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ARTICLE

Development and In-House Validation of the Event-Specific Polymerase Chain Reaction Detection Methods for Genetically Modified Soybean MON89788 Based on the Cloned Integration Flanking Sequence

Jia Liu,[†] Jinchao Guo,[†] Haibo Zhang,[†] Ning Li,[‡] Litao Yang,*[,†] and Dabing Zhang*[,†]

[†]GMO Detection Laboratory, SJTU-Bor Luh Food Safety Center, School of Life Science and Biotechnology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, People's Republic of China, and [‡]Development Center for Science and Technology, Ministry of Agriculture, Building 18, The Maizidian Street, Beijing 100026, China

Various polymerase chain reaction (PCR) methods were developed for the execution of genetically modified organism (GMO) labeling policies, of which an event-specific PCR detection method based on the flanking sequence of exogenous integration is the primary trend in GMO detection due to its high specificity. In this study, the 5′ and 3′ flanking sequences of the exogenous integration of MON89788 soybean were revealed by thermal asymmetric interlaced PCR. The event-specific PCR primers and TaqMan probe were designed based upon the revealed 5′ flanking sequence, and the qualitative and quantitative PCR assays were established employing these designed primers and probes. In qualitative PCR, the limit of detection (LOD) was about 0.01 ng of genomic DNA corresponding to 10 copies of haploid soybean genomic DNA. In the quantitative PCR assay, the LOD was as low as two haploid genome copies, and the limit of quantification was five haploid genome copies. Furthermore, the developed PCR methods were in-house validated by five researchers, and the validated results indicated that the developed event-specific PCR methods can be used for identification and quantification of MON89788 soybean and its derivates.

KEYWORDS: MON89788; event-specific; qualitative and quantitative PCR; in-house validation

INTRODUCTION

Since the first genetically modified (GM) tomato "FLAVR SAVR" was commercialized in the United States in 1994, modern biotechniques have been widely developed and applied in agriculture, and more than 150 GM plant events have been developed and commercialized in the past two decades (*1*). To strengthen the regulation of genetically modified organisms (GMOs), more than 50 countries and areas have published a series of laws and rules for GMO regulation and labeling. For instance, the labeling threshold is defined as 0.9% in the European Union (*2*), 3% in Korea (*3*), 5% in Japan (*4*), and zero in China (*5*).

For implementing the issued GMO labeling regulations, molecular analytical techniques have been developed and used for GMO detection, such as protein-based (enzyme-linked immunosorbent assay and lateral flow strip) and nucleic acid-based detection methods [qualitative and quantitative polymerase chain reaction (PCR)]. To date, the PCR technique has been the primary method for GMO detection because of its high sensitivity and specificity. On the basis of the different kinds of target DNA fragments of exogenous integration, four PCR detection systems were developed for GMO identification and quantification based on the four different kinds of target DNA fragments of exogenous integration, respectively, such as screen-, gene-, construct-, and event-specific DNA fragments. The event-specific PCR method has been the primary trend for GMO identification and quantification because of its high specificity based on the flanking sequence (*6*, *7*).

Soybean is a very important oil crop and food, and it is planted worldwide, especially in America, Brazil, and China. To improve the output and decrease the herbicide dosage in soybean plants, one GM soybean event (GTS 40-3-2) with the glyphosate-tolerant trait has been developed and commercialized worldwide. On the basis of the use and benefits from GTS 40-3-2 soybean planting, the second generation glyphosate-tolerant soybean, MON89788, was developed by the Monsanto Co. to make the control of weeds in soybean plants possible. In MON89788, a single copy of the *CP4 EPSPS* gene, which expresses glyphosate-tolerant protein, was integrated at a single site in the soybean genome (*8*). To date, several event-specific detection methods of GTS 40-3-2 have been published (*9*−*13*). However, no paper of GM soybean MON89788 detection has been reported.

In this study, the 5′ and 3′ integration flanking sequences of MON89788 soybean were revealed by thermal asymmetric interlaced (TAIL)-PCR, and the quantitative and qualitative analysis

*To whom correspondence should be addressed. Tel: +86 21 34205073. Fax: +86 21 34204869. E-mail: yylltt@sjtu.edu.cn (L.Y.) or zhangdb@sjtu.edu.cn (D.Z.).

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Table 1. Primers and TaqMan Probes for PCR Analyses

PCR system	name	sequence (5′−3′)	amplicon length (bp)	ref
TAIL-PCR	FMV-1R	GTCGTCACTGCGTTCGTCATAC		this work
	FMV-2R	TGCCCACTAACTTTAAGTCTTCG		this work
	FMV-3R	GTTGAGGCTTTGGACTGAGAAT		this work
	AD2	NGTCGASWGANAWGAA		*15*
	T9-1F	TATGGTCCTTTTGTTCATTCTC		this work
	T9-2F	TGACCGAAGTTAATATGAGGAG		this work
	T9-3F	AAAAGCTGCAAATGTTACTGAA		this work
qualitative PCR	lectin-1F	GCCCTCTACTCCACCCCCATCC	117	*16*
	lectin-2R	GCCCATCTGCAAGCCTTTTTGTG		
	M1F	TTCCTGCTCCACTCTTCCTT	205	this work
	M2R	TTGAGGCTTTGGACTGAGAA		
real-time PCR	lectin-3F	AACCGGTAGCGTTGCCAG	80	*17*
	lectin-4R	AGCCCATCTGCAAGCCTTT		
	lectin-p	TTCGCCGCTTCCTTCAACTTCACCT		
	M-3F	CGTTACTGCTGCCCCACAAA	145	this work
	M-4R	TTGTCGTTTCCCGCCTTCAG		
	Mp	FAM-CCTCGAAACTTGTTCCTGCTCCACTCTTCCT-BHQ1		

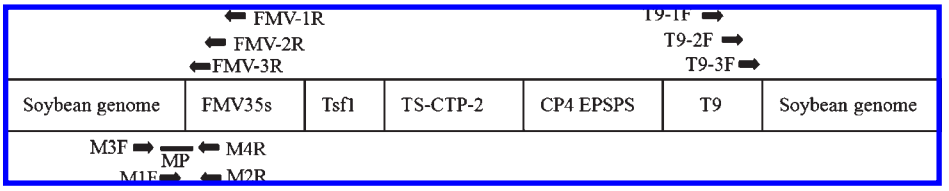

Figure 1. Schematic diagram of the integrated heterologous DNA in GM soybean MON89788.

methods were established based on 5′ integration flanking sequences. Furthermore, the developed methods were in-house validated by five researchers, suggesting that these developed methods are applicable in GMO detection.

MATERIALS AND METHODS

Plant Materials. GM soybean (MON89788 and GTS 40-3-2), GM cotton MON1445, GM maize MON863, and GM canola GT73 were developed and supplied by Monsanto Co. GM tomato Huafan no. 1 seeds were developed and supplied by HZAU. Nontransgenic soybean was purchased from local market in Shanghai, China.

DNA Extraction and Purification. Plant genomic DNA was extracted and purified using a Plant DNA Mini-prep Kit (Shanghai Ruifeng Agrotech Co., Ltd., Shanghai, China) according to the manufacturer's manual. The quantity and quality of DNA in the samples were measured and evaluated according to the absorbance measurements at 260 nm wavelength and 1% agarose gel electrophoresis, and its copy number was calculated from the quantity of DNA and soybean genomic DNA average size (*14*).

Oligonucleotide Primers and Probes. The primers and probes for the qualitative and quantitative PCR assays for MON89788 were designed using Primer Express software version 3.0 (Applied Biosystems, Foster City, CA). The random primer AD2 suitable for plant genome reported by Liu et al. was used for the flanking sequence cloning in TAIL-PCR (*15*). The target-specific primers (FMV-1F/2F/3F and T9-1F/2F/3F) for TAIL-PCR were designed on the basis of the FMV35S promoter and T9 terminator sequences, respectively. The primer pair M1F/2R was designed based on the obtained 5′ integration flanking sequence and used for qualitative detection of MON89788 soybean. The primer pair M3F/4R and TaqMan probe Mp were designed and employed for the quantification of MON89788. The soybean *Lectin* gene was selected as an endogenous reference gene, and its primers (lectin-1F/2R and lectin-3F/4R) and TaqMan probe lectin-p were used in this study (*16*, *17*). All of the primers and probes were synthesized by Invitrogen Co., Ltd. (Shanghai, China) and are listed in **Table 1**.

Determination of the Exogenous Integration Flanking Sequence. The basic molecular character of exogenous integration of MON89788 soybean has been reported, and a single copy of T-DNA, containing the *cp4-EPSPS* gene, was integrated at a single site in MON89788 without any rearrangement (as shown in **Figure 1**) (*8*). The 5′ and 3′ flanking sequences of the MON89788 exogenous insertion were determined using the TAIL-PCR method (*15*), which is comprised of three sequential PCR reactions. The first TAIL-PCR was performed in a total volume of 50 μL containing 1 × PCR buffer with $MgCl_2$ (TaKaRa Biotechnology Co., Ltd., Dalian, China), 400 μM dNTPs, 0.4 μM primer FMV-1F for 5′ or T9-1F for 3′ flanking sequence, 4 μM primer AD2, 2.5 units of *TaKaRa Ex Taq* HS DNA polymerase (TaKaRa Biotechnology Co., Ltd.), and 50000 copies of MON89788 soybean genomic DNA. The PCR program for the primary TAIL-PCR consisted of a denaturation step of 1 min at 94 °C followed by 1 min at 98 °C and the following amplification cycles: 5 cycles of 30 s at 94 °C, 1 min at 60 °C, and 2 min at 72 °C; 1 cycle of 30 s at 94 °C, 3 min at 25 °C, and 2 min at 72 °C; 15 cycles of 30 s at 94 °C, 1 min at 60 °C, 2 min at 72 °C, 30 s at 94 °C, 1 min at 65 °C, 2 min at 72 °C, 30 s at 94 °C, 1 min at 44 °C, and 2 min at 72 °C; and a final step of 10 min at 72 °C. Secondary TAIL-PCR amplification was carried out in a total volume of 50 μL containing 1 × PCR buffer with $MgCl_2$, 400 μM dNTPs, 0.4 μM primer FMV-2F for 5′ or T9-2F for 3′ flanking sequence, 4 μM primer AD2, 2.5 units of *TaKaRa Ex Taq* HS DNA polymerase, and 1 μL of primary PCR products. The secondary TAIL-PCR program was 15 cycles of 30 s at 94 °C, 1 min at 60 °C, 2 min at 72 °C, 30 s at 94 °C, 1 min at 60 °C, 2 min at 72 °C, 30 s at 94 °C, 1 min at 44 °C, 2 min at 72 °C, and a final step of 10 min at 72 °C. Tertiary TAIL-PCR amplification was carried out in a total volume of 50 μL containing 1 × PCR buffer with $MgCl_2$, 400 μM dNTPs, 0.4 μM primer FMV-3F for 5′ or T9-3F for 3′ flanking sequence, 4 μM primer AD2, 2.5 units of *TaKaRa Ex Taq* HS DNA polymerase, and 1 μL of secondary PCR products. The tertiary TAIL-PCR program was 15 cycles of 30 s at 94 °C, 1 min at 60 °C, 2 min at 72 °C, 30 s at 94 °C, 1 min at 60 °C, 2 min at 72 °C, 30 s at 94 °C, 1 min at 44 °C, 2 min at 72 °C, and the last step of 10 min at 72 °C. All PCR reactions were carried out in PTC-100 thermocycler (MJ Research, Waltham, MA).

The second and tertiary PCR amplification products were analyzed by 1.5% agarose gel electrophoresis with GelRed staining, and the amplified fragments with similar sizes in these two PCR amplifications were purified with an AxyPrep DNA Gel Extraction Kit (Axygen Scientific, Inc., CA) and ligated into pMD18-T vector (TaKaRa Biotechnology Co., Ltd.). Sequencing analysis of the cloned DNA was performed using the ABI PRISM 3730 Genetic Analyzer by Shanghai Invitrogen Co., Ltd. Parallel

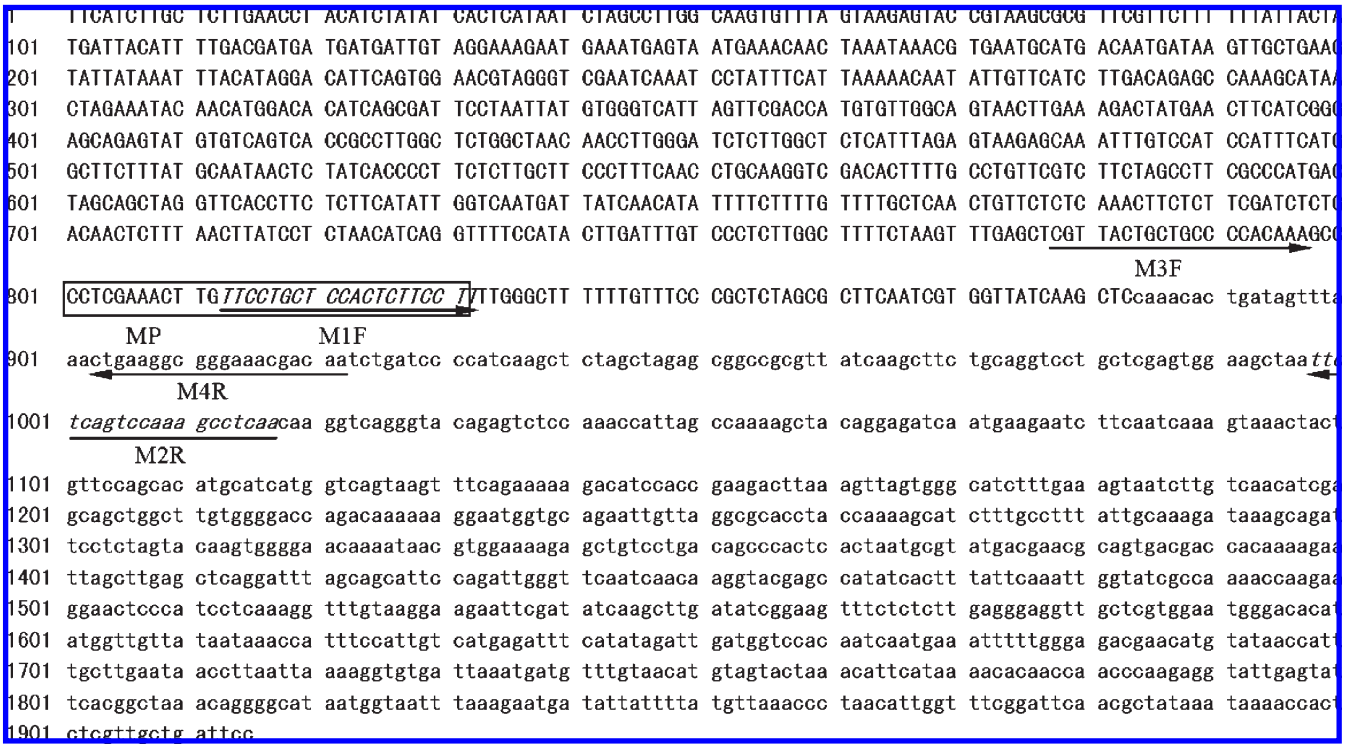

Figure 2. Revealed sequence between the 5′ exogenous integration and the soybean genome of MON89788 soybean. Capital letters represent the flanking soybean genomic sequence, and lowercase letters show the sequence of partial FMV35S promoter and the T-DNA left region. Primers and probe used for qualitative and quantitative PCR amplification in **Table 1** are underlined and boxed.

amplifications with wild-type isogenic DNA were carried out to identify MON89788-specific PCR products.

Qualitative PCR Assay. In qualitative PCR assay, all reactions were carried out in 25 μL volume reactions, which included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, a 0.2 mM concentration of each dNTP, a 0.2 μM concentration of each primer, 1.25 units of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd.), and 2 μL of plant genomic DNA as templates. The PCR was performed in a PTC-100 Thermocycler (MJ Research) with the following program: 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C, and the final step of 7 min at 72 °C. PCR-amplified products were electrophoresed in 2% agarose gels for approximately 30 min at 100 V and stained with GelRed for visualization. Each reaction of one test was repeated three times, and each time with triple parallels.

TaqMan Real-Time PCR Assays. Real-time PCR assays were carried out in a fluorometric thermal cycler Rotor-Gene 3000A (Corbett Research, Australia) with a final volume of 25 μL. The real-time PCR contained the following ingredients: 1 × PCR buffer, 100 μM each of dATP, dGTP, and dCTP, and dUTP, 320 nM primers, 100 nM TaqMan probes, 1.5 U of *Taq* DNA polymerase, 6.5 mM $MgCl_2$, and 5 μL of template DNA samples. Real-time PCR reactions were carried out with the following procedures: 10 min at 94 °C, 45 cycles of 15 s at 94 °C, and 1 min at 60 °C. The fluorescent signal was monitored during every PCR cycle at the annealing step. Data were analyzed with Rotor gene 3000 software version 6.0 (Corbett Research). All of the PCR reagents were purchased from Biocolor Co. (Shanghai, China) except for primers and probes.

Construction of Standard Curves. After optimization of magnesium and primer/probe concentrations, a series of MON89788 soybean genomic DNA dilutions were used for quantification, reproducibility and repeatability tests, and determination of the limits of detection and quantification (LODs and LOQs). Genomic DNA isolated from powdered 100% MON89788 soybean was serially diluted with 0.1 × TE buffer to final concentrations equivalent to 10000, 1000, 100, 10, and 1 copies haploid genome/μL considering 1.155 pg per haploid genome in the case of soybean (*14*). In each reaction, 5 μL of diluted DNA samples was added, and all reactions were repeated three times and each time with triple replicates for each template DNA.

In-House Validation of the Established Qualitative and Quantitative PCR Detection Methods of MON89788 Soybean. When the qualitative and quantitative PCR detection methods of MON89788 soybean were established, we invited five researchers to confirm the developed conventional and real-time PCR methods by means of in-house validation. In the validation of conventional PCR method, the LOD and specificity were tested employing series of diluted MON89788 DNA solutions with different concentrations and different plant species genomic DNA solutions, respectively. In the validation of real-time PCR method, the PCR efficiency and standard curves were validated using a series of diluted MON89788 DNA solutions with different concentrations (50000, 5000, 500, 50, and 5 copy haploid genomes/reaction), and the accuracy of quantification was evaluated employing three practical soybean samples (S1, S2, and S3) with known GM contents (5, 3, and 1%). In the in-house validation, each participant was requested to repeat each reaction three times and each time with three parallels.

RESULTS AND DISCUSSION

Cloning and Determination of the 5′ and 3′ Integration Flanking Sequence of MON89788 Soybean. The combination of the arbitrary primer AD2 and target-specific primer FMV-3F amplified a specific fragment about 2000 bp in size, and another specific DNA fragment about 1500 bp in size was obtained using the primer pair T9-3F/AD2 in genomic DNA from MON89788 soybean. Using the genomic DNA from nontransgenic soybean as the template, no amplified fragment was observed in the parallel experiments. The result of sequence and subsequent analysis showed that one 1915 bp DNA fragment encompassing the 5′ junction region was obtained in TAIL-PCR. The 1915 bp DNA sequence consisted of two parts, one was a 1032 bp sequence of FMV promoter and exogenous T-DNA left border region, and the other was an 883 bp sequence of soybean genome sequence (as shown in **Figure 2**). The sequencing result of the 1500 bp fragment showed that one 1568 bp DNA fragment encompassing the 3′ junction region was obtained, which consisted of one 525 bp sequence of T9 terminator and exogenous T-DNA

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1     ACGGCCAGTG CCAAGCTTGC ATGCCTGCAG GTCGACGATT TGACCGAAGT TAATATGAGG AGTAAAACAC TTGTAGTTGT ACCATTATGC TTATTCACTA
101   GGCAACAAAT ATATTTTCAG ACCTAGAAAA GCTGCAAATG TTACTGAATA CAAGTATGTC CTCTTGTGTT TTAGACATTT ATGAACTTTC CTTTATGTAA
201   TTTTCCAGAA TCCTTGTCAG ATTCTAATCA TTGCTTTATA ATTATAGTTA TACTCATGGA TTTGTAGTTG AGTATGAAAA TATTTTTTAA TGCATTTTAT
301   GACTTGCCAA TTGATTGACA ACATGCATCA ATCGACCTGC AGCCACTCGA AGCGGCCGCA TCGATCGTGA AGTTTCTCAT CTAAGCCCCC ATTTGGACGT
401   GAACGTAGAC ACGTCGAAAT AAAGATTTCC GAATTAGAAT AATTTGTTTA TTGCTTTCGC CTATAAATAC GACGGATCGT AATTTGTCGT TTTATCAAAA
501   TGTACTTTCA TTTTATAATA ACGCTcagac tctagtgact accaccttca ctctcctcaa gcatttcagc ctcttccccg ctcagactcc ttagctttgg
601   gaaccaaatt atcccttacg ttctcgactt caaccatatg tgatagctgc ctatgatacc atggctactt ccccttagtt ctttatcttt cctttccgct
701   ttattccatg ccttaccgat cctctgaagt gtctttgcat tagcttcatt gaaacctcac gcgatgaaag gtgtgatggt ctcctccgat ggcgcacttc
801   tcatagggta acctaattgt cttacgacca acataggatt ataattaata caacccctcg tccctataaa agggacattt ggaaatcctt cacataagca
901   taacactcct acccctcttt ctttccactg tgggaaccaa ctaatggacg ctcctatcat gcctgccaag agttcttccc aatttgcctc gtcctttcct
1001  gagcacatgc gatgaccttg tatggggtag acagatctac tttcatgatt gaagacgtgg gataccaacc acacataaag agcaggcgca caacagaaaa
1101  tcctcgtagt gctcttcttg catcttaagt caaatgtatc atacacttat gctaaaacaa caatgatcgg gctttccttg ctatggtgat aagcaaggaa
1201  agcatcgatt gctactagat ccgccaactc gtctacattc gaaaatagta ctatcccaaa cactagcagt gctaatacgt cgatgaatga tgcccactct
1301  ccttggctgg ccagagtttc cgccttctcc tccaatcact tccttggtat tccccctacc ctattcctac tttgcttcac tcagtctaat tctcatttcc
1401  agatcttgac aactcctgct attctcgcca tagaaggata gtacccagaa aaaaggtatg gcttccttcc tcctatcggg catcctgaga tcccttcgaa
1501  ctcctcatat taacttcggt caaatctcta gaggatcccc gggtaccgag ctcgaattcg taatcatg
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Figure 3. Revealed sequence between the 3′ exogenous integration and the soybean genome of MON89788 soybean. Capital letters represent the sequence of partial T9 terminator and the T-DNA right region, and lowercase letters show the flanking soybean genomic sequence.

right border region and one 1043 bp sequence of soybean genome sequence (**Figure 3**).

Event-Specific Qualitative PCR Assay of MON89788. As expected, in the established qualitative PCR assay, only one single 205 bp DNA fragment from primers M1F/2R was obtained using the MON89788 soybean DNA as template, and no fragment was detected in GM soybean GTS 40-3-2, GM canola GT73, GM cotton MON1445, GM maize MON863, and no template control (NTC) (**Figure 4A**). The target fragment of the *Lectin* gene (117 bp in size) was detected in both two GM soybeans (MON89788 and GTS 40-3-2) and nontransgenic soybean except for other GM plant events and NTC (**Figure 4B**). These data confirmed that the obtained 205 bp DNA fragment was located on the MON89788 event-specific region between the 5′ flanking sequence and soybean genomic DNA.

For one ideal qualitative PCR assay, a high LOD is very important and necessary, especially for the PCR assays of GMO detection. To test the LOD of the established event-specific PCR assay, the MON89788 soybean genomic DNA was diluted using 0.1 × TE with various concentrations of 100000, 10000, 1000, 100, and 10 copy haploid genomes/reaction The test results showed that the lowest tested level was 10 copy haploid genomes/reaction, which approximates to 10 haploid genome copies according to the genome sizes of soybean (**Figure 5**). The results indicate that the established qualitative event-specific PCR detection assay of MON89788 soybean is suitable for the practical detection of GM soybean samples.

Event-Specific Real-Time PCR Assay of MON89788. *Construction of Standard Curves.* After optimization of magnesium and primer/probe concentrations, the quantitative standard curves of the MON89788 event-specific real-time PCR assay were constructed employing the diluted MON89788 genomic DNA samples (50000, 5000, 500, 50, and 5 copy haploid genomes/reaction). The similar high PCR reaction efficiencies of the lectin assay and event-specific assay (0.99 of event-specific PCR assay and 1.00 of *Lectin*) indicated that GM contents could be calculated using these two PCR assays by means of the relative quantitative method. The real-time PCR-amplified results showed that the square regression coefficients (R^2) were both 0.997 for the *Lectin* and the event-specific PCR assay, respectively. The good linearity between DNA quantities and fluorescence values (Ct) indicates that these assays are suitable for quantitative measurements.

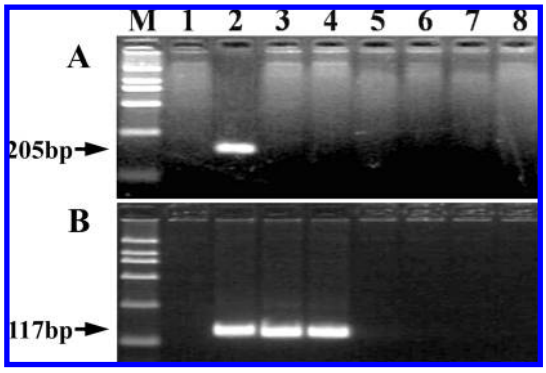

Figure 4. Two percent agarose gel electrophoresis of PCR products amplified with the MON89788 event-specific primers M1F/2R and *Lectin* gene primers lectin-1F/2R: (**A**) MON89788 event-specific PCR-amplified results. (**B**) *Lectin*-specific PCR-amplified results. Lanes: 1, NTC (no template control); 2−8, amplification of MON89788 soybean, GTS 40-3-2, non-GM soybean, GT73 canola, MON1445 cotton, MON863 maize, and Huafan no. 1 tomato, respectively; and M, DL2000 DNA marker.

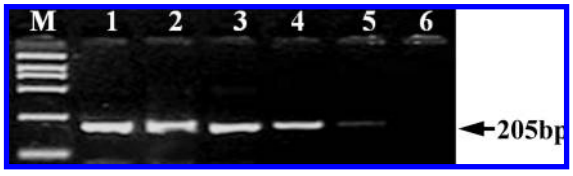

Figure 5. Sensitivity test of MON89788 event-specific assay. PCR products were amplified from MON89788 soybean DNAs with different concentration. Lanes: 1−5, amplification of GM soybean DNAs of 50000, 5000, 500, 50, and 5 copies/μL, respectively; 6, NTC; and M, DL2000 DNA marker.

Repeatability and Reproducibility of the PCR Assays. Repeatability and reproducibility of MON89788 real-time PCR assay were determined and calculated using the described MON89788 genomic DNA dilutions. The standard deviation (SD_r) and relative standard deviation (RSD_r) of repeatability were calculated according to the nine Ct values from three parallels and three replications; the SD_R and RSD_R of reproducibility were calculated from the mean Ct values of each replication. In the test of repeatability and reproducibility of MON89788 real-time PCR assay, the SD_r values ranged from

Table 2. Repeatability and Reproducibility of MON89788 Event-Specific PCR Assays

amount of DNA (copies/reaction)	Ct values 1	2	3	mean	SD_r	SD_R	RSD_r(%)	RSD_R (%)
50000	25.25	25.21	25.19	25.22	0.03	0.29	0.12	1.14
5000	28.83	28.69	28.83	28.78	0.08	0.22	0.28	0.75
500	31.91	31.99	32.04	31.98	0.07	0.25	0.21	0.79
50	35.56	35.39	35.62	35.52	0.12	0.30	0.34	0.86
5	39.26	39.07	38.81	39.05	0.23	0.32	0.58	0.82

Table 3. Amplification Data Used To Determine the Absolute LOD and LOQ

amount of DNA (copies/reaction)	signal rate (positive signals)	mean	SD	RSD (%)
50000	9/9	25.22	0.08	0.31
5000	9/9	28.83	0.06	0.20
500	9/9	31.95	0.08	0.25
50	9/9	35.50	0.14	0.39
5	9/9	39.07	0.22	0.56
2	5/9	ND	ND	ND

0.03 to 0.23, the RSD_r values ranged from 0.12 to 0.58%, the SD_R values ranged from 0.22 to 0.32, and the RSD_R values ranged from 0.75 to 1.14% (**Table 2**). The results of the repeatability and reproducibility tests indicate that the MON89788 event-specific quantitative PCR assay is reliable in GM MON89788 soybean quantification.

LODs and LOQs. In real-time PCR, the LOD and LOQ are the most important parameters, referring to the lowest quantity of the target that can be reliably detected and quantified with a probability of ≥95% (*10*, *18*). The absolute limit is the lowest number of initial template copies that can be detected and quantified. The relative limit refers to the lowest percentage of GMO relative to the species that can be detected and quantified, and the practical limit is the functional limit of the sample during the practical analysis. To determine the LOD and LOQ of the established event-specific real-time PCR assay, a series of MON89788 genomic DNA dilutions (50000, 5000, 500, 50, 5, and 2 copies/reaction) were prepared and tested in three parallel reactions each time and repeated three replicated times (**Table 3**). As expected, the ability to detect MON89788 soybean decreased with decreasing genomic DNA copy numbers, and two copies of MON89788 genomic DNA have only been detected five times in nine repeated reactions. The results indicated that the LOD value was about two copies. The data also showed that the SD values of the nine reactions with the same template concentration increased with decreasing copy number. To obtain reliable quantification results under ideal conditions, approximately five initial template copies were required, and we concluded that the LOQ of the event specific real-time PCR assay was five copies of haploid genome.

In-House Validation of the Qualitative PCR Method. The specificity of the qualitative PCR method was tested employing different plant species genomic DNA solutions. The specificity test results from five particpants showed that only one 205 bp DNA fragment was obtained from the amplification of MON89788 soybean DNA, and no fragment was detected in other GM plants, such as GTS 40-3-2 soybean, GT73 canola, MON1445 cotton, MON863 maize, and Huafan no. 1 tomato (**Table 4**). These data confirmed that the validated qualitative PCR assay of MON89788 was highly specific for MON89788 soybean.

In the LOD test, a series of diluted MON89788 DNA solutions with different concentrations (100000, 10000, 1000, 100, and 10 copies/reaction) were used, and the test results from five participants showed that the lowest tested level was 10 haploid genome copies (**Table 4**). These above results indicated that the established qualitative event-specific PCR method was suitable for the detection of MON89788 soybean and its derivates.

Table 4. Specificity and LOD Test of Qualitative PCR Method by Five Participants[a]

validation item	DNA template	participants 1	2	3	4	5
specificity test	NTC	−	−	−	−	−
	GTS 40-3-2	−	−	−	−	−
	non-GM	−	−	−	−	−
	GT73	−	−	−	−	−
	MON1445	−	−	−	−	−
	MON863	−	−	−	−	−
	Huafan no. 1	−	−	−	−	−
	MON89788	+	+	+	+	+
sensitivity test	10^5 copies/reaction	+	+	+	+	+
	10^4 copies/reaction	+	+	+	+	+
	10^3 copies/reaction	+	+	+	+	+
	10^2 copies/reaction	+	+	+	+	+
	10^1 copies/reaction	+	+	+	+	+

[a] "+" represents that the corresponding target band was amplified, and "−" represents that corresponding target band was not amplified.

Table 5. Values of Standard Curve Slope, PCR Efficiency, and Linearity (R^2)

participants	slope	PCR efficiency (%)	linearity (R^2)
1	−3.159	107	0.998
2	−3.361	98	0.999
3	−3.318	100	0.999
4	−3.337	99	0.998
5	−3.329	100	0.998

In-House Validation of the Quantitative Real-Time PCR Method. *PCR Efficiency and Linearity of Standard Curve*. Each of five participants was requested to construct standard curves using a series of diluted MON89788 DNA solutions with concentrations of 50000, 5000, 500, 50, and 5 copies/μL. The PCR efficiency and linearity of the constructed standard curves were employed to evaluate the quantitative real-time PCR assays. Those values obtained from each participant were presented in **Table 5**. The values of PCR efficiency ranged from 0.98 to 1.07. The linearity values of the regression were all above 0.998. Therefore, we could believe that the high PCR efficiency and good linearity of the standard curves indicate that this real-time PCR assay system is reliable and suitable for MON89788 genomic DNA quantification.

Blind Sample Quantification. To validate the applicability of MON89788 quantitative PCR assay in practical samples quantification, each participant received three blind soybean DNA samples (S1, S2, and S3) with the GM MON89788 contents of 5.0, 3.0, and 1% and quantified these three samples using the developed real-time PCR method. The mean values of three replicates for each sample as provided by all participants are shown in **Table 6**. The results of the in-house validation were statistically analyzed and are reported in **Table 7**. The mean

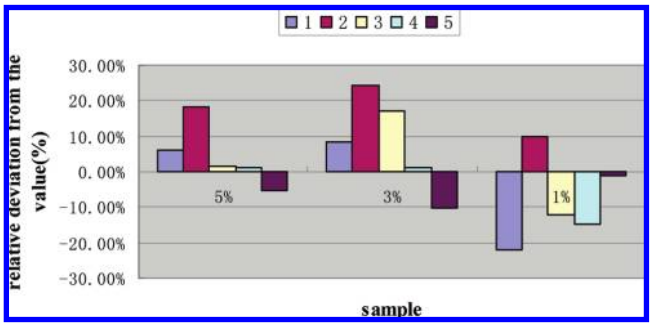

Figure 6. Relative deviation (%) from the true value of MON89788 for all participants. Five colors represent separate results obtained from five participants.

Table 6. Replicate's Mean Value of Three Blind Samples by Five Participants

	sample GM content (%)								
	5.0			5.0			1.0		
participants	REP1	REP2	REP3	REP1	REP2	REP3	REP1	REP2	REP3
1	5.58	5.01	5.42	3.09	3.04	3.62	0.80	0.83	0.70
2	4.84	4.71	4.51	2.79	3.25	3.10	1.01	0.89	0.99
3	4.45	4.39	4.45	2.95	2.96	3.56	0.81	0.79	0.92
4	4.57	4.51	4.60	2.82	2.78	2.88	0.81	0.90	0.84
5	5.36	5.58	5.28	3.47	3.44	2.70	1.09	1.21	1.37

Table 7. Quantitative Analysis of the Mixed MON89788 Soybean Samples

	expected value (GM %)		
unknown sample (GM %)	5.0	3.0	1.0
participants having returned results	5	5	5
sample per participant	3	3	3
no. of outliers			
mean value	4.88	3.10	0.93
repeatability relative standard deviation (%)	9.10	9.79	19.19
repeatability standard deviation	0.44	0.30	0.18
reproducibility relative standard deviation (%)	9.31	5.45	19.01
reproducibility standard deviation	0.46	0.17	0.18
bias (absolute value)	−0.12	0.10	−0.07
bias (%)	−2.40	3.33	−7.00

quantitative results of these three DNA sample (S1, S2, and S3) were 4.88, 3.10, and 0.93%, respectively. The average bias between quantified values and true values of blind samples ranged from 2.32 to 6.90%. The relative deviation from the true value for the three samples of each participant was calculated and is shown in **Figure 6**. As it can be observed, all of the average relative deviations at all of the GM levels are below the limit of the trueness acceptance level (bias ≤ 25%) (*19*). In in-house validation, the RSDs of repeatability ranged from 9.10 to 19.19%, and the RSDs of reproducibility ranged from 5.45 to 19.01%.

We should discuss the sequence information, as no sequencing information was related to the target sequence in JRC (Joint Research Center), while we provided the information, which is critical for GMO analysis and further confirmation. Therefore, this point is helpful for GMO analysis as well as the monitoring of food safety.

As compared to the previous reports by the JRC (*13*), in which the LOD and LOQ were 0.045 and 0.1% in 200 ng of DNA, and the LOD and LOQ were two and five haploid genome copies, respectively, in this study. These results showed that the developed method has better sensitivity than that of the JRC method, and this sensitivity is sufficient to quantify 0.01−100% MON89788 soybean in 100 ng of genome DNA. Concerning to the accuracy and precision of the reported data about soybean, maize, and corn samples (*20*−*22*), the average bias between quantified values and true values ranged from 0.60 to 24.68%. As compared to these above data with that of this study, the developed method in this study was more accurate and precise. All of these validated that the developed MON89788 real-time PCR assay is creditable and reliable for practical GM MON89788 soybean sample quantification.

Conclusion. In summary, we revealed the 5′ and 3′ flanking sequences of exogenous integration of MON89788 soybean and established the event-specific qualitative and quantitative PCR assays based on the 5′ integration flanking sequence. The LOD of qualitative PCR was 10 copies, and the LOD and LOQ of quantitative PCR assays were two and five copies, respectively. Furthermore, the developed qualitative and quantitative PCR methods were in-house validated by five researchers, and the results demonstrated that the developed event-specific qualitative and quantitative PCR assays for MON89788 soybean were suitable for the identification and quantification of GM MON89788 soybean and its derived products.

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