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Event-Specific Detection of Stacked Genetically Modified Maize Bt11 × GA21 by UP-M-PCR and Real-Time PCR

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More and more stacked GMOs have been developed for more improved functional properties and/or a stronger intended characteristic, such as antipest, improved product efficiency etc. Bt11 × GA21 is a new kind of stacked GM maize developed by Monsanto Company. Since there are no unique flanking sequences in stacked GMOs, up to now, no appropriate method has been reported to accurately detect them. In this passage, a novel universal primer multiplex PCR (UP-M-PCR) was developed and applied as a rapid screening method for the simultaneous detection of five target sequences (NOS, 35S, Bt11 event, GA21 event, and IVR) in maize Bt11 × GA21. This method overcame the disadvantages rooted deeply in conventional multiplex PCR such as complex manipulation, lower sensitivity, self-inhibition and amplification disparity resulting from different primers. What's more, it got a high specificity and had a detection limit of 0.1% (approximates to 38 haploid genome copies). Furthermore, real-time PCR combined with multivariate statistical analysis was used for accurate quantification of stacked GM maize Bt11 × GA21 in 100% GM maize mixture (Bt11 × GA21, Bt11 and GA21). Detection results showed that this method could accurately validate the content of Bt11, GA21 and Bt11 × GA21 in 100% GM mixture with a detection limit of 0.5% (approximates to 200 haploid genome copies) and a low relative standard deviation <5%. All the data proved that this method may be widely applied in event-specific detection of other stacked GMOs in GM-mixture.

KEYWORDS: Stacked GM maize Bt11 × GA21; event-specific detection; universal primer multiplex PCR; real-time PCR; multivariate statistical analysis

INTRODUCTION

With worldwide commercialization and the growth of genetically modified organisms (GMOs), transgenic crops are being developed with a trait of multiple genes for more and greater characteristics expected to solve problems like disease, weed management etc. According to the definition of OECD, a stacked GMO could be a retransformation of an existing transgenic line or hybridization between two or more GM transgenic lines (*1*). In this passage, plants obtained from hybridization of GM events are only taken into consideration when speaking of stacked GM events. United States has ratified more than twenty stacked GM crops such as corn and cotton, which were assayed as "new" GMOs for approval in EU (*2*). Molecular characterization as the unique identifier of every stacked GMO species is a main aspect of GMO risk assessment (*3*). For a single GM event, data are provided on the actually inserted sequences or flanking sequences, but more information is necessary for a stacked GMO.

Labeling regulations have been widely accepted to protect consumers' rights, widely approved to improve healthy development of biotechnology and guarantee the benefits of countries. Currently accurately estimating the content of GMO derived ingredients in foods is important since many national governments or governing bodies require the labeling of foods that originate from GMOs. For example, the European Union (EU) has established a 0.9% content threshold for the presence of approved GMO-derived material in food, food ingredients and feed. To support the labeling regulations of GMOs, the analytical methods for GMO detection must be developed with GMO technology. The most widely used GMO detection methods are based on DNA amplification, because of the high stability of genes in both raw materials and processed products and more information than protein detection (*4*). There are four different

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Table 1. Information of Compound Specific Primers Used in UP-M-PCR[a]

primer name	primer sequence	length	ref
GA21-466-F	*CCTTCCTTCCTTCCCCCC*TCTCGATCTTTGGCCTTGGTA	466	*19*
GA21-466-R	*CCTTCCTTCCTTCCCCCC*TGCAGCCCAGCTTATCGTCTA	466	
Bt11-277-F	*CCTTCCTTCCTTCCCCCC*CAATCCCACTATCCTTCGCAA	277	*18*
Bt11-277-R	*CCTTCCTTCCTTCCCCCC*GTAGACGTCGGTGTGGCAGA	277	
NOS-216-F	*CCTTCCTTCCTTCCCCCC*GAATCCTGTTGCCGGTCTTG	216	this study
NOS-216-R	*CCTTCCTTCCTTCCCCCC*TTATCCTAGTTTGCGCGCTA	216	
IVR-175-F	*CCTTCCTTCCTTCCCCCC*GTCGTGGTCTCCCGTGATCC	175	this study
IVR-175-R	*CCTTCCTTCCTTCCCCCC*GCGCTTCCTCTCGTTTTCCC	175	
35S-137-F	*CCTTCCTTCCTTCCCCCC*ATTGATGTGATATCTCCACTGACGT	137	this study
35S-137-R	*CCTTCCTTCCTTCCCCCC*CCTCTCCAAATGAAATGAACTTCCT	137	
UP	*CCTTCCTTCCTTCCCCCC*		this study

[a] The table shows the details of primer sequences, expected DNA fragment length and the source of primer used in UP-M-PCR. Each primer pair originates from the corresponding specific primer set (sequence in straight matter) and has a common sequence *CCTTCCTTCCTTCCCCCC* (18 bp) at its 3′-end in italics, which is also the sequences of the universal primer (UP) used in this developed new way.

strategies to detect GMOs: screening, gene-specific, construct-specific and event-specific detection, the specificity of which to discriminate GM and non-GM derived DNA increases in this order. Event specific detection has the highest specificity in GMO detection, since this method could flank the junction regions between exogenous DNA and host lines, which is also unique for a single transformation event (*5*). Nowadays, event-specific PCR is available for the detection of Bt11 (*5*), MON810 (*6*), GA21 (*7*), NK603 (*8*), MON863 (*9*), T25 maize (*10*); Roundup Ready soybean (*11*); MON1445, MON531 (*12*), MON15985 (*13*) cotton; GT73 (*14*) canola, Topas 19/2 Rapeseed (*15*) etc. For stacked materials by hybridization, event-specific PCR methods cannot be applied for the detection of stacked GMOs, which do not have a unique flanking sequence. Though PCR has many advantages, a single PCR could not distinguish the stacked maize from the mixture of parent line maize (for example of maize Bt11 × GA21 and the mixture of 50% maize Bt11 and 50% maize GA21), and it is proved to overestimate the GMO level in the sample containing the stack variety in GM mixture (*16*). With more reactions, it could be used to detect multiplex-events maize in individual kernels, but it is time-consuming and laborious. Multiplex PCR allows for simultaneous detection of more than one gene in a genome or different genes in a mixture, theoretically convenient for the identification of individual kernels with two or more GM traits to save considerable time and effort, but it always involves complex manipulation and lower sensitivity, and it cannot avoid self-inhibition and amplification disparity resulting from different primers. Both methods are limited to be used in single kernel identification and not available for complex materials. Therefore rapid sensitive and specific PCR systems that simultaneously amplify multiple target genes are considered advantageous. GM maize is one of the most extensively cultivated GM plants, since now thirty varieties of maize including fourteen stacked GM maize have been authorized by the European Commission (EC) (*17*). Bt11 × GA21 maize is a new kind of stacked genetically modified maize. Up to now, there is no report on methods superior to conventional multiplex PCR that could simultaneously detect all the exogenous genes in its lines. This paper describes an improved multiplex PCR technique (UP-M-PCR) for event-specific detection of maize Bt11 × GA21, which simplified traditional multiplex PCR, avoided the disparity of different primers in traditional multiplex PCR and got a higher specificity and sensitivity. Real-time PCR has the advantage over conventional PCR for figuring out the percent of GMO-derived content in samples to be estimated, which is being used increasingly for event-specific detection of GMOs. For the quantification of stacked maize Bt11 × GA21 in 100% GM mixture, real-time PCR combined with multivariate statistical analysis was used in this paper.

MATERIALS AND METHODS

Maize Samples. Four kinds of maize seeds were used in this study, including maize Bt11 × GA21, maize Bt11, maize GA21 and non-GM maize. Maize Bt11 × GA21 derives from the hybridization of maize Bt11 and maize GA21. All these four kinds of seeds were kindly provided by Bayer BioScience and Syngenta Company. Before the extraction of DNA, they were ground respectively into powder with the size of 200 mesh avoiding cross-contamination. Mixtures of the three GM materials at a set of different ratios were prepared according to weight percentage.

Preparation of DNA Template. Genomic DNA extraction from the finely ground powder was performed using the DNeasy 96 Plant kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The DNA was dissolved for 1 h in 0.1 × TE buffer (1 mmol L^{-1} Tris, 0.1 mmol L^{-1} EDTA, pH 8.0). Then it was quantified with the picogreen dsDNA quantification kit (Molecular Probes, Leiden, The Netherlands). Fluorescence was detected using the FL×800 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT) and analyzed by KC4 software 2000. The number of molecules was measured according to the DNA quantity and DNA average size. DNA purity was evaluated on the basis of the UV absorption ratios of A_{260}/A_{280}, A_{260}/A_{230} and 1% agarose gel. Only the samples with A_{260}/A_{280} ratio ranging from 1.6 to 1.9, A_{260}/A_{230} ratio ranging from 1.8 to 2.0 and a single band on gel were taken as pure DNA. DNA extraction was diluted 10-fold with 0.1 × TE buffer into solutions from 5×10^5 copies/μL to 5×10^0 copies/μL for the preparation of standard molecules. These templates were stored at −20 °C before amplification avoiding degradation.

Primers and Probes. Primer pairs for the universal primer multiplex PCR and real-time PCR are listed in **Table 1** and **Table 2** respectively. All the primers were synthesized and purified by Shanghai Sangon Company (Beijing, P. R. China). Primer sets used in UP-M-PCR target for maize *IVR* gene, *35S* gene, *NOS* gene, Bt11 event-specific sequence and GA21 event-specific sequence were described in previous papers (*18, 19*). The UP primer was designed using the ABI PRISM Primer Express Version 2.0 software (Applied Biosystems company, Foster City, CA) with an optimal melting temperature (T_m) of 60 °C. The transgenic maps of maize Bt11 and maize GA21 and the targeting positions of primers in UP-M-PCR are shown in **Figure 1**. Primers used in real-time PCR and their corresponding Taq-man probes were from the CRL calibrations (*20, 21*) and Chinese calibration (*22*), targeting for *Adh* gene (a maize endogenous gene target), *NOS* gene, Bt11 event-specific gene, GA21 event-specific gene. All the probes were labeled with fluorescent report dye FAM (6-carboxy-fluorescein) at its 5′-end and fluorescent quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) at its 3′-end.

Universal Primer-Multiplex PCR (UP-M-PCR). In this novel multiplex PCR approach, a set of primers were designed to amplify the target sequences and the endogenous sequences in maize Bt11 ×

Table 2. Information of Primers and Probes Used in Real-Time PCR[a]

primer name	primer sequence	length (bp)	ref
GA21-112-F	5′-CTTATCGTTATGCTATTTGCAACTTTAGA-3′	112	
GA21-112-R	5′-TGGCTCGCGATCCTCCT-3′	112	*20*
GA21-112-P	5′-TAMRA-CATATACTAACTCATATCTCTTTCTCAACAGCAGGTGGGT-FAM-3′		
Bt11-97-F	5′-GCGGAACCCCTATTTGTTTA-3′	97	
Bt11-97-R	5′-TCCAAGAATCCCTCCATGAG-3′	97	*21*
Bt11-97-P	5′-TAMRA-AAATACATTCAAATATGTATCCGCTCA-FAM-3′		
NOS-166-F	5′-ATCGTTCAAACATTTGGCA-3′	166	
NOS-166-R	5′-ATTGCGGGACTCTAATCATA-3′	166	*22*
NOS-166-P	5′-TAMRA-CATCGCAAGACCGGCAACAGG-FAM-3′		
Adh1-70-F	5′-CCAGCCTCATGGCCAAAG-3′	70	
Adh1-70-R	5′-CCTTCTTGGCGGCTTATCTG-3′	70	*20*
Adh1-70-P	5′- TAMRA-CTTAGGGGCAGACTCCCGTGTTCCCT-FAM-3′		

[a] The table shows the details of primer used in real-time PCR including sequence, amplicon length, corresponding probe sequence and their references.

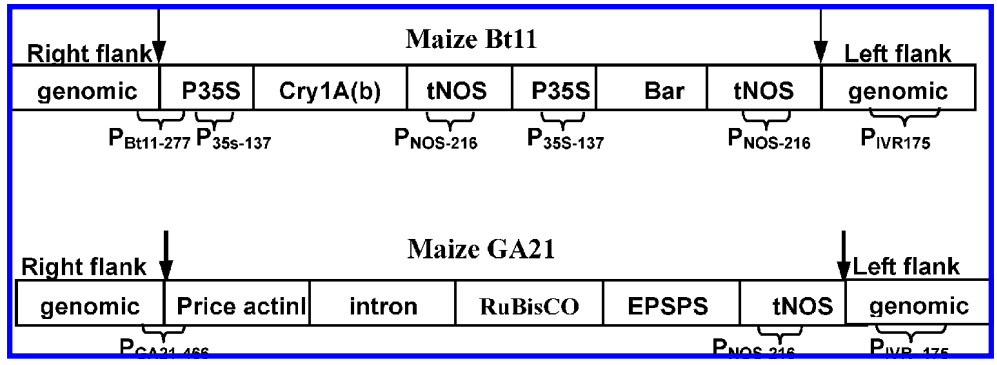

Figure 1. The transgenic map of maize Bt11; maize GA21 and the target positions of primer sets used in UP-M-PCR. Schematic represents the maize Bt11 and maize GA21 transgenic map. The primers' target positions are indicated with brackets. $P_{35S\text{-}137}$: primer pair 35S-137-F/R targeting for the Caullinus Mosaic Virus promoter. $P_{NOS\text{-}216}$: primer pair NOS-216-F/R targeting for *Agrobacterium tumefaciens L.* terminator. $P_{Bt11\text{-}277}$: primer pair Bt11-277-F/R for event-specific detection of Bt11. $P_{GA21\text{-}466}$: primer pair GA21-466-F/R for event-specific detection of GA21. $P_{IVR\text{-}175}$: primer pair IVR-175-F/R target for endogenous gene *IVR*.

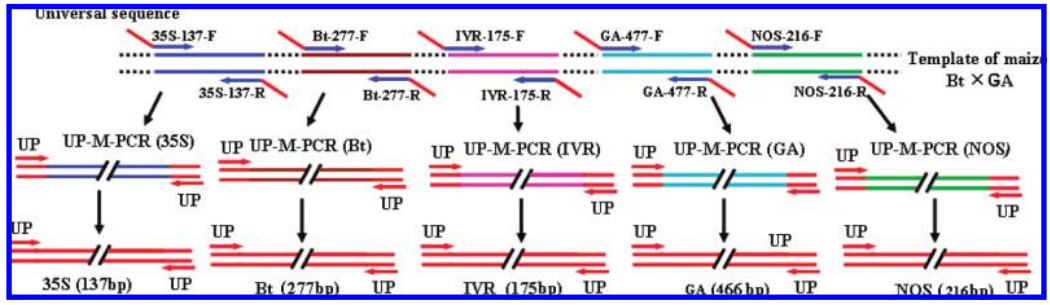

Figure 2. Amplification routine of UP-M-PCR. Each compound specific primer contained a specific primer of at the 5′-end (blue) and the universal sequence at the 3′-end (red). The amplified fragments with the primer pairs of 35S-137-F/R, NOS-216-F/R, IVR-175-F/R, Bt11-277-F/R, and GA21-466-F/R are individually marked in blue, brown, pink, cyan and green. The amplified fragments only by the universal primer are marked in red.

GA21. All the primers include a common sequence at its 5′-end, which is also the sequence of the universal primer (UP). The amplification routine of UP-M-PCR is shown in **Figure 2**. In the former ten cycles, compound specific primers take action for amplification of these target sequences and the endogenous sequences, while the universal primer almost has no amplification. With the compound specific primers used up and the amplified products increasing, the universal primer takes the amplicons as templates and shows its ability to amplify the fragments of five different lengths according to the compound specific primers.

Based on the conventional multiplex PCR assay (*23*), the annealing temperature of primers was optimized from 52 to 62 °C. The concentration of universal primer (from 50 nmol L^{-1} to 500 nmol L^{-1} at an interval of 50 nmol L^{-1}) and compound specific primers (from 200 nmol L^{-1} to 0.2 nmol L^{-1} with a 10-fold gradient dilution) was evaluated. The specificity of compound specific primer pairs was also tested individually according to Xu's melting temperature-based SYBR Green I PCR method (*24*). All the PCR reactions were carried out using a Peltier Thermal Cycler Controller (MJ Research, BioRad Laboratories, MA) in a 30 μL reaction volume, containing 1.5 mmol L^{-1} magnesium chloride, 200 μmol L^{-1} dNTPs and 1.5 units of Taq DNA polymerase (Promega Company USA), 2 nmol L^{-1} of each compound specific primers and 300 nmol L^{-1} universal primer. The thermal cycling program included an initial 5 min denaturation at 95 °C; and then 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by a final extension for 10 min at 72 °C.

Real-Time PCR Combined with Multivariate Statistical Analysis. PCR based Taqman probe was chosen as the quantification technology and performed in an ABI PRISM 7000 Sequence Detection System. After analysis of the genome structure of maize Bt11, maize GA21 and maize Bt11 × GA21, four separate sequences (*Adh*, *NOS*, Bt11 event, GA21 event) were chosen as the detection genes, which have definite content in each maize genome. When the three kinds of GM-maize were mixed together, the percentage of each GM-maize satisfied the four equations in **Chart 1** (a, b, c, d), any three ones of which composed a three linear equation system, and its coefficient was determined by gene composition of three GM-maize materials. The copy number of the four target genes could be figured out by real-time

Chart 1[a]

$$\begin{cases} X+Y+Z=100\% & (a) \\ X+Y=C_{Bt11}/C_{ivr} & (b) \\ 3X+2Y+Z=C_{NOS}/C_{ivr} & (d) \end{cases} \quad \text{Equation I}$$

$$\begin{cases} X+Y+Z=100\% & (a) \\ X+Z=C_{GA21}/C_{ivr} & (c) \\ 3X+2Y+Z=C_{NOS}/C_{ivr} & (d) \end{cases} \quad \text{Equation II}$$

$$\begin{cases} X+Y+Z=100\% & (a) \\ X+Y=C_{Bt11}/C_{ivr} & (b) \\ X+Z=C_{GA21}/C_{ivr} & (c) \end{cases} \quad \text{Equation III}$$

[a] C_{Bt11}: copy number of Bt11 event-specific sequence. C_{GA21}: copy number of GA21 event-specific sequence. C_{NOS}: copy number of *NOS* gene. C_{IVR}: copy number of *IVR* gene. *X*: the content of maize Bt11 × GA21 in 100% GM mixture. *Y*: The content of maize Bt11 × GA21 in 100% GM mixture. *Z*: The content of maize Bt11 × GA21 in 100% GM mixture.

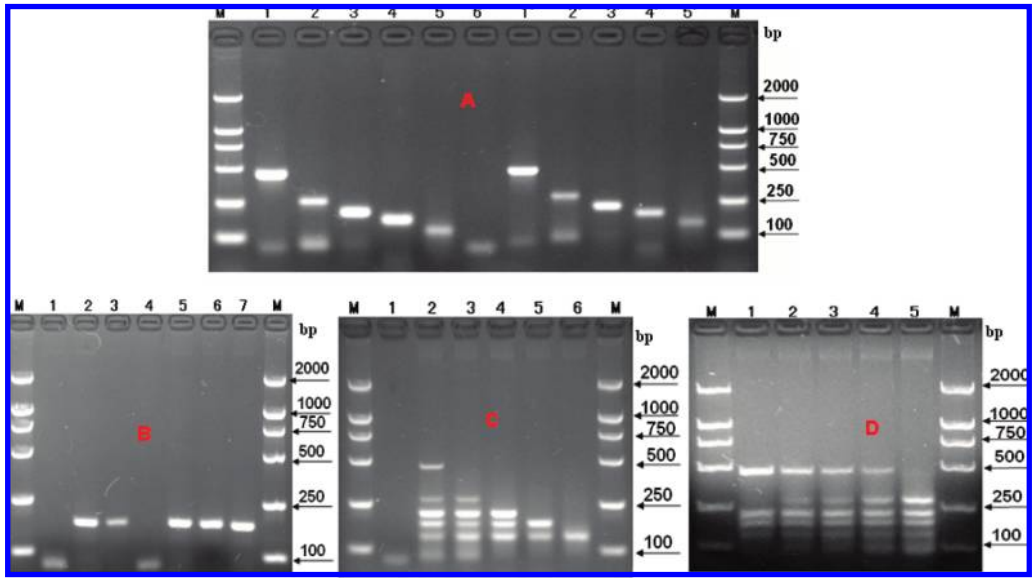

Figure 3. Test for the feasibility of UP-M-PCR. (**A**) Comparison of specificity between specific primers and compound specific primers. Lanes 1, 2, 3, 4, 5, amplicon fragments of specific primer *35S*, *IVR*, *NOS*, Bt11 event, GA21 event; lanes 1′, 2′, 3′, 4′, 5′, amplicon fragments of UP and compound specific primer for *35S*, *IVR*, *NOS*, Bt11, GA21; lane 1, negative control without template; lane M, molecular weight makers (DL 2000 bp). (**B**) Impact of concentration of universal primer on single PCR. Lane 1, negative control without template; lanes 2, 3, 4, amplicon fragments by compound specific primer pair *IVR-175* at the concentration of 200 nmol L^{-1}, 20 nmol L^{-1}, 2 nmol L^{-1} respectively; lanes 5, 6, 7, amplicon fragments by UP (300 nmol L^{-1}) and compound specific primer IVR-175 at a series concentrations of 200 nmol L^{-1}, 20 nmol L^{-1}, 2 nmol L^{-1}; lane M, molecular weight makers (DL 2000 bp). (**C**) Detection of the specificity of UP-M-PCR. All the reactions were performed with the same amount of template (20 ng). Amplified fragments including 137bp, 175bp, 216bp, 277bp, 466bp products, which are corresponding to the targeting genes *35S*, *NOS*, *IVR*, event Bt11 and event GA21 respectively. Lane 1, negative control without template; lanes 2, 3, 4, 5, 6 are the results of five-plex, four-plex, triplex, duplex and singleplex of UP-M-PCR; lane M, molecular weight makers (DL 2000 bp). (**D**) Impact of compound specific primers' concentration on UP-M-PCR. All the reactions were performed with the same amount of template (20 ng), containing six primer pairs 35S-137-F/R, NOS-216-F/R, IVR-175-F/R, Bt11-277-F/R, GA21-466-F/R and UP, from lanes 1−5 the concentration of primer pair GA21-466-F/R decreased from 200 nmol L^{-1} to 0.2 nmol L^{-1} in 10-fold, in contrast, the concentration of primer pair Bt11-277-F/R increased from 0.2 nmol L^{-1} to 200 nmol L^{-1} in 10-fold, while other three primer pairs kept at the concentration of 2 nmol L^{-1}.

PCR as the known data for equations, the contents of each GM-maize in mixture are taken as the three unknown variables of ternary linear equations (I, II, III), the average value of solutions from three equations (I, II, III) is taken as the accurate content of each GM-maize in mixture.

Each reaction was carried out in a total volume of 30 μL, consisting of 15 μL of 2× reaction buffer (Amplitaq Gold, Applied Biosystem ABI, 100 mmol L^{-1} Tris-HCl, pH 8.3, 500 mmol L^{-1} KCl, 1.5 μL of Taq DNA polymerase, 6 mmol L^{-1} $MgCl_2$, 200 mmol L^{-1} dNTPs, 0.2 unit of AmpErase uracil N-glycosylase), 200 nmol L^{-1} primer, 100 nmol L^{-1} corresponding probe and 1 μL of DNA template. The reaction program was described as follows: 5 min denaturation at 95 °C, and then 35 cycles of 30 s at 95 °C; 30 s at 60 °C, and 30 s at 72 °C, followed by a final extension of 10 min at 72 °C. Only when amplification curves in detection were clearly observed after 15 cycles, we considered the sample as positive.

Standard curves for the four target genes (Bt, GA, IVR, NOS) were established with five dilutions of DNA from 100% stacked maize Bt-GA. Five serially diluted concentrations (500000, 50000, 5000, 500, 50 copies per reaction, respectively) of DNA were used for the preparation of standard curves, DNA (10 ng mL^{-1}) from non-GM maize was used as no-template control (NTC). Each test sample was analyzed by PCR with three repetitions. Standard curves with efficiencies from 90% to 110% and corresponding $R^2 > 99\%$ were taken as the final results. PCR efficiency $= 10^{-\text{slope}} - 1$.

RESULTS

Specificity of Compound Specific Primers. The new designed compound primer pairs originating from specific primers have been tested to get equivalent intensities of bands on gels with the same template concentration (**Figure 3A**, lanes 1, 2, 3, 4, 5 to lanes 1′, 2′, 3′, 4′, 5′), which showed that the set of compound specific primers worked efficiently and had the same specificity as the specific primers from reference. Because the compound specific primers contained a common sequence (18 bp) at the 3′-end, as a result they got a higher annealing temperature and generated amplicons larger of 36 bp than the products amplified by corresponding specific primers, so the bands on gel were a little higher too. That there were no unexpected bands showed there was no unexpected reaction, which also proved that the specificity of compound specific primers was high.

Feasibility of Universal Primer (UP). Keeping the concentration of templates at 20 ng, with the amount of the specific primers for *IVR* gene decreasing (200 nmol L^{-1}, 20 nmol L^{-1}, 2 nmol L^{-1}), the intensity of band fell down markedly (primer 20 nmol L^{-1}) until to nothing (primer 2 nmol L^{-1}) in conventional single PCR (**Figure 3B**, lanes 2, 3, 4), which showed that the concentration of amplified fragments became lower and lower. While in the novel singleplex PCR, for the addition of universal primer (300 nmol L^{-1}), though there is a down gradient concentration of compound specific primers IVR-175-F/R from 200 nmol L^{-1} to 2 nmol L^{-1}, the PCR system above worked efficiently and got an equivalent amount of amplified products (**Figure 3B**, lanes 5, 6, 7). Similar results were achieved from the compound specific primers GA21-466-F/R, Bt11-277-F/R, NOS-216-F/R, 35S-137-F/R (data not shown) with UP in novel singleplex PCR. The sharp contrast showed that the universal primer was well designed to work efficiently for the PCR amplification and had a high feasibility to amplify the amplicons produced by compound specific primers.

Optimization of the UP-M-PCR. The concentrations of primers strongly influence the efficiency and disparity of PCR reaction, which is very important for the PCR reaction especially in multiplex PCR. The final optimized concentration of universal primer (UP) was 300 nmol L^{-1} in UP-M-PCR, which is the same as in normal single PCR, while the other five compound specific primers GA21-466-F/R, Bt11-277-F/R, NOS-216-F/R, IVR-175-F/R, 35S-137-F/R were 2 nmol L^{-1} respectively (about 1/10 of the normal concentration). To find the best annealing temperature, a gradient temperature PCR from 52 to 62 °C has been performed (data not shown). At last the optimum annealing temperature was chosen at 60 °C. **Figure 3C** showed the amplification results by UP-M-PCR on 2.0% agarose gel. Lanes 2−6 were the results of five-plex PCR with primer pairs 35S-137-F/R, IVR-175-F/R, NOS-216-F/R, Bt11-277-F/R, GA21-466-F/R, four-plex PCR with primer pairs 35S-137-F/R, IVR-175-F/R, NOS-216-F/R, Bt11-277-F/R, triplex PCR with primer pairs IVR-175-F/R, NOS-216-F/R, Bt11-277-F/R, duplex PCR with primer pairs 35S-137-F/R, IVR-175-F/R, singleplex PCR with primer pair 35S-137-F/R. All the reactions were performed with the same amount of template (20 ng). Each compound specific primer pair in the mixture was sensitive and specific enough to amplify the corresponding sequence and generated the expected length of amplicons the same as in the single PCR and no unexpected PCR products were detected. There was less or even no disparity between various primers as in UP-M-PCR. Similar duplex, triplex and four-plex PCR results were achieved with arbitrary combination of compound specific primer pairs (data not shown).

Impact of Concentrations of Compound Specific Primers on UP-M-PCR. In five-plex PCR, primer pairs (35S-137-F/R, IVR-175-F/R, NOS-216-F/R, Bt11-277-F/R, GA21-466-F/R) were mixed together with the proportion of 1:1:1:0.0001:1, 1:1:1:0.001:0.1, 1:1:1:0.01:0.01, 1:1:1:1:0.1:0.001 (1 represents 200 nmol L^{-1}) in separate reaction. Amplification results indicated the relationship between primer amounts and band intensity (**Figure 3D**). With the amount of primer pair Bt11-277-F/R increased while that of primer pair GA21-466-F/R decreased, the band of 277 bp fragment became intense, the 466 bp fragment became faint until to nothing visible under the UV light and the concentration of other amplicons did not change for the same amount of corresponding primers (**Figure 3D**, lanes 1−5). The results showed that amplicon intensity had a positive correlation with the concentration of compound specific primers in UP-M-PCR. When the amount of Bt11-277-F/R primer pair was lower than 2 nmol L^{-1} PCR reaction system, there was too little corresponding product to present a band visible on the gel under the UV light. This limit for compound primer was consistent with that in singleplex PCR system. For the detection of multiplex genes with UP-M-PCR, 300 nmol L^{-1} UP and 2 nmol L^{-1} specific primer were recommended to be taken.

Table 3. Test Result of Universal Primer Four-plex PCR as a Screening Method[a]

real proportion (Bt-GA:Bt:GA:non)	Bt11	GA21	NOS	IVR
0.05%:0%:0%:99.95%	−	−	+	+
0.05%:0.05%: 0%:99.9%	+		+	+
0.05%:0%:0.05%:99.9%		+	+	+
0.1%:0%:0%:99.9%	+	+	+	+
0.5%:1%:10%:88.5%	+	+	+	+
1%:10%:10%:79%	+	+	+	+
2.5%:20%:20%:57.5%	+	+	+	+
5%:10%:10%:75%	+	+	+	+
10%:10%:10%:10:60%	+	+	+	+
50%:0%:0%:50%	+	+	+	+
0%:50%:0%:50%	+	−	+	+
0%:0%:50%:50%	−	+	+	+
0%:0%:0%:100%	−	−	−	+

[a] Bt-GA: maize Bt11 × GA21. Bt: maize Bt11. GA: maize GA21. +: positive result. −: negative result.

Application of UP-M-PCR in Detection as a Rapid Screening Method. For most incidents, the detection samples are complicated by more than one GM material accompanied with multiple characteristics. To test the feasibility of UP-M-PCR in real detection, 13 samples of GM mixture were prepared with the content of maize Bt11, maize GA21 and maize Bt11 × GA21 in a mixture ranging from 0.05 to 50% (w/w). Each sample was tested with three duplicates from DNA extraction to novel four-plex PCR targeting for *NOS* gene, *IVR* gene, Bt11-event gene, GA21-event gene, so actually there were nine duplications for each sample. Results showed that the detection limit of UP-M-PCR is 0.1% (about 38 haploid genome copies); when the content of gene in the mixture was lower than the limit, the result was negative and could not reflect fact correctly (**Table 3**). In addition, 0.1% is far lower than the GM labeling limit of the European Union and satisfied with the requirements of all the countries, so this method could be recommended as a new efficient way to detect genetically modified materials. This is the fundamental base to perform UP-M-PCR before quantification of stacked genetically modified materials.

Application of Real-Time PCR Combined with Multivariate Statistical Analysis to Quantify Stacked Maize Bt11 × GA21. As showed by **Figure 4**, the average R^2 value of the four calibration lines (standard curves Bt, GA, NOS, IVR) were higher than 99.9%, which indicated a good correlation between the amount of target genes and the threshold (C_t) value after amplification. In addition, the average values of the slope for the standard curves were between −3.365 and −3.345, which were within the requirements of CRL (−3.1 > value of slope > −3.6.). The coefficient of our test variation was lower than ±25%, which was also acceptable by CRL. **Table 4** shows the detection results of real-time PCR combined with multivariate statistical analysis to quantify stacked maize Bt11 × GA21 in GM mixture, and all the data showed that it could substantially figure out the accurate content of stacked genetically modified maize Bt11 × GA21 in mixture. The detection limit is as low as 0.5%; when the content of stacked maize was lower than the detection limit, the test result was far away from the true value,

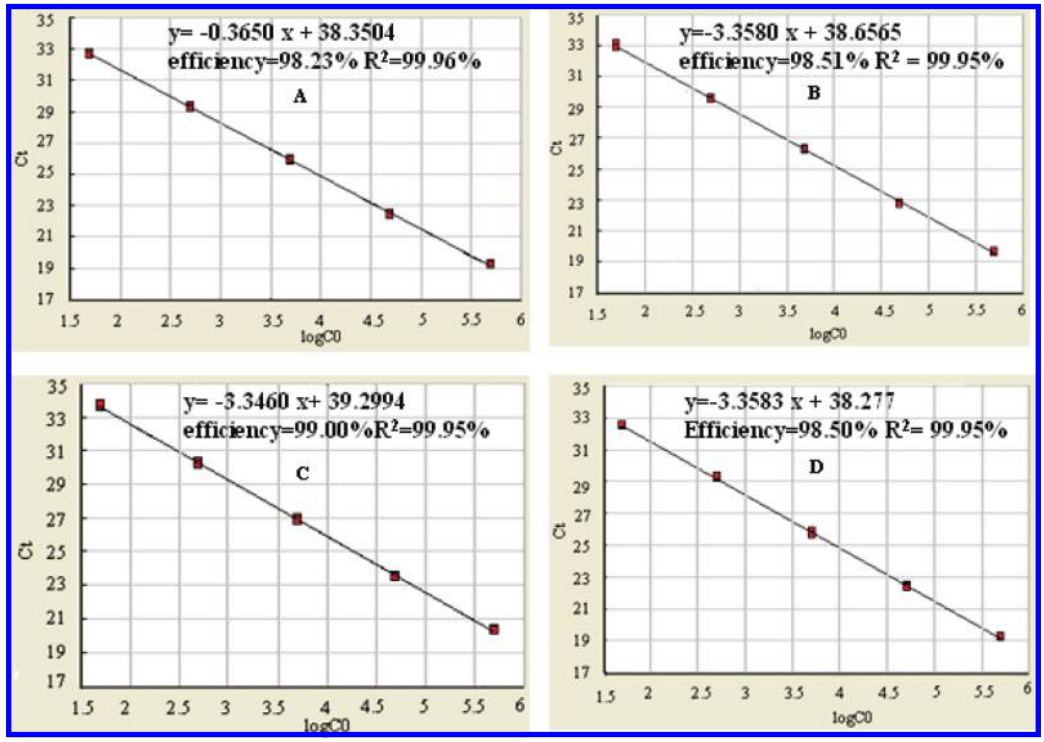

Figure 4. Calibration curves of the four target genes. (**A**) Amplification graph for the Bt11 event-specific sequence assay. (**B**) Standard curve for the and GA21 event-specific sequence assay. (**C**) Amplification graph for the *NOS* gene assay. (**D**) Amplification graph for the *IVR* gene assay. Five serially diluted concentrations (500000, 50000, 5000, 500, 50 copies per reaction, respectively) of DNA were used for the preparation of standard curves, DNA (10 ng mL^{-1}) from non-GM maize was used as no-template control (C).

Table 4. Test Results of Bt11 × GA21 Maize in Mixture by Real-Time PCR and Multivariate Statistical Analysis[a]

real proportion (Bt-GA:Bt:GA)	Bt copies		GA21/copies		NOS/copies		Ivr/copies		test proportion (Bt-GA:Bt:GA)
	real value	test value	real value	test value	real value	test value	real value	test value	
0.05%:89.95%:10%	36000	36012	4020	4021	76020	76033	40000	40033	0%:89.96%:10.04%
0.1%:10%:89.9%	4040	4033	36000	36004	44080	44091	40000	40015	0.09%:10%:89.91%
0.5%:94.5%:5%	38000	38015	2200	2207	78200	78220	40000	40010	0.51%:94.49%:5%
1%:5%:94%	2400	2411	38000	38002	42800	42811	40000	40025	0.96%:5.06%:93.99%
2.5%:95%:2.5%	39000	39008	2000	1998	80000	80009	40000	40011	2.48%:95.01%:2.51%
5%:2.5%:92.5%	3000	3007	39000	39005	45000	45033	40000	40028	4.97%:2.55%:92.48%
10%:1%:89%	4400	4411	39600	39605	48400	48401	40000	40014	9.97%:10.4%:88.99%
50%:49%:1%	39600	39602	20400	20411	99600	99626	40000	40019	49.48%:48.99%:1.04%
0%:0.5%:99.5%	200	203	39800	39812	40200	40212	40000	40005	0.02%:4.9%:99.19%
0%:99.5%:0.5%	39800	39807	200	188	79800	79810	40000	40021	0%:99.44%:0.56%
50%:0.1%:49.9%	20040	20036	39960	39971	80040	80034	40000	40028	49.91%:0.14%:49.95%
50%:49.9%:0.1%	39960	39952	20040	20047	99960	99972	40000	40005	50.01%:49.87%:0.12%
50%:0.05%:49.95%	20020	20015	39980	39982	80020	80017	40000	40012	49.96%:0.07%:49.98%
50%:49.95%:0.05%	39980	39971	20020	20023	99980	99982	40000	40038	49.87%:49.97%:0.16%
33.33%:33.33%:33.33%	26664	26657	26664	26674	79992	80001	40000	40056	33.16%:33.4%:33.45%

[a] Bt-GA: maize Bt11 × GA21. Bt: maize Bt11. GA: maize GA21.

as can be seen from the results (real content of maize Bt11 × GA21 is 0.05% and 0.1%, test results are 0% and 0.09% with high relative standard deviation (RSD) of 100% and 15%, shown on rows one and two, column four in **Table 5**). Similar results were gotten with maize Bt11 (real content is 0.05%, 0.1%, test result is 0.07% and 0.14% with high RSD of 33.33% and 43.33% shown on rows thirteen and eleven, column eight in **Table 5**) and GA21 (real content is 0.05%, 0.1%, test result is 0.12%, 0.16%, with high RSD of 21.67% and 223.33%, shown on rows twelve and fourteen, column twelve in **Table 5**). When the content of a single GM-maize is higher than 0.5%, the RSD value was always lower than 5%, the maximum RSD value is 4.5% with Bt11-GA21 maize (row four, column four in **Table 5**), 4% with Bt11 maize (row seven, column eight in **Table 5**), 3.5% with GA21 maize (row eight, column twelve in **Table 5**). All these results showed the credibility of this method.

DISCUSSION

In optimized UP-M-PCR system, the universal primer has a concentration of 300 nmol L^{-1} at normal degree, while the concentration of every compound specific primer was as low as 2 nmol L^{-1}, therefore, the total amount of all the primers was almost equal to that of conventional single PCR and only about one-third to one-fifth of that in conventional multiplex PCR, in which all the primers are mixed with a normal concentration about 300 nmol L^{-1}. In a word, it really simplified the multiplex PCR reaction system, which was also the reason why it could circumvent the amplification disparity resulting from different primers in traditional multiplex PCR. Similar results were gotten from the detection of maize Mon 810 and Mon 863 (data not shown). Higher sensitivity (2 nmol L^{-1}) and

Table 5. Comparison of Separate Solution and Average Value of Results by Real-Time PCR and Ternary Linear Equations

Bt-GA/ test result and (RSD)				Bt/ test result and (RSD)				GA/test result and (RSD)			
soln I	soln II	soln III	av value	soln I	soln II	soln III	av value	soln I	soln II	soln III	av value
0%	0%	0%	0%	89.96%	89.96%	89.96%	0.8996%	10.04%	10.04%	10.04%	0.100%
(100%)	(100%)	(100%)	(100%)	(0.01%)	(0.01%)	(0.01%)	(0.01%)	(0.4%)	(0.40%)	(0.40%)	(0.40%)
0.11%	0.09%	0.06%	0.09%	9.97%	10.02%	10.02%	10.00%	89.92%	89.90%	89.92%	89.91%
(10%)	(40.00%)	(40.00%)	(15.00%)	(0.30%)	(0.20%)	(0.20%)	(0.03%)	(0.02%)	(0.01%)	(0.02%)	(0.01%)
0.49%	0.51	0.53%	0.51%	94.52%	94.48%	94.48%	94.49%	4.99%	5.01%	4.99%	5.00%
(2.00%)	(2.00%)	(6.00%)	(2.00%)	(0.02%)	(0.02%)	(0.02%)	(0.01%)	(0.20%)	(0.20%)	(0.20%)	(0.07%)
0.94%	0.96%	0.97%	0.96%	5.08%	5.05%	5.05%	5.06%	93.98%	94.00%	93.98%	93.99%
(6.00%)	(6.00%)	(3.00%)	(4.50%)	(1.60%)	(1.00%)	(1.00%)	(1.20%)	(0.02%)	(0.01%)	(0.02%)	(0.02%)
2.48%	2.18%	2.48%	2.48%	95.01%	95.01%	95.01%	95.01%	2.51%	2.51%	2.51%	2.51%
(0.80%)	(0.80%)	(0.80%)	(0.80%)	(0.01%)	(0.01%)	(0.01%)	(0.01%)	(0.40%)	(0.40%)	(0.40%)	(0.40%)
4.99%	4.97%	4.95%	4.97%	2.52%	2.56%	2.56%	2.55%	92.49%	92.47%	92.49%	92.48%
(0.2%)	(1.00%)	(1.00%)	(0.60%)	(0.80%)	(2.40%)	(2.40%)	(1.86%)	(0.01%)	(0.03%)	(0.01%)	(0.02%)
9.94%	9.97%	10.00%	9.97%	1.08%	1.02%	1.02%	1.04%	88.98%	89.01%	88.98%	88.99%
(0.6%)	(0.00%)	(0.00%)	(0.30%)	(8.00%)	(2.00%)	(2.00%)	(4.00%)	(0.02%)	(0.01%)	(0.02%)	(0.01%)
49.99%	49.98%	49.96%	49.98%	48.97%	49.00%	49.00%	48.99%	1.04%	1.02%	1.04%	1.04%
(0.02%)	(0.08%)	(0.08%)	(0.05%)	(0.06%)	(0.00%)	(0.00%)	(0.02%)	(4.00%)	(2.50%)	(4.00%)	(3.50%)
0.01%	0.02%	0.03%	0.02%	0.50%	0.48%	0.48%	0.49%	99.49%	99.50%	99.49%	99.49%
(100%)	(101%)	(102%)	(109%)	(0.00%)	(4.00%)	(4.00%)	(2.67%)	(0.01%)	(0.00%)	(0.01%)	(0.01%)
0.05%	0.05%	0.05%	0.05%	99.53%	99.53%	99.53%	99.53%	0.52%	0.52%	0.52%	0.52%
(100%)	(101%)	(102%)	(110%)	(0.03%)	(0.03%)	(0.03%)	(0.03%)	(4.00%)	(4.00%)	(4.00%)	(4.00%)
49.90%	49.91%	49.91%	49.91%	0.15%	0.14%	0.14%	0.14%	49.95%	49.96%	49.95%	49.95%
(0.2%)	(0.18%)	(0.19%)	(0.19%)	(50.00%)	(40.00%)	(40.00%)	(43.33%)	(0.10%)	(0.11%)	(0.10%)	(0.10%)
50.03%	50.01%	49.98%	50.01%	49.84%	49.89%	49.89%	49.87%	0.13%	0.11%	0.13%	0.12%
(0.06%)	(0.04%)	(0.04%)	(0.01%)	(0.12%)	(0.02%)	(0.02%)	(0.05%)	(30.00%)	(5.00%)	(30.00%)	(21.67%)
49.96%	49.96%	49.95%	49.96%	0.06%	0.07%	0.07%	0.07%	49.98%	49.97%	49.98%	49.98%
(0.08%)	(0.10%)	(0.10%)	(0.09%)	(20.00%)	(40.00%)	(40.00%)	(33.33%)	(0.06%)	(0.05%)	(0.06%)	(0.06%)
49.89%	49.87%	49.84%	49.87%	49.94%	49.99%	49.99%	49.97%	0.17%	0.15%	0.17%	0.16%
(0.22%)	(0.32%)	(0.32%)	(0.27%)	(0.02%)	(0.08%)	(0.08%)	(0.05%)	(240.00%)	(190.00%)	(240.00%)	(223.33%)
33.17%	33.16%	33.14%	33.16%	33.38%	33.41%	33.41%	33.40%	33.45%	33.44%	33.45%	33.45%
(0.48%)	(0.57%)	(0.57%)	(0.53%)	(0.15%)	(0.24%)	(0.24%)	(0.21%)	(0.36%)	(0.32%)	(0.36%)	(0.35%)

specificity of UP-M-PCR made it serve as a rapid screening method before real-time PCR detection.

Nowadays the most efficient way to detect stacked maize is the application of individual kernel analysis. For example, the European Union takes real-time PCR for detection of stacked maize MON 863 × NK603, maize 59112 × 1507 × NK603, MON810 × NK603, et al. (*25*−*27*). These methods are restricted to identify the individual kernel accompanied and proved to overestimate the GMO level in the sample containing the stack variety in GM mixture (*16*). Traditional multiplex PCR has also been used to detect stacked event GMOs (*28*). It is convenient for saving time, but traditional multiplex PCR could not avoid amplification disproportionate resulting from disparity of different primers and always with lower sensitivity. Other researchers developed this traditional multiplex PCR method incorporated by capillary gel electrophoresis (CGE) for simultaneous detection of combined-trait genetically modified maize kernel (*29, 30*). The CGE method involved complex and cute performance, furthermore it could not detect out stacked maize in GM maize mixture either.

Real-time PCR is credibly used in GM detection and has been widely accepted by worldwide scientists due to its speed, sensitivity, specificity, high degree of automation and the possibility of target quantification (*31*). This paper first developed a new way to detect stack GM-maize Bt11 × GA21 in GM maize mixture by real-time PCR combined with multivariate statistical analysis. Four equations (a, b, c, d) were designed according to the content of target genes in each GM-maize. Theoretically 2b + c = d, so only three meaningful ternary linear equations were gotten from the four equations with one composed by equations b, c, d deposited. The RSD of each test value was lower than 5%, (data not shown), which showed the low test variation of this method. The RSD of the solution of a single ternary linear equation from the true value was higher and not steady, while the average value of solutions of three ternary linear equations was lower and more stable with RSD values always less than 5% as seen in **Table 5** (column 4 with 1, 2, 3, column 8 with column 5, 6, 7; column 12 with column 9, 10, 11). The method could accurately quantify the mixture containing any three kinds of maize among the above four kinds of maizes, such as "maize Bt × GA, maize Bt and non-GM maize", "maize Bt × GA, maize GA and non-GM maize", "Bt, GA and non-GM maize" etc. (data not shown). It is really a progress when comparing with single kernel detection. However, the limitation of the method is that it could not accurately quantify the mixture containing four kinds of maizes (Bt × GA, Bt, GA and non-GM maize).

Both the detection limit of UP-M-PCR (0.1%) and real-time PCR (0.5%) were lower than the GM-labeling limit of the European Union and satisfied the qualifications of all the countries for labeling test, and all the results showed the superiority and feasibility of these two methods. Although the calculation procedure is a little complicated, the test results were proved close to the real value and it did solve the problem to detect stacked maize in GM mixture. It may find wide application in detection of other GM maize crops, other stacked GM plants like soybean, rice and even food products containing stacked events.

ABBREVIATIONS USED

UP-M-PCR, universal primer multiplex PCR; GMOs, genetically modified organisms; LOD, limit of detection; Adh, maize invertase gene; nos, terminator of nopaline synthase gene from *Agrobacterium tumefaciens*; ivr, intron from the maize alcohol dehydrogenase gene; 35s, promoter from the cauliflower mosaic virus; FAM, 6-carboxy-fluorescein; TAMRA, tetramethyl-6-carboxyrhodamine.

LITERATURE CITED

(1) Halpin, C. Gene stacking in transgenic plants-the challenge for 21st century plant biotechnology. *Plant Biotechnol. J.* **2005**, *3*, 141–155.

(2) De Schrijver, A.; Devos, Y.; Van den Bulcke, M.; Cadot, P.; Reheul, D.; De Loose, M.; Sneyers, M. Risk assessment of GM stacked events obtained from crosses between GM events. *Trends Food Sci. Technol.* **2007**, *18*, 101–109.

(3) European Food Safety Authority EFSA-Q-2003-005D. Guidance Document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants containing stacked transformation events. 2007, Vol. 512, pp 1−5.

(4) Wolf, C.; Burgener, M. M.; Hübner, P.; Lüthy, J. PCR-RFLP analysis of mitochondrial DNA: differentiation of fish species. *Lebensm-Wiss. -Technol.* **2000**, *33*, 144–150.

(5) Rønning, S. B.; Va1tilingom, M.; Holst-Jensen, A.; Berdal, K. G. Event specific real-time quantitative PCR for genetically modified Bt11 maize (*Zea mays*). *Eur. Food Res. Technol.* **2003**, *216*, 347–354.

(6) Hernandez, M.; Pla, M.; Esteve, T.; Prat, S.; Puigdomenech, P.; Ferrando, A. A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard based on the 3′-transgene integration sequence. *Transgenic Res.* **2003**, *12*, 179–189.

(7) Hernández, M.; Esteve, T.; Prat, S.; Pla, M. Development of real-time PCR systems based on SYBR® Green I, Amplifluor and TaqMan® technologies for specific quantitative detection of the transgenic maize event GA21. *J. Cereal Sci.* **2004**, *39*, 99–107.

(8) Huang, H. Y.; Pan, T. M. Detection of genetically modified maize MON810 and NK603 by multiplex and real-time polymerase chain reaction methods. *J. Agric. Food Chem.* **2004**, *52*, 3264–3268.

(9) Pan, A. H.; Yang, L. T.; Xu, S. C.; Yin, C. S.; Zhang, K. W.; Wang, Z. Y.; Zhang, D. B. Event-specific qualitative and quantitative PCR detection of MON863 maize based upon the 3′-transgene integration sequence. *J. Cereal Sci.* **2006**, *43*, 250–257.

(10) Heide, B. R.; Heir, E.; Holck, A. Detection of eight GMO maize events by qualitative multiplex PCR and fluorescence capillary gel electrophoresis. *Eur. Food Res. Technol.* **2008**, *227*, 527–535.

(11) Berdal, K. G.; Holst-Jensen, A. Roundup ready soybean event-specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analyses. *Eur. Food Res. Technol.* **2001**, *213*, 432–438.

(12) Yang, L.; Pan, A.; Zhang, K.; Yin, C.; Qian, B.; Chen, J.; Huang, C.; Zhang, D. Qualitative and quantitative PCR methods for event-specific detection of genetically modified cotton Mon1445 and Mon531. *Transgenic Res.* **2005**, *14*, 817–831.

(13) Joint Research Centre (JRC) of the European Commission. A quantitative PCR detection method for MON-15985-7 cotton. Available at http://gmo-crl.jrc.it/statusofdoss.htm, **2008**, 1−11.

(14) Demeke, T.; Ratnayaka, I. Multiplex qualitative PCR assay for identification of genetically modified canola events and real-time event-specific PCR assay for quantification of the GT73 canola event. *Food Control* **2008**, *19*, 893–897.

(15) Wu, G.; Wu, Y. H.; Xiao, L.; Lu, C. M. Event-specific qualitative and quantitative PCR detection of genetically modified rapeseed topas 19/2. *Food Chem.* **2008** [doi: 10.1016/j.foodchem.2008.05.105].

(16) Akiyama, H.; Watanabe, T.; Wakabayashi, K.; Nakade, S.; Yasui, S.; Sakata, K.; Chiba, R.; Spiegelhalter, F.; Hino, A.; Maitani, T. Quantitative detection system for maize sample containing combined-trait genetically modified maize. *Anal. Chem.* **2005**, *77*, 7421–7428.

(17) Biotechnology & GMOs Unit CRL-GMFF. GM food and feed status of dossiers. **2008**, available at http://gmo-crl.jrc.it/statusofdoss.htm.

(18) Peano, C.; Bordoni, R.; Gulli, M.; Mezzelani, A.; Samson, M. C.; De Bellis, G.; Marmiroli, N. Multiplex polymerase chain reaction and ligation detection reaction/universal array technology for the traceability of genetically modified organisms in foods. *Anal. Biochem.* **2005**, *346*, 90–100.

(19) Cao, J. J.; Zhu, Y. F.; Cao, Y. Y. Establishment of the real-time fluorescent PCR detection method for genetically modified GA21 maize. *J. Chin. Biotechnol.* **2003**, *23* (8), 87–95.

(20) Biotechnology & GMOs Unit. CRL-GMFF. Event-specific method for the quantification of maize line GA21 using real-time PCR (validation report). **2003**, available at http://gmo-crl.jrc, 1−10.

(21) Biotechnology & GMOs Unit. CRL-GMFF. GMO specific real-time PCR system protocol for event-species quantification of Bt11 in maize. 2003, 1−6.

(22) China calibration. Protocol of the real-time PCR for detecting genetically modified plants and their derived products. 2004, SN/T 1204-2003, 1−7.

(23) Kim, J.; Demeke, T.; Clear, R. M.; Patrick, S. K. Simultaneous detection by PCR of *Escherichia coli*, *Listeria monocytogenes* and *Salmonella typhimurium* in artificially inoculated wheat. *Int. J. Food Microbiol.* **2006**, *111*, 21–25.

(24) Xu, W. T.; Huang, K. L.; Wang, Y.; Zhang, H. X.; Luo, Y. B. A cotton-specific gene, stearoyl-ACP desaturase, used as a reference for qualitative and real-time quantitative polymerase chain reaction detection of genetically modified organisms. *J. Sci. Food Agric.* **2006**, *86*, 1103–1109.

(25) Biotechnology & GMOs Unit CRL-GMFF. Verification of performances of MON 863 and NK603 event-specific methods on the hybrid MON863 × NK603 using Real-time PCR validation report. CRLVL21/04VR. **2006**, available at http://gmo-crl.jrc.it/doc/Method20%requirements.pdf.

(26) Biotechnology & GMOs Unit CRL-GMFF. Report on the Verification of the performances of 59122, 1507 and NK603 event-specific methods on the hybrid 59122 × 1507 × NK603 using Real-time PCR. CRLVL19/05VR. **2008**, available at http://gmo-crl.jrc.it/doc/Method20%requirements.pdf.

(27) Biotechnology & GMOs Unit. CRL-GMFF. Verification of Performances of NK603 and MON810 Event-specific Methods on the Hybrid NK603 × MON 810 using Real-time PCR. CRLVL21/04VR. **2006**, available at http://gmo-crl.jrc.it/doc/Method20%requirements.pdf.

(28) Akiyama, H.; Watanabe, T.; Wakabayashi, K.; Nakade, S.; Yasui, S.; Sakata, K.; Chiba, R.; Spiegelhalter, F.; Hino, A.; Maitani, T. Quantitative detection system for maize sample containing combined-trait genetically modified maize. *Anal. Chem.* **2005**, *77*, 7421–7428.

(29) Garcia-Canas, V.; Gonzalez, R.; Cifuentes, A. Sensitive and simultaneous analysis of five transgenic maizes using multiplex polymerase chain reaction, capillary gel electrophoresis and laser-induced fluorescence. *Electrophoresis* **2004**, *25*, 2219–2226.

(30) Cankar, K.; Chauvensy-Ancel, V.; Fortabat, M. N.; Gruden, K.; Kobilinsky, A.; Žel, J.; Bertheau, Y. Detection of non-authorized genetically modified organisms using differential quantitative polymerase chain reaction: application to 35S in maize. *Anal. Biochem.* **2008** [doi: 10.1016/j.ab.2008.02.013].

(31) Mackay, I. M.; Arden, K. E.; Nitsche, A. Real-time PCR in virology. *Nucleic Acids Res.* **2002**, *30* (6), 1292–1305.

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