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A High-Throughput Multiplex Method Adapted for GMO Detection

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A high-throughput multiplex assay for the detection of genetically modified organisms (GMO) was developed on the basis of the existing SNPlex method designed for SNP genotyping. This SNPlex assay allows the simultaneous detection of up to 48 short DNA sequences (~70 bp; "signature sequences") from taxa endogenous reference genes, from GMO constructions, screening targets, construct-specific, and event-specific targets, and finally from donor organisms. This assay avoids certain shortcomings of multiplex PCR-based methods already in widespread use for GMO detection. The assay demonstrated high specificity and sensitivity. The results suggest that this assay is reliable, flexible, and cost- and time-effective for high-throughput GMO detection.

KEYWORDS: SNPlex; GMO; detection; multiplex; high-throughput

INTRODUCTION

In the context of food safety concerns, there is an increasing need to be able to detect the presence of foreign DNA in complex samples. This is particularly true for the detection of pathogenic or allergen-producing organisms, such as viruses, fungi, bacteria, and plants in different applications (food, water). Consumer distrust means there is an increasing need to be able to monitor the presence of foreign organisms and the corresponding DNA in food.

In the context of food quality, such needs are also present and have led the European Union (EU) to develop a set of regulations mandating labeling of food and feed products containing, or derived from, genetically modified organisms (GMOs). Thus, there is an ever-increasing need for reliable, low cost, high-throughput standardized assays that can quickly and accurately reveal the presence of any undesired organism or DNA to be detected.

Due to the increased demand for quality and/or safety, single-target detection methods already in widespread use currently are not considered to be sufficient to fulfill the remarkable need for sample analysis in a cost- and time-effective way in private and enforcement laboratories. Accordingly, alternatives aiming to increase sample number and to reduce the time and cost of analysis are urgently sought. DNA-based methods are favored, as DNA is more process resistant and as these methods are more sensitive as well as more flexible. PCR is generally the method of choice in the GMO detection domain as exemplified by the European CRL laboratory (http://gmo-crl.jrc.it/statusofdoss.htm/). Until now the only approach that has been intensively employed is "multiplex" PCR (*1, 2*) combined or not with further microarray hybridization (http://bgmo.jrc.ec.europa.eu/home/documents/report-JRC-EAT.pdf). It has been used for the detection of nine targets simultaneously in one sample (*3*), sometimes with lower sensitivity than simplex PCR.

Two major drawbacks with these multiplex PCR methods, namely, the appearance of amplification artifacts, such as primer-dimers, truncated fragments, or nonspecific amplification products, giving rise to unexpected nonspecific products and thus false positives and the amplification of minor targets. Therefore, the number of simultaneous qualitative PCR amplification reactions is still limited. All of the qualitative and quantitative multiplex methods used in GMO analysis are described in **Table 1**. As seen in in this table, multiplex methods for conventional PCR with agarose gel analyses (*4*) succeeded in one case in the detection of nine targets (nonaplex) at one time that included eight GM maize events and an endogenous reference gene (*ssIIb*) (*5*) and, in another case, seven targets (heptaplex) including five GM maize events (*6*).

Using real-time PCR for quantitative analyses, generally no more than two targets were detected routinely in one tube, due to the technical problems faced, such as the limited number of potential dyes and their fluorescence spectra overlaps (*7, 8*). Furthermore, in most cases, current methods have not been

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Table 1. State of the Art Multiplex Methods Used in GMO Detection[a]

	Assays	Number of multiplexed events	Number of multiplexed targets	References
Qualitative methods	PCR and agarose gel analysis	-	2 (intrinsic gene *papain* and event specific test) using papaya line 55-1	(29)
		-	2 (Pat and Cry1Ab genes) using maize MON810, Bt176, Bt11 and T25	(30)
		-	2 (EPSPS and Tnos) using the soybean GTS40-3-2	(31)
		-	4 (β-actin, P35S, CP4EPSPS, Tnos and 1 endogenous reference gene : lectin) using soybean GTS40-3-2	(31)
		-	3 (barnase, pat and 1 endogenous reference gene : cruciferin) using canola MS8/RF3, MS1/RF2	(31)
		-	3 (TrnL, cruciferin and gox247) using canola GT73	(31)
		8 maize events (NK603, Bt176, T25, GA21, MON863, MON810, TC1507 and Bt11)	9 (8 construct specific: hsp70int/CP4EPSPS, PEPC/Cry1Ab, Pat/Puv18, OTP/mEPSPS, Cry3Bb1/tahsp17, hsp70int/Cry1Ab, CryIFa2/3'polyA, IV6/cry1Ab and 1 endogenous reference gene : ssIIb)	(3)
		5 maize events (Bt176, GA21, MON810, T25 and bt11)	5 construct specific targets (PEPCpro/Cry1Ab, IV6/Cry1Ab, Pat/P35S, hsp70/Cry1Ab, OTP/mepsps)	(32)
		5 (4 maize events: GA21, Bt176, Bt11, MON810 and 1 soybean event GTS40-3-2)	7 (1 screening target: Cry1Ab, 1 event specific target: P35S/Plant, 3 construct specific targets: OTP/mEPSPS, IVS2/Pat and P35S/hsp70 and 2 endogenous genes: Lectin and zein	(6)
	SYBR Green I real time PCR	3 maize events (Bt176, GA21 and MON810)	-	(33)
	PCR coupled with oligonucleotide microarray	7 (6 maize: MON810, Bt176, Bt11, GA21, T25, MON863 and 1 soybean: GTS40-3-2)	3 (2 screening targets: (2 Cry1Ab) and 1 construct specific target: (P35S/hsp70int)	(34)
	PCR coupled with membrane hybridization	-	4 screening targets (nptII, P35S, Cry1Ab, Tnos and 18sRNA using maize event Bt1 and soybean event RR.	(35)
	PCR and ligation detection reaction/universal array technology	4 (3 maize events: Bt11, GA21 and CB351 and 1 soybean event RR)	-	(27)
	PCR coupled with CGE	5 (4 maize events: Bt11, GA21, Bt176 and MON810 and 1 soybean event RR)	7 (5 construct specific: Tnos/CP4EPSPS, P35S/hsp70, Pract/OTP, P35S/Bar, P35S/IVS2) and 2 endogenous reference genes: lectin and zein	(36)
	DNA microarray chip	4 maize events: GA21, Bt11, NK603, MON810	-	(2)
Quantitative methods	Real-time PCR	2 maize events (NK603 and MON810)	2 event specific targets (P35S/Plant and rice actin1 promotor/Plant)	(37)
		2 plant species (Wheat and Barley)	1 endogenous reference gene: PKAB1	(38)
	Real-time PCR with attached universal template probe	-	2 (1 screening target: Cry1Ab and 1 endogenous reference gene: invertase 1 using maize event Bt176	(39)
	Ligation-dependent amplification reaction.	2 (Maize event MON810 and soybean event RR)	2 event specific (P35S/plant) using maize event MON810 and soybean event RR	(40)
	Quantitative DNA array based PCR (MODA-PCR)	7 maize events : Bt176, Bt11, Mon810, T25, GA21, CBH351 and DBT418	-	(1)

[a] The table describes two types of public multiplex studies: the detection of multiple GMO events in the same reaction and the simultaneous detection of many targets using one sample.

adequately evaluated for performance criteria such as the specificity and the sensitivity.

Today, as previously stated by numerous authors [see, for instance, Davison and Bertheau (*9*)], there is a clear need to develop new assays capable of simultaneously detecting multiple target DNA sequences. Assays must be reliable and efficient and show high levels of sensitivity and specificity. Furthermore, to lower costs, they should be adapted for high sample throughput and multiplexing. Accordingly, all new multiplex methods currently developed are qualitative methods, mostly using the "matrix approach" described in the GMOchip European program (www.gmochips.org).

In this framework, our goal was to develop such an assay for GMO detection, considered to be an urgent need for the European community facing a growing number of approved and unapproved GMOs to be detected. For this, we chose to adapt an existing method designed for SNP genotyping, SNPlex, for the qualitative detection of short DNA sequences ("signature sequences") corresponding to GM events and related taxa. The SNPlex technology was developed by Applied Biosystems (*10, 11*) as a high-throughput genotyping method (*12, 13*). Through the use of a set of universal primers and a set of single-nucleotide polymorphism (SNP) specific ligation probes, it is capable of performing genotyping up to a 48-plex level (48 SNPs genotyped in a single reaction). Here, in the place of SNPs, 48 signature sequences are detected that correspond to sequences of GMO construction (screening targets, construct specific targets, and event-specific targets), sequences of plant reference genes, and sequences of donor organisms such as *Agrobacterium tumefaciens*, *Bacillus thuringiensis*, and cauliflower mosaic virus. The sensitivity and specificity were then evaluated, demonstrating the utility of SNPlex for GMO detection.

MATERIALS AND METHODS

Biological Material. Species used in this study included cotton, rice, maize, sugar beet, tomato, potato, rapeseed, and soybean. Twenty-eight samples of GM plants including Certified Reference Material (CRMs) were involved in the collection of samples.

GM Samples. CRMs used in this study were RRS (IRMM-410; 100% GMO), Bt176 (IRMM-411, 5, 1, 0.5, and 0.1%), Bt11 (IRMM-412; 5, 2, 1, 0.5, and 0.1%), MON810 (IRMM-413; 5, 2, 1, 0.5, and 0.1%), GA21 (IRMM-414; 4.3, 1.7, 1, 0.5, and 0.1%), NK603 (IRMM-415; 4.3, 1.7, 1, 0.5, and 0.1%), MON863 (IRMM-416; 9.85, 5, 2, 1, and 0.5%), stacked maize MON863 × MON810 (IRMM-417; 5, 2, 1, 0.5, and 0.1%), and TC1507 (ERM-BF418c; 9.88, 5, 2, 1, 0.5, and 0.1%). Samples were purchased as dried powder from Fluka (Buchs, Switzerland). Maize event Bt10 was isolated from routine samples, the rice event LLrice62 was provided by the DGCCRF (Fraud Repression services, France), and cotton events (LLcotton25, MON531, 1445) were provided by Dr. Dabing Zhang (Shanghai Jiao Tong University, Shanghai, China). Tomato events (NCIMB40015, NCIMB40135) were purchased from the NCIMB collection http://www.ncimb.com/), and sugar beet events (GTSB77 and T120-7) and presumed OXY235, GT73, T45 were verified using already published tests targeting screening elements of the GM construction. MS1, RF1, RF2, RF3, and MS8 canola lines were provided by Bayer Crop Sciences. NK603 grains (Dekalb RR DK 684 RR2) were provided by INTA (Argentina).

Donor Organism Samples. *A. tumefaciens* (At) strain EHA105 and *B. thuringiensis* (Bt) strain DSM6101 were provided by the CFBP, the French collection of phytopathogenic bacteria (http://www-intranet.angers.inra.fr/cfbp/) and from the DSMZ (Deutsche Sammlung von Mikrooganismen and Zellkulturen GmbH). Cauliflower mosaic virus (CaMV) strain CabSS was provided by Dr. P. Yot (CNRS, Strasbourg, France) (*4*). Those samples were included in the panels to avoid false positives resulting from infection(s) of plant material.

Genomic DNA Extraction and Quantification. DNA was extracted from GM and non-GM plants either from frozen leaves or CRM powder. The CTAB method was used for DNA extraction as described in EN ISO 21571 (*14*). The concentration of genomic DNA was determined by Nanodrop (ND-1000 spectrophotometer, Chatsworth, CA).

Conventional PCR Conditions. All qualitative PCR amplifications (see Supporting Information Table 1 for primers) were run on MJ Research thermocyclers (Bio-Rad) in a final volume of 25 μL. Each reaction mixture had 1× PCR buffer, 0.2 mM dNTP, 0.4 μM of each primer, 5 ng/μL of each DNA sample, and 1 unit of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). Gels were scanned by Image Master VDS (Amersham Pharmacia Biotech, Amersham, U.K.).

Sequencing of PCR Products. The same primer pairs used for PCR were used for sequencing. The PCR products were purified using P100 (beads for DNA filtration) (Bio-Rad Bio-Gel R P-100 Gel Fine 45−90 μm). Sequencing was performed in a 10 μL reaction containing 10 nmol/L of one primer (forward or reverse), 1 μL of BigDye Terminator Cycle Sequencing mix (Perkin-Elmer), 1 μL of BigDye buffer, 5 μL of H_2O, and 2 μL of the purified PCR product. Purification of this reaction was performed using G50 gel filtration [Sephadex TM G-50 superfine (Amersham Biosciences AB)] and loaded onto an ABI3730XL 96 capillary sequencer. Sequence alignments was performed using the Multialign program (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html), and detection of polymorphism was performed using the software GENALYS, available at http://software.cng.fr (*15*). All of the sequences used in this study will be available in databases in the context of the European Coextra project.

SNPlex Assay. Sequence alignments were used to choose target sequences to be detected. These were submitted to Applied Biosystems (http://www.appliedbiosystems.com/) for panel design.

The SNPlex assay (Applied Biosystems) was carried out using the manufacturer's instructions (http://www.appliedbiosystems.com), taking care to perform pre-PCR and post-PCR steps in different locations. This protocol was modified as follows:

DNA concentration was increased and ranged from 38 ng/μL to 1000 ng/μL. DNA fragmentation was performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems) with 5 min at 99 °C followed by incubation overnight at room temperature.

To increase the specificity and sensitivity of the assay, the oligonucleotide ligation assay (OLA) reaction was performed using double the quantity of probes called for in the Applied Biosystems protocol.

Once the hybridization was achieved, universal PCRs were carried out using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems) with 10 min at 95 °C and 35 cycles of 15 s at 95 °C and an extension step at 63 °C for 1 min. The quantity of PCR universal primers was also doubled to increase the amplification efficiency.

Ninety-six ZipChute probes (Applied Biosystems, Foster, CA), labeled with two different fluorophores (green and red) were used. In our case, only 48 were detected, because our aim is not genotyping but signature sequence detection. This revealing step was performed as follows: 4 μL of the biotinylated amplicons was transferred into a streptavidin-coated microplate using the automated robot TAP (The Automation Partnership, Hertfordshire, U.K.) and incubated for 1 h at 20 °C. The plate was then loaded into an ABI 3730XL 96 capillary sequencer (Applied Biosystems), and results were analyzed using GeneMapper software.

Study of the Sensitivity (Absolute and Relative Limits of Detection, LODa and LODr). For the determination of LODa, several independent DNA dilution series of different GMO events were tested and ranged from 1 μg/μL to 0.5 ng/μL. To optimize sensitivity, some assay parameters were slightly adjusted as follows: DNA concentrations were increased from 50 ng/μL to 1 μg/μL in the final reaction, and fragmentation time was 4 min instead of 3 min. In the OLA reaction, concentrations of probes and universal primers were doubled, and the number of PCR cycles was increased from 30 to 35. Increase in probe and universal primer concentrations led to stronger fluorescence signals and therefore better sensitivities at low target concentrations.

For the determination of the LODr, artificial mixtures of DNA containing known percentages of GMO in the presence of non-GMO "ballast" plant DNA were performed. Percentages ranged from 10 to 0.01% of input DNA. In addition, all of the certified reference materials (CRMs) available in our laboratory with percentages ranging from 0.1 to 5% GMO percentages were used.

RESULTS

Adaptation of SNPlex to Sequence Detection. The principle of the SNPlex technology (**Figure 1**) relies on three main steps: the OLA using target-specific probes and two common steps including PCR amplification with universal primers and the decoding using universal probes called ZipChutes containing fluorophores and unique structures allowing their differentiation during electrophoresis. The first adaptation concerned the detection of signature sequences instead of SNPs, as seen in the example of P35S described in **Figure 1**. For this, in a given sequence to be detected, a nucleotide was randomly designated an SNP. The second adaptation concerned the modification of some parameters of the protocol, as described under Materials and Methods, to increase the sensitivity and the specificity of the method.

Choice of Targets. The signature sequences to be detected include three types of sequences: sequences used in GMO constructions (screening, construct-specific, and event-specific targets), sequences from plant taxa-specific reference genes (for a discussion on reference genes, see ref *16*), and, finally, sequences derived from donor organisms that allow the detection of false-positive results such as those arising from the presence of P35S, *Cry1A*(b) or Tnos elements from a possible plant infection with the cauliflower mosaic virus, *Bacillus thuringiensis*, or *Agrobacterium tumefaciens*, respectively.

Target sequences to be detected in this SNPlex experiment were chosen among all of the different PCR tests commonly used for the detection and quantification of specific taxa, GMO, or donor organisms. The corresponding signature sequences were chosen to represent a maximum of public primers corresponding to transgenic sequences available in databases such as NCBI and databases developed in previous European projects [for example, the GMOchip project (http://www.bats.ch/

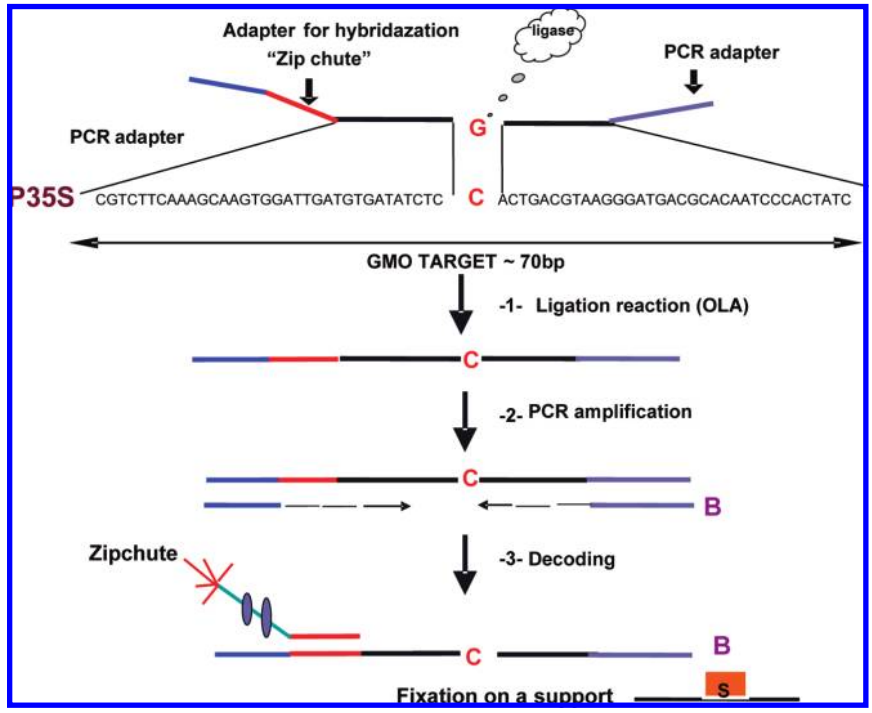

Figure 1. Schematic representation of the SNPlex assay for the detection of a common screening target, the promoter 35S. Three main reactions are described: the OLA (oligonucleotid ligation assay) reaction, PCR amplification with the universal primers, and the final detection called "decoding" using the ZipChute probes. Two probes are used in the first reaction, OLA. The first one is composed of three parts: from 5′ to 3′, the PCR adapter (blue and gray), the adapter for hybridization of the ZipChute probes used in the final step of detection (red), and the complementary nucleic acid sequence (~35 bp) (black). The second probe of the OLA reaction is composed only of the complementary sequence and the PCR adapter. During the OLA reaction, the two probes hybridize to the selected target due to their complementary parts. Once the correct recognition of the allele is performed, the ligation takes place with a "ligase". After that, PCR amplification is performed using universal primers (one of which is biotinylated), generating a chimeric DNA fragment. This biotinylated amplicon is then captured onto a support containing streptavidin (S). Finally, ZipChute probes labeled with two different fluorophores (green and red colors) that specifically anneal to their corresponding adapter in the chimeric DNA fragment are loaded in the 3730XL sequencer and generate fluorescent signal. These ZipChute probes contain a unique structure that enables their size differentiation during electrophoresis.

gmochips/)] and allowed us to sequence all of the targets used in this study and finally assemble 79 sequences used in previous PCR-based methods.

Sequencing and Polymorphism. Because tests already described for some GMO detection use different primers with different amplicon sizes, it was important to verify the exact sequences of each target amplicon. Additionally, these tests have been conducted using few GMO events. For example, for detecting the terminator *nos*, different PCR-based methods were developed using (1) maize events Bt11, MON809, and GA21 (*17, 18*), (2) maize Bt11, MON802, MON810, and GA21 (*19*−*21*), soybean event RRS (*3, 22, 23*), and (3) tomato Zeneca and Huafan No. 1 and (4) using transgenic tobacco (*24*). Few of the tests have been validated through interlaboratory studies (*25*) or by the Community Reference Laboratory (http://gmo-crl.jrc.it/statusofdoss.htm).

Signature sequences were therefore amplified and sequenced. Alignments performed using Multialign software (*26*) did not reveal any polymorphism in comparison with the sequences in the databases except for two common screening targets: *Cry1A*(b) in the three maize transformation events Bt11, Bt176, and MON810 and CP4 EPSPS in soybean RR, presumed canola GT73, sugar beet GTSB77, and cotton 1445. Because of the observed polymorphism, it was impossible to use a consensus sequence for the detection of *Cry1A*(b) and CP4 EPSPS in the corresponding GMO events. Finally, three sequences of *Cry1A*(b) and two sequences of CP4 EPSPS were included for the detection of the corresponding GMO event. The observed polymorphism pointed out that sequencing is required when new sequence-based assays such as PCR, microarray, or SNPlex are designed.

The designed panels were submitted to Web software (Applied Biosystems) to test the compatibility of the chosen sequences and to provide a maximum number of sequences to be multiplexed. In our case, the virtual SNPs chosen were not fixed, and therefore their positions could be moved. Thus, an allele was chosen empirically and designated arbitrarily as an SNP and submitted to ABI. Finally, of 79 SNPs (here, signature sequences to be detected) submitted for validation, one panel of 47 sequences (panel 1) and one of 48 (panel 2) were retained and ordered. Because of the redundancy of the signature sequences (several sequences to detect one GMO element), each of the two panels covered the totality of the sequences to be detected. All of the elements of the designed panels are described in **Table 2**.

Assessment of the Performance Criteria. *Specificity of the SNPlex Assay.* The first tests were performed using all of the plant collection in duplicate. The first panel (47 probes) was used to assess specificity. Specific fluorescent signals were obtained for 41 of 47 probes in the first panel. The first panel harbored three nonspecific probes (junction P35S/Pat, P35S/plant of RRS event, and maize EPSPS of the GA21 event) and three nonfunctional probes (junction IV6/P35S, *Cry1A(b)*/hsp70, and P35S/plant of the 1445 cotton event) that could be due to probe design, failure in probe synthesis, or the assay itself. Due to the nonspecific and nonfunctional probes observed in the first

Table 2. Description of the Multiplexed Targets Grouped into the Two Studied Panels[a]

	TARGETS				
PANEL 1 (47 markers)	**Taxa**	**Screening**	**Construct specific**	**Event specific**	**Donor organisms**
	Potato (*UGpase*)	Cry1Ac	P35s/Bar	RRS (P35S/plant)	CaMV
	Tomato (*lat52*)	NptII	P35s/CP4EPSPS1	MON810 (Cry1Ab/plant)	*Bacillus thuringiensis*
	Maize (*adh1*)	Pat	P35s/Pat (SS3)	Bt11 (Tnos/plant)	*Agrobacterium tumefaciens*
	Sugarbeet (*gln. synthase*)	T35S	PG/Tnos	Cotton MON531 (Cry1Ac/plant)	
	Rapeseed (*BnACCg8*)	Bar	Tnos/Pract	Cotton 1445 (P35S/plant)	
	Cotton (*sad*)	CP4EPSPS1*	Pract/mEPSPS	MON863 (P35S/plant)	
	Rice (*ppi-PPF*)	P35S (SS1)	Ivs6/P35S	T25 (P35S/plant)	
	Wheat (*wax*)	Tnos	Cry9c/P35S	CBH351 (Tnos/plant 1)	
		Pnos	OTP/mEPSPS	Bt10 (Tnos/plant)	
		Nitrilase	Cry1Ab/hsp70	Bt176 (Bar/plant)	
		Barstare		TC1507 (Pat/plant)	
		PFMV		T25 (pUc18/plant)	
		mEPSPS		CBH351 (Tnos/plant 2)	
	TARGETS				
PANEL 2 (48 markers)	**Taxa**	**Screening**	**Construct specific**	**Event specific**	**Donor organisms**
	Potato (*UGpase*)	Cry1Ac	P35S/Bar	RRS (P35S/plant)	CaMV
	Tomato (*LAT52*)	NptII	P35S/CP4EPSPS1	MON810 (Cry1Ab/plant)	*Bacillus thuringiensis*
	Maize (*adh1*)	Pat	P35S/Pat (SS4)	Bt11 (Tnos/plant)	*Agrobacterium tumefaciens*
	Soybean (*lectin*)	T35S	Tnos/Nitrilase	Cotton MON531 (Cry1Ac/plant)	
	Sugarbeet (*Gln synthase*)	Bar	PG/Tnos	Cotton 1445 (P35S/plant)	
	Rapeseed (*BnACCg8*)	CP4EPSPS1*	Tnos/Pract	MON863 (P35S/plant)	
	Cotton (*Sad*)	CP4EPSPS2*		T25 (P35S/plant)	
	Medicago (*acc*)	Cry1Ab1*		CBH351 (Tnos/plant)	
	Barley (*γ-hordein*)	Cry1Ab2*		Bt10 (Tnos/plant)	
	Rice (*ppi-PPF*)	Cry1Ab3*			
	Sunflower (*hel*)	Cry9C			
	Wheat (*Wx-D1*)	P35S (SS2)			
		Tnos			
		Pnos			
		Ntitrilase			
		Barnase			
		Barstare			
		PFMV			

[a] CP4EPSPS1 (soybean event RR sequence), CP4EPSPS2 (sugar beet event GTSB77 sequence); Cry1Ab1, 2 and 3 correspond to Bt11, Bt176, and MON810 sequences. SS, signature sequence. For the targets P35S and P35S/pat, the signature sequences were different between the first and second panels.

panel, a second panel was designed containing 48 probes (panel 2). All results presented below concern only the second panel. The latter showed only two nonspecific probes (CP4 EPSPS2 and junction PG/Tnos), which probably can be explained by the presence of other similar sequences present in the genomes of the assayed plants (respectively, EPSPS and PG proteins). For example, the CP4 EPSPS2 sequence derived from *A. tumefaciens* used in our study showed around 80% of homology to EPSPS protein I from *Arabidopsis thaliana* (accession no. AK227120). In the second panel, 45 of 48 probes gave fluorescent signals only with the expected targets, showing the high specificity of the assay. For example, the probe corresponding to the sequence of *bar* (**Figure 2A**) gave specific signals only with the expected GMO events (Bt176, RF1, RF2, MS8, and LLrice62). For the construct-specific element (**Figure 2B**), the probe corresponding to P35S/*bar* hybridized only to expected events (Bt176 and LLrice62). For the event-specific T25 probe (**Figure 2C**), we observed a specific hybridization to its specific target. Also, the rice endogenous reference gene (*ppi-PPF*) probe (**Figure 2D**) hybridized only with rice samples. Finally, **Figure 2E** shows the specific detection of *A. tumefaciens* using the *ChVh* probe. These examples illustrate the level of specificity obtained for all of the functional probes in the two panels.

Nonspecific fluorescent signals are sometimes observed. A value known as the "cutoff" value for the signal is calculated from the mean value of the nonspecific signals plus 2.5 times the standard deviation (SD) (*2*). Positive signals were also compared to "no template control" (NTC) values. Signal intensities (SI), standard deviations (SD), and the measured cutoff (Co) values using the example of the canola GMO event Topas19/2 are given in **Table 3**. This example demonstrated that up to seven targets could be detected in one tube. The signal intensity differed among the targets of the panel. Relatively low signals were observed with T35S in maize T25 or canola Topas19/2 events. This is probably due to the fact that the specific target is an "AT" rich sequence (low melting temperature). In the second panel, the "taxa probes" corresponding to publicly available reference genes allowed the recognition of 12 different taxa (maize, soybean, rapeseed, rice, cotton, tomato, potato, sugar beet, sunflower, wheat, barley, and alfalfa). None of the taxa probes hybridized with nontarget DNA when the

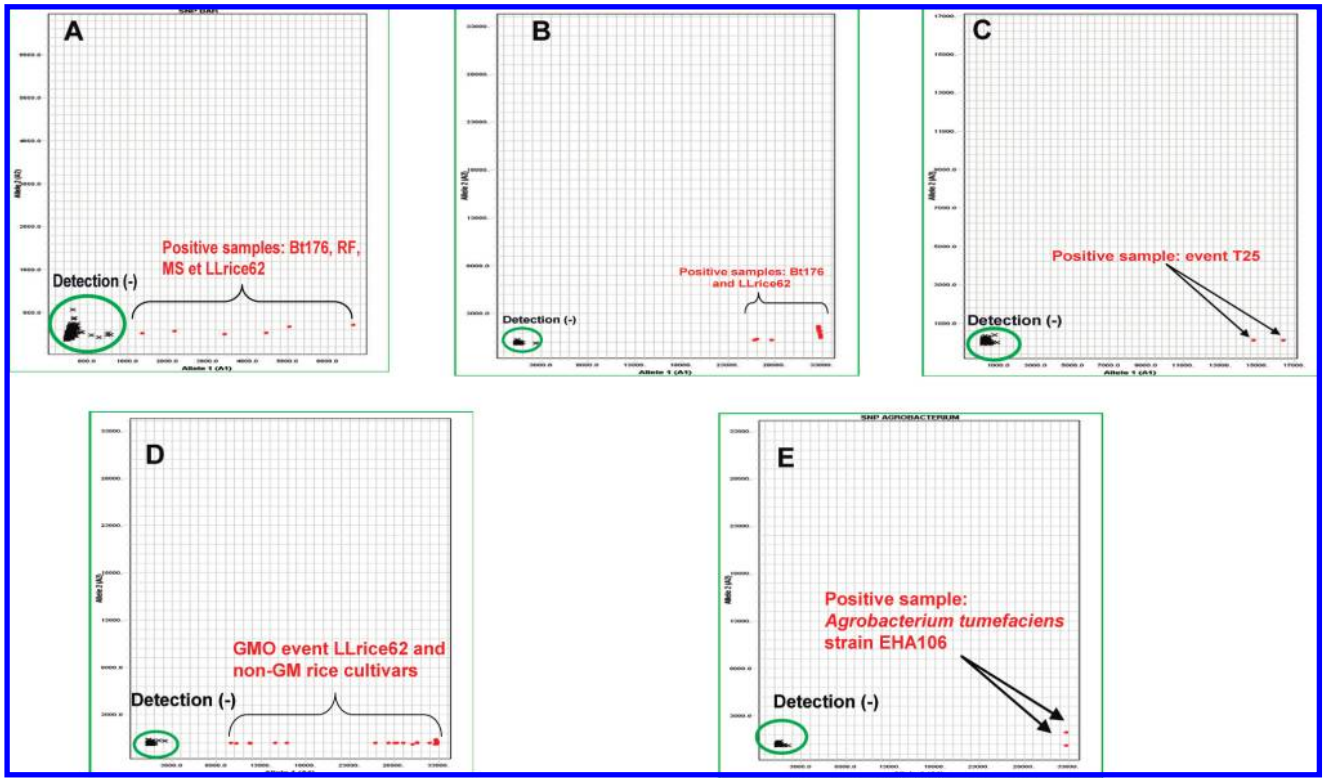

Figure 2. Examples of specificity of GMO detection with the SNPlex assay (panel 2): (**A**) positive and specific detection (red points) of a GMO common screening target (bar) in maize event Bt176, rice event LLRICE62, and canola events RF1, RF2, MS1, and MS8; (**B**) positive and specific detection of a GMO construct target (junction between the promoter 35S and the gene *bar*) in events Bt176 and LLRICE62; (**C**) positive and specific detection of a GMO identification target (junction between the promoter 35S and the plant genome) in event T25; (**D**) positive and specific detection of the rice endogenous reference gene (*ppi-PPF*) in rice event LLRICE62 and all of the tested rice cultivars; (**E**) positive and specific detection of the gene *ChVh* in *Agrobacterium tumefaciens*.

Table 3. Example of Results Generated for the Identification of Canola Event TOPAS19/2 Using 50 ng of DNA[a]

Endogenous references

Targets	Potato (*UGpase*)	Tomato (*LAT52*)	Maize (*adh1*)	Soybean (*lectin*)	Sugarbeet (*Gln synthase*)	Canola (*BnACCg8*)	Cotton (*Sad*)	Medicago (*acc*)	Barley (*γ-hordein*)	Rice (*ppi-PPF*)	Sunflower (*hel*)	Wheat (*Wx-D1*)
Mean SI	421	442	461	419	525	2147	414	463	552	459	487	458
Mean SD	29	55	63	22	36	156	28	11	2	6	15	51

Screening sequences

Targets	Cry1Ac	NptII	PAT	T35S	Bar	CP4EPSPS1	CP4EPSPS2	PFMV	Cry9C
Mean SI	411	2016	1236	1596	452	458	6125	466	436
Mean SD	90	288	365	177	11	74	187	24	43

Targets	Ntitrilase	Barnase	Barstare	Cry1A(b)1	Cry1A(b)2	Cry1A(b)3	P35S	Tnos	Pnos
Mean SI	467	386	499	454	491	485	2214	426	1633
Mean SD	13	23	45	20	36	17	453	2	512

Construct specific sequences

Targets	P35S/Bar	P35S/CP4EPSPS1	P35S/PAT	Tnos/Nitrilase	PG/Tnos	Tnos/Pract
Mean SI	621	541	1332	531	2964	532
SD	85	102	362	66	85	13

Event specific sequences

Targets	RRS (P35S/plant)	MON810 (Cry/plant)	Bt11 (Tnos/plant)	Coton 531 (Cry/plant)	Coton 1445 (P35S/plant)	MON863 (P35S/plant)	T25 (P35S/plant)	CBH351 (Tnos/plant)	Bt10 (Tnos/plant)
Mean SI	489	445	478	458	463	512	632	547	528
Mean SD	14	52	47	12	26	43	22	28	36

Donor organisms sequences

Targets	CaMV	Bac. thuringiensis	Agro. tumefaciens
Mean SI	525	558	445
Mean SD	15	13	27

[a] Data for the seven expected targets of the event assayed were detected, and their corresponding mean SI values are in red. The cutoff and NTC values here are 483 and 35, respectively. Two targets, CP4EPSPS2 and PG/Tnos, gave rise to nonspecific signals (blue) and were excluded from the analysis.

following plants were tested: eggplant, pepper, *A. thaliana*, cauliflower, cabbage, tobacco, and white bean (Supporting Information Table 2). The high specificity demonstrates the potential of this SNPlex assay as a reliable, precise, and rapid qualitative detection method. The experiment presented here also demonstrates the versatility of the SNPlex assay for multiplex detection of a large number of GMO events, thereby reducing labor and expense.

Sensitivity Determination: Absolute and Relative Limits of Detection (LODa and LODr). The limit of detection (LOD) is

Table 4. Determination of the Absolute Limit of Detection (LODa) Using All GMO Signature Sequences of the Second Panel[a]

	GMO events	Mean $LODa_{(SI)}$	SD_{LODa}	Mean $LODa_{(ng/\mu l)}$	$SD_{(LODa)}$
Maize	Bt176	328	25.41	8	4.47
	T25	660.4	73.37	**10**	0
	GA21	706	45.6	8	3.46
	NK603	366	72.06	7.25	3.2
	MON863	313.4	49.17	**14**	14.74
	Bt11	420.4	94.37	8.8	2.68
	Bt10	432.6	72.74	**10**	0
	MON863xMON810	528	57.37	16.4	3.77
	MON810	311.6	26.14	**12**	2.73
	TC1507	189.25	63.4	11.25	2.5
	CBH351	382.57	45.67	16.42	4.75
		Mean – 421.49	Mean – 58.59	**Mean= 11.1**	Mean – 3.06
Canola	TOPAS19/2	589.57	99.72	8.57	2.43
	T45	452.5	24.44	11.25	**4.7**
	OXY235	490	150.62	8.5	3
	GT73	572	228.4	**10**	0
	RF1	459	71.32	**10**	0
	RF2	439.6	86.6	**10**	0
	RF3	607.2	79.37	**10**	0
	MS8	729.25	102.53	8.75	0
		Mean= 542.39	Mean = 105.37	**Mean = 9.63**	Mean = 1.26
Soybean	RRS	460.6	55.34	**9.16**	2.04
Rice	LLRICE62	426	110.5	**8.8**	2.68
Cotton	Cotton event 1445	430.33	65.53	**15**	4.08
	Cotton event 531	430	65	21.6	9.3
	LLcotton25	614	218.44	10	0
		Mean= 495	Mean = 145.48	**Mean = 15.53**	Mean = 4.46
Tomato	NCIMB40015	455.5	52.57	**10**	0
	NCIMB40134	499.26	43.36	**10**	0
		Mean – 701.41	68.76	**Mean = 10**	Mean – 0
Sugarbeet	GTSB77	250.4	77.6	**10**	0
	T120-7	200.33	27.71	11.66	2.58
		Mean = 225.36	52.65	**Mean = 10.83**	**Mean = 1.29**

[a] All LODa mean values range from 10 to 15 ng/μL (bold). SD, standard deviation; SI, signal intensity.

the lowest amount or concentration of analyte in a sample that can be reliably detected with an acceptance criterion; the LOD should be less than $^1/_{20}$ the target concentration. In this study, the LOD was considered as the lowest DNA concentration for which signal was observed superior to the cutoff value. Two LODs were assessed, the LODa, using independent dilution series of the template, and the LODr, using independent dilution series (percentages) in the presence of non-GMO DNA. The LODr assesses whether the assay is reliable for the detection of GMO in complex samples and whether the presence of foreign DNA affects the detection.

Using the dilution series and the optimized conditions described under Materials and Methods, the LODa was calculated for all of the GMO elements of the second panel including screening, construct-specific, and event-specific targets. As shown in **Table 4**, the mean LODa was 11.1 ng/μL for GM maize, 9.63 ng/μL GM canola, 9.16 ng/μL GM cotton, 8.8 ng/μL for GM soybean, 10.83 ng/μL for GM sugar beet, 8.8 ng/μL for GM rice, and 10 ng/μL for GM tomato. Supporting Information Table 3 shows the values obtained for the determination of the LODa of all the GMO elements of the second panel including screening, construct-specific, and event-specific targets.

The signature sequences were clearly detected from samples with a mean LODr of 0.65% for GM maize, 0.36% for GM canola, 0.67% for GM cotton, 0.18% for GM soybean, 0.78% for GM sugar beet, 0.2% for GM rice, and 0.15% for GM tomato, as shown in **Table 5**. Supporting Information Table 4 shows the LODr of all the GMO elements of the second panel. The sensitivity observed is acceptable to monitor the reliability of the current European 0.9% labeling system. All LODa and LODr values including mean SI, cutoff, and mean SD values are given in the Supporting Information (Tables 5−32 for LODa determination and Tables 33−60 for LODr determination). As described in **Tables 4** and **5**, variability was observed when the LODa and LODr values were calculated, which can be explained by the different levels of hybridization of the target depending on the sequence itself and on the samples (method of extraction, uncertainties in DNA quantification...).

DISCUSSION

Given the requirements for international trade of GMOs and new labeling regulations, there is an urgent need to develop high-throughput, multiplex GMO assays that provide accurate, repeatable, and specific results at a reasonable cost. Our objective was to develop such an assay. Here, we describe an adapted SNPlex protocol that is capable of detecting many signature sequences for the qualitative detection of GMOs.

SNPlex, developed by ABI for SNP genotyping, relies on an oligonucleotide ligation assay (OLA) using target specific probes, followed by amplification using a set of universal primers and, finally, a "decoding" step that uses patented ZipChutes, modifications that permit electrophoretic separation

Table 5. Determination of the Relative Limit of Detection (LODr) Using All GMO Signature Sequences of the Second Panel[a]

	GMO events	Mean $LODr_{(SI)}$	$SD_{(LODr)}$	Mean $LODr_{(\%)}$	$SD_{(LODr)}$
Maize	Bt176	854.75	225.34	0.5	0
	T25	711.8	150.61	0.52	0.31
	GA21	775.5	176	0.5	0
	NK603	1045	292.26	0.5	0
	MON863	546.33	120.68	**1**	0.86
	Bt11	497.6	102.7	0.23	0.23
	Bt10	634.33	75.22	0.36	0.23
	MON810	686.33	110.81	0.5	0
	MON863xMON810	611.16	165.95	0.6	0.34
	TC1507	562.66	116.76	0.83	0.28
	CBH351	949.6	116.65	0.66	0.25
		Mean = 653.52	Mean = 150.27	**Mean = 0.65**	0.21
Canola	TOPAS19/2	543.33	116.59	0.15	0.17
	T45	636.6	141.14	0.36	0.23
	OXY235	794.5	192.16	0.3	0.23
	GT73	519	108.89	0.5	0
	RF1	760	75.31	0.36	0.23
	RF2	713.25	129.38	0.4	0.2
	RF3	784.3	163	0.36	0.23
	MS8	701.3	22.59	0.5	0
		Mean = 681.53	Mean = 118.63	**Mean = 0.36**	0.16
Soybean	RRS	772.4	191.78	**0.18**	0.17
Rice	LLRICE62	423.5	65.19	**0.2**	0.2
Cotton	Cotton event 1445	805.83	211.88	0.6	0.34
	Cotton event 531	803.2	57.03	0.9	0.65
	LLcotton25	697.3	230.07	0.53	0.45
		Mean = 768.77	Mean = 166.32	**Mean = 0.67**	0.48
Tomato	NCIMB40015	459.5	133.65	0.2	0.17
	NCIMB40134	609.75	88.09	0.1	0
		Mean = 534.62	Mean = 110.87	**Mean = 0.15**	0.08
Sugarbeet	GTSB77	964.75	239.86	0.87	0.25
	T120-7	930.4	231.16	0.7	0.27
		Mean = 947.57	Mean = 235.51	**Mean = 0.78**	0.26

[a] All of the observed LODr (bold) are lower than the threshold required for GMO labeling (0.9%). SD, standard deviation; SI, signal intensity.

of the different products. We have described an assay in which an "artificial" SNP is designated in a target sequence, thereby allowing its detection by SNPlex.

This assay is ideal for our purpose because up to 48 signature sequences are detected in a sample, and four 384-well plates can be processed simultaneously in just 3 days. This will permit the "bulk sample" to be divided into subsamples and consequently increase the probability of GMO detection and also to increase the number and size of laboratory samples analyzed. Its applicability in routine detection laboratories still needs further studies using a cost-benefit analysis approach.

The oligonucleotide ligation (OLA), which can occur only if the correct sequence is present, ensures specificity, even in the case that signature sequences (and not SNPs) are to be detected. No signal was observed when nontarget DNAs was used, except in two cases, probably due to the presence of similar sequences in the genomes of the assayed plants (EPSPS and PG). Thus, the ligation step is considered to be key for the specificity of our assay.

Another important factor in the success of this assay is the use of universal amplification primers. This avoids constraints faced in classic multiplex PCR in which a large set of primer sequences is assembled to achieve suitable differences in amplicon molecular weights. The use of universal primers avoids overlaps of unextended primers and the possible generation of nonspecific products, and the use of ZipChutes allows electrophoretic discrimination of all products. This, in turn, allows multiplexing that greatly surpasses what has previously been reported (*26*), but which is required to detect the large number of authorized and nonauthorized GMO currently in use, as well as those in development.

In addition to assessing specificity, we looked at another important performance criterion, the sensitivity. This was measured using two different parameters, the absolute and the relative limits of detection (LODa and LODr). For all of the GMO samples used, the mean LODa was estimated to range from 8 ng/μL to 15 ng/μL and the mean LODr from 0.2 to 0.8%. The level of sensitivity of the SNPlex assay is therefore suitable for routine GMO analysis scoring below the Russian and EU set threshold, where the maximum level of fortuitous presence of GMO has been set at 0.9%. Nonetheless, when calculating the mean values of the signal intensities, cutoff, and standard deviations, we observed variability that can affect the sensitivity. The origin of this variability is thought to be related to DNA quality and/or quantity, which can be influenced by the source of the material used for DNA extraction (leaves, fruit, flour, root...), the method used for DNA extraction, and the method used for DNA quantification. This will require further investigation to eliminate sources of variability.

This assay, in addition to showing high sensitivity and specificity, offers the advantage of being extremely cost-efficient. Experimental costs are amortized by the high degree

of multiplexing involved, resulting in a very inexpensive assay. We compared costs per sample to those based on PCR methods. Using the qualitative SNPlex assay when screening at least 384 samples is 10-fold less expensive than qualitative simplex PCR per data point. This is clearly advantageous when a large number of samples are to be analyzed (*27*).

The SNPlex assay presented here thus offers several advantages over conventional multiplex PCR. However, as is the case for any "sequence-based" assay, knowing the exact nucleic acid sequence of the target is crucial. In this case, an incorrect sequence will lead to the design of probes in the OLA reaction that cannot hybridize to the correct target, leading to false-negative results. For this reason, all potential targets were resequenced, and in some cases, new probes were designed as a function of the polymorphism present. Differences were observed between theoretical descriptions and our sequenced constructions, for example, for the maize event T25 (*28*). This highlights the importance of databases, such as the Biosafety Clearing House of the Cartagena protocol, that should contain accurate sequences of not only approved transgenic constructs but also unapproved ones.

The challenge of GMO detection is the ability to detect GMO sequences in complex samples that include highly processed samples such as food- and feedstuffs containing degraded DNA. For this, our SNPlex assay offers the further advantage that the total length of the sequences flanking the SNP to be detected is ~70 bp. This feature should permit the detection of fragmented nucleic acid sequences and, thus, will increase the probability of detecting DNA traces in such complex samples. Moreover, its high multiplexing capacity means that numerous targets can be detected in a single sample—targets that correspond to multiple different elements that make up a construction, including screening, construct-specific sequences, and identification sequences as well as taxa-specific reference genes and donor organism sequences.

In conclusion, we have demonstrated the successful adaptation of the SNPlex assay for high-throughput GMO and taxa detection in one step. Due to its flexible multiplexing capacity, SNPlex is a highly cost-effective alternative technique to the current state of the art techniques for GMO detection purposes. This technology is an obvious choice for this application because it combines a ligation step, required for specificity, and an amplification step, required for sensitivity, critical parameters to ensure the reliability of the method. The reported assay was shown to be feasible for GMO detection complying with the regulatory "threshold". Furthermore, to our knowledge, no such level of multiplexing for sequence detection has been reported so far. We expect that "SNPlexing" using panels such as those in this study will progressively replace qualitative multiplex PCR-based methods for routine GMO analysis. Other technologies should be tested in the near future for a higher level of multiplexing such as Illumina "GoldenGate assay" or "Veracode", genotyping techniques, which are able to detect more than 1536 SNP (http://www.illumina.com/).

Finally, the SNPlex assay should be adapted for a variety of applications for the detection of sequences derived from other organisms such as pathogens (viruses, fungi, and bacteria), because many of their complete genome sequences are available through Web databases.

ABBREVIATIONS USED

Acc, acetyl-CoA carboxylase; Adh1, alcohol dehydrogenase-1 intron I; At, *Agrobacterium tumefaciens*; Bar, phosphinothricin acetyltransferase, gene issue from *S. hygroscopicus*; Barnase, protein with ribonuclease activity from *Bacillus amyloliquefaciens* that is toxic in the absence of its inhibitor, Barstar; Barstar, inhibitor of Barnase derived from *B. amyloliquefaciens*; Bt, *Bacillus thuringiensis*; BnACCg8, *Brassica napus* acetyl-CoA carboxylase; CAE, capillary electrophoresis; CaMV, cauliflower mosaic virus; ChVh, chromosomal virulence genes derived from *A. tumefaciens*; Co, cutoff; CP4-EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase, isolated from *Agrobacterium* sp. (strain CP4); Cry1Ab δ-endotoxin, a synthetic version of the δ-endotoxin insecticidal protein, Cry1Ab, derived from *B. thuringiensis* subp. *kurstaki* strain HD-1; Cry1Ac δ-endotoxin, a modified gene (cry1Ac) that encodes an insecticidal Cry1Ac δ-endotoxin protein, derived from the soil bacterium *B. thuringiensis* subsp. *kurstaki* (B.t.k) strain HD-73; Cry9C δ-endotoxin, a chimeric modified insecticidal gene (cry9C.PGS2a) derived from *B. thuringiensis* subsp. *Tolworthi*; CTAB, cetyltrimethyl ammonium bromide; DNA, deoxyribonucleic acid; EC, European Commission; EDTA, ethylenediaminetetraacetic acid; ENGL, European network of GMO laboratories; EU, European Union; GMO, genetically modified organism; Gln, glutamine; hel, helianthine; hsp70, heat-shock protein 70, intron from the hsp70 gene (heat-shock protein); IRMM, Institute for Reference Material and Measurements; LODa, absolute limit of detection; IV6, intervening sequence 6 intron derived from the maize gene adh1 (alcohol dehydrogenase-1S gene); LODr, relative limit of detection; maize-EPSPS, a modified form of wild type 5-enolpyruvyl-3-phosphoshikimate synthase gene from *Zea mays* that encodes an insensitive enzyme to inactivation by glyphosate; NHC, negative hybridization controls; Nitrilase, also called oxy or BXN, gene isolated from *K. pneumoniae* subspecies *ozaenae*; NptII, neomycin phosphotransferase:aminoglycoside (3′) phosphotransferase type II gene from *Escherichia coli* transposon Tn5 (or kanamycin resistance gene); NTC, no template control; OLA, oligonucleotide ligation assay; OTP, N-terminal chloroplast transit peptide (CTP); P-35S, promoter 35S originating from cauliflower mosaic virus; PAT, gene coding for a phosphinothricin acetyltransferase from *Streptomyces viridochromogenes*, homologue to bar; PCR, Polymerase Chain Reaction; PFMV, a promoter derived from figwort mosaic virus (FMV); PG, polygalacturonase; PHC, positive hybridization controls; ppi-PPF, pyrophosphate-phosphofructokinase; Pract, 5′ region of the rice actin 1 gene containing the promoter and first intron; pUC18, sequence of high copy *E. coli* plasmid pUC18 used for cloning of DNA sequences; RRS, Roundup Ready soybean; Sad, stearoyl-ACP desaturase; SD, standard deviation; SDS, sodium dodecyl sulfate; SNP, single-nucleotide polymorphism; SPR, surface plasmon resonance; T35S, terminator 35S originating from cauliflower mosaic virus; Tnos, terminator nopaline synthase; Pnos, promoter nopaline synthase originating from *A. tumefaciens*; UGpase, UDP-glucose pyrophosphorylase.

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Supporting Information Available: Sequences of all primers used to obtain sequences for constructed panels, non-GM samples used for specificity assessment, and determination of absolute and relative LODs. This material is available free of charge via the Internet at http://pubs.acs.org.

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