTTT  
 TCAGTTCTGAAGTGGGTAAAGCTGAGGGGCTTACAAACCAAAGTTGGCATTAACTCA  
 AGGTTAACTCAGTGTTCAATATGGGCATGCCTAAGCCTATTTGTGTAAATCAAGGGGAT  
 CCGACCAAGGTAAAGTCACAATTGGGGCAAGTAAGAGCGGTGAGCTCTTCAATCGAGA  
 TGTTGAAAAGAGAGAAATGGTTTGAAGTATAGACGACGGTAGAGACATAAGTTGAGGCAAA  
 Sad1 2R  
 GGTTCGGAAGGAA

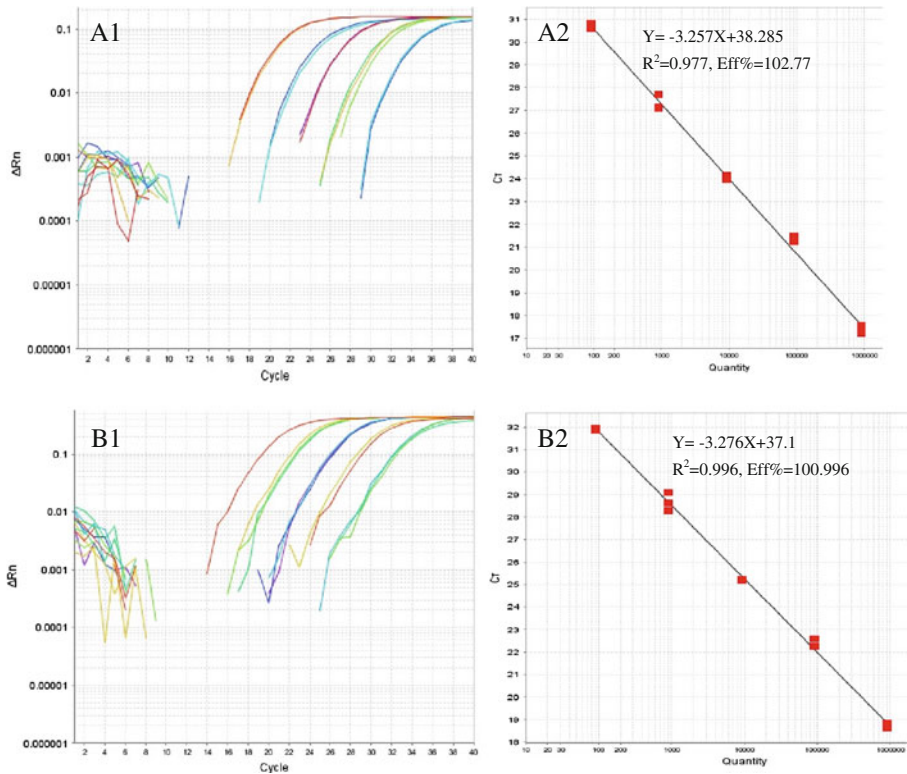
**Fig. 2** Nucleotide sequences of the integrated fragments (*Cry1Ab/c* gene and *Sad1* gene) in pMD19-T Simple Vector. *Cry1Ab/cF*, the forward primer of *Cry1Ab/c* gene for fusion PCR and real-time PCR; *Cry1Ab/c1R*, the reverse primer of *Cry1Ab/c* gene for real-time PCR; *Cry1Ab/c2R*, the reverse primer of *Cry1Ab/c* gene for fusion PCR; *Cry1Ab/c probe*, the probe of *Cry1Ab/c* gene for real-time PCR; *Sad12F*, the forward primer of *Sad1* gene for fusion PCR; *Sad12R*, the reverse primer of *Sad1* gene for fusion PCR; *Sad11F*, the forward primer of *Sad1* gene for real-time PCR; *Sad11R*, the reverse primer of *Sad1* gene for real-time PCR; *Sad1 probe*, the probe of *Sad1* gene for real-time PCR

**Fig. 3** The electrophoresis analysis of the PCR assay of cottonseed meal and maize from seven districts. 1 Weifang, 2 Zhumadian, 3 Tacheng, 4 Weixian, 5 Sanhe, 6 Beijing, 7 Qingdao, 8 wild cottonseed, 9 non-transgenic maize, 10 blank control, M marker I (700; 600; 500; 400; 300; 200; and 100 bp), (A) *Cry1Ab/c* gene, (B) *Sad1* gene



### Fusion of the *Cry1Ab/c* and *Sad1* Genes

The appropriate annealing temperature for the target gene was determined from 55.3°C to 61.2°C. In the first PCR step, a segment of 52-bp nucleotides including *Sma*I site was



**Fig. 4** Amplification plot and standard curves of the *Cry1Ab/c* and *Sad1* genes. A1: amplification plot of *Cry1Ab/c* gene; A2: standard curve of *Cry1Ab/c* gene; B1: amplification plot of *Sad1* gene; B2: standard curve of *Sad1* gene

**Table 2** Repeatability of the standard curve

Copy number	<i>CryIAb/c</i> gene			<i>SadI</i> gene		
	C <sub>T</sub> mean	C <sub>T</sub> SD	C <sub>T</sub> CV, %	C <sub>T</sub> mean	C <sub>T</sub> SD	C <sub>T</sub> CV, %
Test 1						
91.00	30.94	0.08	0.25	30.93	0.07	0.21
910.00	28.40	0.05	0.16	28.02	0.15	0.54
9,100.00	24.87	0.08	0.31	24.83	0.08	0.31
91,000.00	21.58	0.04	0.16	21.36	0.05	0.22
910,000.00	18.36	0.03	0.18	17.99	0.06	0.33
Test 2						
91.00	31.12	0.21	0.66	30.78	0.24	0.79
910.00	27.70	0.27	0.97	28.57	0.01	0.04
9,100.00	25.00	0.13	0.50	25.23	0.35	1.39
91,000.00	21.37	0.31	1.43	21.60	0.11	0.50
910,000.00	18.20	0.15	0.82	17.89	0.05	0.28
Test 3						
91.00	31.18	0.09	0.30	30.90	0.04	0.12
910.00	27.83	0.30	1.09	27.66	0.41	1.48
9,100.00	24.54	0.06	0.23	24.08	0.25	1.06
91,000.00	22.02	0.18	0.80	21.39	0.13	0.63
910,000.00	17.87	0.15	0.84	17.73	0.06	0.34

inserted between the *CryIAb/c* and *SadI* genes as an overlap primer. In the second PCR step, no primer was added. After denaturation, the overlap of the *CryIAb/c* and *SadI* genes was hybridized and extended to produce the recombinant fusion segment from both genes. In the third step, this fusion segment was amplified under both outer primers. Finally, DNA bands with the desired size as the fused segment of 1656 bp (*CryIAb/c-SadI*, lane 3) and their donor segment 999 bp (*CryIAb/c*, lane 1) and 709 bp (*SadI*, lane 2) were observed with the naked eye (Fig. 1). The product of *CryIAb/c-SadI* is of significant yield (1.55 µg/50 µL PCR reaction) and purity (>95%) without side products, which suggested that the primers for the target gene had high specificity.

**Table 3** Repeatability in different batches

Copy number	<i>CryIAb/c</i> gene							<i>SadI</i> gene				
	C <sub>T</sub> test 1	C <sub>T</sub> test 2	C <sub>T</sub> test 3	C <sub>T</sub> mean	C <sub>T</sub> SD	C <sub>T</sub> CV, %	C <sub>T</sub> test 1	C <sub>T</sub> test 2	C <sub>T</sub> test 3	C <sub>T</sub> mean	C <sub>T</sub> SD	C <sub>T</sub> CV, %
91.00	30.94	31.12	31.18	31.08	0.12	0.40	30.93	30.78	30.90	30.87	0.08	0.26
910.00	28.40	27.70	27.83	27.98	0.37	1.32	28.02	28.57	27.66	28.08	0.46	1.62
9,100.00	24.87	25.00	24.54	24.80	0.23	0.94	24.83	25.23	24.08	24.71	0.58	2.36
91,000.00	21.58	21.37	22.02	21.65	0.33	1.53	21.36	21.60	21.39	21.45	0.13	0.61
910,000.00	18.36	18.20	17.87	18.15	0.25	1.37	17.99	17.89	17.73	17.87	0.13	0.74



**Table 4** Quantitative results of the unknown samples using the established system

Cottonseed meal	Zhu madian	Weixian	Tacheng	Qingdao	Beijing	Sanhe	Weifang
<i>Cry1Ab/c</i> copy number mean	1,194.8	2,792.1	247.9	755.2	2,604.8	2,881.9	2,631.5
<i>Sad1</i> copy number mean	4,540.2	11,714.7	11,562.5	3,885.3	8,820.42	20,977.96	21,455.7
Copy number ratio of <i>Cry1Ab/c</i> and <i>Sad1</i> gene (%)	26.32	23.83	2.14	19.44	29.53	13.74	22.97

### Identification of Recombinant Plasmid

Clones containing the insert were screened by PCR amplification with the outer primers. The positive plasmid pMDCS was extracted and sequenced. The sequence of the inserted DNA was shown in Fig. 2. A tenfold dilution series of the linearized plasmid pMDCS was prepared ranging from 910,000 to 91 copies  $\mu\text{L}^{-1}$  for further establishment of the standard curve.

### Specificity of Primers for Real-time PCR

In order to verify the validity of the primers for real-time PCR, nine samples from different regions were detected by qualitative PCR. Seven samples except non-transgenic maize and wild cottonseed showed an expected band of 161 bp-amplicon for *Sad1* and a single 118 bp-band for *Cry* demonstrating the high specificity of the both primer pairs for real-time PCR (Fig.3).

### Establishment of Standard Curve

The serially diluted pMDCS samples (910,000, 91,000, 9,100, 910 and 91 copies per reaction) were used to build the standard curves for *Cry* and *Sad1* gene. The real-time PCR results showed that the PCR reaction efficiencies were 1.03 and 1.01 for the standard curves of *Cry* and *Sad1* gene in GM cotton ( $R^2 > 0.977$ ) (Fig. 4). High reaction efficiencies and linearities between copy numbers and fluorescence values demonstrated that the standard curves on the basis of the plasmid pMDCS were suitable for further quantitative measurements. A similar result was reported by Caliendo et al [18]. The limits of detection and quantitation of the *Cry* and *Sad1* genes were 9 and 91 copies of pMDCS, respectively.

### Repeatability in Inter- and Intra-run Tests

To validate the repeatability of real-time PCR systems based on pMDCS, five plasmid DNA dilutions from 91 to 910,000 copies per reaction were employed. Each test was performed three times with triplicate reactions each time. The result was analyzed by 7500 V2.03. Tables 2 and 3 show the repeatability of the five plasmid levels in replicates of three for each of the three batches. The inter- and intra-run CVs were less than 1.48% and 2.36%, respectively. The inter-run and intra-run SD values were ranged from 0.01 to 0.41 and from 0.08 to 0.58, respectively. These results indicate that the *Cry* target gene should be detectable with a good accuracy by the method established in this study.

## Determination of Copy Number Percentage of GM Cotton Samples

A summary of the results obtained for determination of percentage GM cotton is shown in Table 4. The mean percentage of GM component of seven samples (from Zhumadian, Weixian, Tacheng, Qingdao, Beijing, Sanhe, and Weifang, China) were 26.32%, 23.83%, 2.14%, 19.44%, 29.53%, 13.74%, and 22.97%, respectively. At present, hundreds of Bt cotton varieties are authorized and have been planted in China. Cottonseed meal in feedstuff as commercial goods from the market is probably a mixture of different varieties of GM Bt cotton. Therefore, it is difficult to quantify the Bt cotton in an event-specific level [19, 20] and track the accurate origin of Bt cottonseed meal especially from Henan, Shandong, Hebei, and Xinjiang provinces as the main areas of cotton. In comparison, the copy number percentage of the exogenous gene of Bt cotton (*CryIAb/c*) and the endogenous gene (*Sad1*) may better reflect the GMO content in cotton meal. Therefore, this assay for the copy number percentage of the target GM gene provides a feasible approach in practice for labeling policies regarding GM feed.

## Conclusion

In this study, a novel reference molecule pMDCS and its quantitative PCR assay for GM Bt cotton were developed. The reference molecule containing the tandem fragments of *CryIAb/c* and *Sad1* genes could be used as calibrator for the identification and quantification of GM Bt cotton by measurement of the *CryIAb/c* and *Sad1* copy numbers.

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