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Development of a Real-Time PCR Method Based on Duplo Target Plasmids for Determining an Unexpected Genetically Modified Soybean Intermix with Feed Components

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The occurrence of intermixing, especially that resulting from genetically modified (GM) species, is increasingly becoming a problem in the delicate chain of feed and food quality control. Thus, a strategy is needed for precisely quantifying the presence of intermixing. An analytical assay based on real-time PCR has been developed; it can ascertain the extent of unexpected intermixing of GM soybean with maize meal. Three soybean−maize mix levels, with soybean intermix percentages of, respectively, 0.1, 0.5, and 1%, were prepared to simulate samples containing traces of soybean. As calibrator standards, ad hoc multiple-target pGEM-T plasmids containing soybean and maize reference genes in a 1:1 ratio were constructed. Four different maize endogenous genes, alcohol dehydrogenase 1 (*adh1*), high-mobility group protein a (*hmg*a), invertase 1 (*ivr1*), and zein (*zein*), were assessed, each combined with the soybean endogenous lectin 1 (*lect1*) gene. Plasmids containing *adh1*−*lect1* and *zein*−*lect1* genes were found to be the most reliable calibration systems for this analysis, providing precise and accurate quantification results. Measuring the percentage of GM soybean intermixing makes it possible to calculate the actual transgenic component of the total sample.

KEYWORDS: Genetically modified organism; intermix; feed; soybean; maize; real-time PCR; endogenous gene; duplo target plasmid

INTRODUCTION

Maize (*Zea mays* L.) and soybean (*Glycine max* L.) raw stuffs and derived products are primary components of animal feed. Considered to elite energy and protein sources for animal nutrition (*1*, *2*), they are consistently employed in a diverse range of complete, complementary, and concentrated feed formulations. Various alternatives have been proposed for a proper protein supply, including sunflower, canola, broad bean, pea, faba, and lupin (*3*), but the use of soybean remains an essential aspect of today's animal husbandry (*1*, *4*, *5*).

In Europe, whereas domestic production of maize satisfies local demand, only a tiny percent of the soybean used is grown and processed within the European Union (EU). Thus, overall, at least 95% of raw and processed soybean used for feed preparation is imported, mostly from the United States, Argentina, Canada, and Brazil (*6*). These are countries where cultivation and commercialization of genetically modified (GM) plants and their products is allowed and where traceability regulation is peculiar for each state and is less restrictive with respect to Europe's (*7*−*12*).

Trade in the raw material of a variety of agrofood goods is a complex procedure often affected by the occurrence of unwanted intermixing. Intermixing is the result of careless handling in various steps of product importation during the transportation, stocking, and distribution (*13*). As a consequence, traces of GM soybean may be present in a stock of supposedly GM-free soybean. Accordingly, several laboratories in charge of food security analysis commonly detect GM soybean in food and feed where the species should not be present (personal communications). In addition, our experience in analyzing samples of individual feed components (maize, pea, wheat, barley, sunflower, and flax) and formulations of fodder of different sources (mixtures of maize and barley) confirms the frequent presence of soybean that is not declared on the label and which is often transgenic at high percentages. In maize matrixes, for instance, we have often detected the presence of transgenic sequences that are not event-specific, for example, the 35S-CaMV promoter. Here, further assays excluded origin from maize and proved that soybean was the cause of the transgenic occurrence (unpublished data).

Such unexpected intermixing is emerging as a problem in the delicate chain of feed and food quality control. EU regulations on GM food and feed, while establishing precise traceability and labeling rules that also contemplate the occurrence of accidental GMO contamination on stuff of the same species (*14*, *15*), at present do not consider this kind of mixing. The increasing relevance of its occurrence and stakeholder concern (*16*), however, are expected to push authorities and laboratories in charge of official GMO analysis to also consider

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this problem in official food and feed analysis. This matter represents an interesting example of the close link between the evolution of regulation and technological advances and focuses on the duty of institutions to take into account the concerns of the public.

Specifically, there is a need for a strategy to precisely quantify the amount of nondeclared species in a sample, characterize the GM material, and finally calculate the percentage of the transgenic component in the whole sample. Although quantification of intermixing is becoming a crucial part of GM detection, no literature is available on the matter, although several protocols are already established for GMO detection (*17*). Thus, within the framework of a project that aims to develop suitable analytical methodologies for tracing GMOs in the food and feed chains, we developed an analytical assay based on real-time PCR that is capable of ascertaining the extent of soybean intermix in a feed sample. To develop a model to systematically detect soybean intermix, we simulated various mixture levels (0.1, 0.5, and 1%) with a single-component feed (maize). This matrix was chosen because maize is the main energy source in animal nutrition (*6*).

For building up the standard curves, we constructed ad hoc multiple-target plasmids (*18*). In particular, we used duplo target plasmids, also called dual amplicons (*19*). This type of plasmid has been described in the literature, and their use in qualitative and quantitative detection of GM material in food and feed sample has been documented (*19*, *20*). The duplo target plasmids that we used are DNA plasmids that contain a hybrid amplicon (*21*) carrying, in tandem orientation, the selected sequences of the reference species-specific endogenous genes respectively for soybean and maize, in a 1:1 ratio. Plasmids are progressively becoming valuable calibration standards for standard curve construction during real-time PCR analysis and have proved to be reliable and practical alternatives to genomic DNA extracted from the certified material (*18*−*20*, *22*, *23*).

Moreover, we assessed four different maize endogenous genes, with the aim of exploiting the most suitable to be present in the same plasmid with the proper soybean one. Accordingly, four kinds of plasmids were prepared, all of them containing the soybean gene for lectin 1 (*24*) with, alternatively, the maize genes for the alcohol dehydrogenase 1 (*25*), the high-mobility group protein a (*26*), the invertase 1 (*27*), and the zein (*28*).

MATERIALS AND METHODS

Preparation of Soybean−Maize Mix Test Samples. Maize of an old autochthonous variety of the Garda lake area of the province of Trento (Italy) was kindly provided by the Technical Assistance Centre (CAT) of IASMA. Kernels were milled to a fine powder in an electric grinder, and the obtained meal was sieved through a 40 mesh screen sieve (Sigma). Comparison of the DNA extraction efficiencies from the ground maize and the certified maize standard Fluka was performed on the genomic DNA extracted from six aliquots of 150 mg for both meals. DNA concentrations were measured with a spectrophotometer (Biophotometer, Eppendorf), and extraction yield was calculated according to the following formula: micrograms of total extracted DNA/grams of maize meal quantity used for extraction.

To assemble the samples that simulate soybean intermix with maize meal, three 1 g aliquots of powdered maize were mixed thoroughly in plastic bags with 1, 5, and 10 mg of certified soybean powder (standard Fluka 5% RR Soybean) to obtain, respectively, 0.1, 0.5, and 1% mix levels. Each of these blends was homogenized in 5 mL of extraction buffer composed of 50 mM CTAB, 1.4 M NaCl, 100 mM Tris-HCl, and 20 mM EDTA for 30 min at 65 °C with shaking. The whole homogenized mixture was centrifuged (10 min at 13200 rpm), the supernatant was divided into three identical aliquots, and all of them were used for the subsequent DNA extraction.

DNA Extraction. Genomic DNA was independently extracted from the three aliquots of each soybean−maize mix test sample (0.1, 0.5, and 1%), as well as from the crushed maize meal and the certified soybean powder (standard Fluka 5% RR Soybean), according to the cetyltrimethylammonium bromide (CTAB) extraction protocol (*29*), resuspended in distilled water, and quantified by UV spectrophotometer (Biophotometer, Eppendorf). The extracted DNA was stored in aliquots at −20 °C.

Qualitative Check for Soybean Presence. The qualitative PCR reactions for *lectin 1* gene amplification were performed on genomic maize DNA extracted from the crushed kernels (expected soybean-free meal) and the ad hoc contaminated test samples (soybean−maize mix levels of 0.1, 0.5, and 1%), using the Thermocycler (Tgradient, Biometra) in a final volume of 25 μL, containing 0.625 unit of the Ampli*Taq* Gold (Applied Biosystems), 0.5 μM of each primer (primer Fw, gccctctactccaccccatcc; primer Rv, gcccatctgcaagcctttttgtg, according to ref *30*), 200 μM of each dNTP, 2.5 mM $MgCl_2$, and 100 ng of DNA. The PCR thermal protocol consisted of a first denaturing step of 9 min at 95 °C followed by 40 cycles of denaturation, annealing, extension of, respectively, 30 s at 95 °C, 30 s at 65 °C, and 60 s at 72 °C, and a final extension of 5 min at 72 °C. The PCR products (10 μL) were electrophoresed at a constant voltage (100 V) with loading buffer (Promega) and Sybr Gold 10 X (Molecular Probes) in a 2% agarose gel (Sigma), and the gel was scanned by Gel Doc 2000 (Bio-Rad).

Quantitative Real-Time PCR. The real-time PCR reactions were performed in 96-well reaction plates on the iCycler iQ Thermocycler (Bio-Rad), in a 25 μL final volume containing 1× Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 200 ng of genomic DNA, 0.3 μM primers, and 0.2 μM specific Taqman probe (5′ FAM−3′ TAMRA, Sigma) as shown in **Table 1**. The thermal protocol was as follows: UDG PCR decontamination for 2 min at 50 °C and for 2 min at 95 °C, followed by 50 cycles of denaturation, annealing/extension of, respectively, 15 s at 95 °C and 1 min at 60 °C. Fluorescence signals were collected during the annealing/extension phase. For each calibration system, three distinct PCR runs were performed; the standard curves were built using five decreasing concentrations of each plasmid (described below) in a serial dilution of 1:6 (500 000, 83 333, 13 888, 2314, and 385 copies). Nuclease-free water was used as negative control. Plasmids and samples were analyzed in triplicates. The copy numbers of soybean and maize endogenous genes were calculated by the iCycler iQ optical System software version 3.0a (Bio-Rad) as mean values of the three replicate threshold cycles (Ct) on the basis of the standard curves obtained. The percentages of soybean present in the three soybean−maize mix levels were calculated with the following formula: [soybean gene copy number/(maize gene copy number × 2.4)] × 100, where the corrective value 2.4 is the ratio between the maize (2.73 pg) and soybean (1.13 pg) haploid genome (*32*) sizes (Plant DNA C-values Database, Royal Botanic Garden, Kew, U.K., 2005).

Construction of the Duplo Target Plasmids. As calibrators in the real-time PCR assays, four different plasmids were developed, each containing both the maize and soybean sequences of selected reference endogenous genes in tandem orientation. In this first PCR step, the genes for the soybean lectin 1 (*lect1*) and for the maize alcohol dehydrogenase 1 (*adh1*), high-mobility group protein a (*hmg*a), invertase 1 (*ivr1*), and *zein* (*zein*) were separately amplified using specific primers sets, as reported in **Table 2**. The *lect1* forward primer contained a 20 nt 5′-terminal sequence extension complementary to the 20 nt 5′-overhangs of the *adh1*, *hmg*a, *ivr1*, and *zein* reverse primers to allow the hybridization of the complementary ends during the second PCR step that creates the hybrid amplicon (*21*). The first-step PCRs were performed in a final volume of 25 μL, containing 0.625 unit of the Ampli*Taq* Gold (Applied Biosystems), 0.5 μM of each primer (**Table 2**), 200 μM of each dNTP, 2 mM $MgCl_2$, and 100 ng of DNA extracted from maize and soybean meals. Amplifications were carried out in the PCR Thermocycler (Tgradient, Biometra), with the following thermal protocol: a first denaturing step of 9 min at 95 °C followed by 45 cycles of denaturation, annealing, extension of, respectively, 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C, and a final extension of 5 min at 72 °C. PCR products were analyzed on a 2% agarose gel as described above. Assembly of the four couples of the maize and soybean

Table 1. Sequences of the Primers and Dual-Labeled Taqman Probes Employed in the Real-Time PCR Assays[a]

gene	accession no.		sequence	bp	ref
lect1	K00821	Pr.Fw	5′CGGCACCCCAAAACCC3′	79	Bio-Rad real-time PCR OGM Course, Torino, Italy, 2001
		Pr.Rv	5′GCTACCGGTTTCTTTGTCCCA3′		
		probe	5′CTCTTGGTCGCGCCCTCTACTCCAC3′		
adh1	X04050	Pr.Fw	5′CGTCGTTTCCCATCTCTTCCTCC3′	136	Hernandez et al. (*34*)
		Pr.Rv	5′CCACTCCGAGACCCTCAGTC3′		
		probe	5′AATCAGGGCTCATTTTCTCGCTCCTCA3′		
hmga	AJ131373	Pr.Fw	5′TTGGACTAGAAATCTCGTGCTGA3′	79	Hernandez et al. (*34*)
		Pr.Rv	5′GCTACATAGGGAGCCTTGTCCT3′		
		probe	5′CAATCCACACAAACGCACGCGTA3′		
ivr1	U16123	Pr.Fw	5′CGCTCTGTACAAGCGTGC3′	104	Hernandez et al. (*34*)
		Pr.Rv	5′GCAAAGTGTTGTGCTTGGACC3′		
		probe	5′CACGTGAGAATTTCCGTCTACTCGAGCCT3′		
zein	X07535	Pr.Fw	5′TGCAGCAACTGTTGGCCTTA3′	72	Bio-Rad real-time PCR OGM Course, Torino, Italy, 2001
		Pr.Rv	5′TCATGTTAGGCGTCATCATCTGT3′		
		probe	5′CATCACTGGCATCGTCTGAAGCGG3′		

[a] The accession numbers of the genes are according to EMBL/GenBank official numbers.

Table 2. Sequences of Primers for the Two-Step Hybrid Amplicon Preparation[a]

PCR step	gene		sequence	bp
first	*lect1*	Pr.Fw	5′**ACAGTTGAGCTCGACGCATT**AAACGGCACCCCAAAACC3′	116
		Pr.Rv	5′GCGAAGCTGGCAACGCTA3′	
	adh1	Pr.Fw	5′CGTCGTTTCCCATCTCTTCCTCC3′	156
		Pr.Rv	5′**AATGCGTCGAGCTCAACTGT**CCACTCCGAGACCCTCAGTC3′	
	ivr1	Pr.Fw	5′CGCTCTGTACAAGCGTGC3′	124
		Pr.Rv	5′**AATGCGTCGAGCTCAACTGT**GCAAAGTGTTGTGCTTGGACC3′	
	hmga	Pr.Fw	5′TTGGACTAGAAATCTCGTGCTGA3′	99
		Pr.Rv	5′**AATGCGTCGAGCTCAACTGT**GCTACATAGGGAGCCTTGTCCT3′	
	zein	Pr.Fw	5′TGC-AGC-AAC-TGT-TGG-CCT-TA3′	92
		Pr.Rv	5′**AATGCGTCGAGCTCAACTGT**TCATGTTAGGCGTCATCATCTGT3′	
second	*adh1-lect1*	Pr.Fw	5′CGTCGTTTCCCATCTCTTCCTCC3′	252
		Pr.Rv	5′GCGAAGCTGGCAACGCTA3′	
	ivr1–lect1	Pr.Fw	5′CGCTCTGTACAAGCGTGC3′	220
		Pr.Rv	5′GCGAAGCTGGCAACGCTA3′	
	hmga–lect1	Pr.Fw	5′TTGGACTAGAAATCTCGTGCTGA3′	195
		Pr.Rv	5′GCGAAGCTGGCAACGCTA3′	
	zein–lect1	Pr.Fw	5′TGC-AGC-AAC-TGT-TGG-CCT-TA3′	188
		Pr.Rv	5′GCGAAGCTGGCAACGCTA3′	

[a] Complementary 5′ overhangs for the hybridization of the complementary ends in the further hybrid amplicon construction are in bold.

endogenous genes (*adh1*−*lect1*, *hmg*a−*lect1*, *ivr1*−*lect1*, and *zein*−*lect1*) was performed in a second step of PCR reactions in 25 μL reaction mixtures, using 0.625 unit of the Ampli*Taq* Gold (Applied Biosystems), 0.3 μM of each primer (**Table 2**), 200 μM of each dNTP, 2 mM $MgCl_2$, and 0.2 μL of the two PCR products obtained during the first PCR step. All amplifications were performed with the following thermal protocol: a first denaturing step for 9 min at 95 °C followed by 45 cycles of denaturation, annealing, extension for, respectively, 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C, and a final extension of 5 min at 72 °C. Aliquots of the PCR products were run on a 2% agarose gel, as described above, and quantified in comparison with a standard quantity of Lambda DNA (New England Biolabs-Celbio). The four couples of amplified reference genes were cloned into the pGEM-T Easy vector (Promega) with the T4 DNA ligase (Promega) at an optimal 1:3 vector/insert molar ratio, during a 4 °C overnight incubation, and transferred to *Escherichia coli* strain JM109, according to the Promega technical manual. The plasmids were extracted and purified from the selected colonies with the QIAprep[R] Spin Miniprep Kit (Qiagen) and eluted in 100 μL of nuclease-free water (Promega). Plasmid concentrations were measured at 260 nm with the BioPhotometer (Eppendorf). Their respective molarities in the solutions were calculated according to the nomogram for double-stranded DNA (*31*). The inserted fragments were checked by sequencing (CRIBI, Padova). Plasmid solutions were diluted in TE buffer (pH 8.0) at a concentration of 10^8 copies/μL, subdivided into 100 μL aliquots, and stored at −20 °C.

RESULTS AND DISCUSSION

We propose an analytical assay for estimating the soybean rate and its GM percentage in a maize meal, as a consequence of an unexpected soybean intermix.

First of all, the method makes it possible to quantify the contaminating soybean rate in the maize sample. Then, in a real sample, the percentage of the GM soybean in the contaminating soybean would be quantified according to already established protocols available in the literature (*17*). In our model system, conversely, this latter analysis has been skipped, being the minor aspect of the assay. Accordingly, to simulate the contaminating soybean, the certified standard Fluka at known RR percentage has been used.

Of the overall strategy, we detail here the method we developed for determining the intermix occurrence of soybean with maize meal, this being the innovative part of the assay and the object of the present paper.

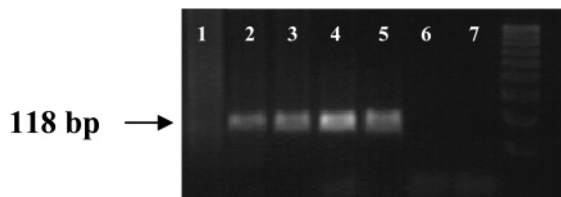


Figure 1. Qualitative check of soybean presence. PCR products of the lectin 1 gene amplification were electrophoresed on a 2% agarose gel. Lanes: 1, expected soybean-free crushed maize kernels; 2, 3, 4, soybean–maize test samples with mixes at, respectively, 0.1, 0.5, and 1% mixing levels; 5, certified standard Fluka 5% RR as positive control; 6, 7, nuclease-free water as negative control.

Preparation of Soybean−Maize Mix Test Samples. The preparation of the test samples simulating the intermixing of soybean with maize meal was a crucial preliminary step of our analysis. In the view of simulating a realistic mingling level that would involve small amounts—usually traces—of soybean, three maize−soybean mixing levels, 0.1, 0.5, and 1%, were carefully prepared. To increase their homogeneity, each mixture was entirely dissolved in the extraction buffer and subsequently divided into three aliquots, and all of them were used for the following DNA extraction.

To obtain test samples adequate for the analysis, soybean and maize powdery base materials need to be pure and sufficiently homogeneous. As for soybean, certified standard Fluka 5% RR that has a verified rate of purity and homogeneity was employed. For maize, we used kernels collected from a variety of the Garda lake area of the Trento Province (Italy) that are cultured according to the market trend of recovering valued genotypes of the local tradition. Maize kernels were ground to ensure a homogeneous distribution of their different parts and sieved to select the particles with the smallest size possible. Moreover, to evaluate the adequacy of the maize meal particle size, DNA extraction efficiencies from the ground maize and the certified maize standard Fluka were compared. Their average extraction yields were, respectively, 196 and 61 μg/g, showing a higher value for the "home-made" powder and proving its suitable granulometry. Moreover, maize meal purity was confirmed by a qualitative PCR on genomic DNA where the amplification of soybean *lectin 1* gene proved the absence of contaminating soybean (**Figure 1**).

Soybean and Maize Endogenous Genes. In our method, the estimation of the entity of soybean intermix with maize is based on the calculation of the ratio between the endogenous species-specific reference genes, respectively, for maize and soybean, assessed with the real-time PCR analysis.

A crucial aspect of this strategy is the selection of the suitable endogenous genes that have to fit some basic requirements, such as species specificity, no intraspecific variability, and the presence in one or a low copy number in the genome (prEN ISO 2426 and 21569). For soybean, we employed lectin 1, a widely studied endogenous gene (*24*, *33*) nowadays mainly used in the routine and official GMO detection (prEN ISO 21570, *11*).

Regarding maize, currently, among the various taxon-specific genes studied, there is still the need to find the endogenous gene considered to be the most suitable one for species identification and quantification in the different cultivars. A variable copy number presence seems to be one of the major problems (*34*). Among the various systems described in the literature, we exploited the genes for the alcohol dehydrogenase 1, invertase 1, high-mobility group protein a, and zein. These four maize genes were described as suitable for real-time PCR assays in many maize varieties, proving low variability among cultivars, species specificity, and low copy number for haploid genome (*34*, *35*). PCR systems specific for the maize endogenes have been developed within the framework of a project coordinated by the Institute for Health and Consumer Protection of the European Commission (Joint Research Centre, Ispra, Italy) (*34*).

Real-Time PCR Setup. The real-time PCR amplification performances of the four maize reference endogenous genes systems were compared on 100 ng of DNA extracted from the soybean-free maize meals. The reaction conditions applied were the ones that proved to be optimal for the lectin 1 gene, as described under Materials and Methods. These, in fact, needed to be optimal for both soybean and maize reference endogenous genes, being simultaneously amplified during the same PCR run. As shown in **Figure 2**, the mean values of the threshold cycles (Ct) obtained in three replicates for the *adh1*, *hmg*a, and *zein* genes were similar (Ct ∼ 24). Conversely, the value obtained for the *ivr1* gene was higher (Ct = 27.5).

Duplo Target Plasmids. A relevant aspect of our method is the development of dual amplicon plasmids to be used as calibrators to establish the standard curves in the real-time PCR assays. Four different sets of plasmids were constructed, where the soybean lectin 1 gene was alternatively linked to the maize genes for the alcohol dehydrogenase 1, invertase 1, high-mobility group protein a, and zein, with the view of exploiting the best dual system. The target sequences for the maize and soybean endogenous genes were linked together during a two-step PCR reaction producing a hybrid amplicon (*21*). In our experience, during GMO detection linear hybrid amplicons present low molecular stability, resulting in being less adapted as standard calibrators. This limitation was overcome by inserting these sequences into the pGEM-T easy plasmids and using them in the circular configuration to be more protected against degradation effects. Moreover, to ensure a high stability and long-lasting preservation, they were stored at −20 °C in TE buffer (pH 8.0) (*19*).

The precision and stability performances of the circular pGEM-T easy plasmid calibrators were assessed in two distinct real-time PCR assays, where decreasing amounts of the lectin 1 gene inserted in pGEM-T plasmid were amplified. This gene was chosen as it was the endogenous reference gene present in each of the four calibration plasmids exploited in this study.

The method showed high precision levels in the range of 10 000 to 50 gene copy numbers (**Figure 3**). In fact, the repeatability standard deviation values (RSDr %) calculated for the 10 000, 1000, 200, and 50 copy numbers were <25%, thus fitting the minimum performance requirements recognized by the European Network of GMO Laboratories (*36*) for estimating the proficiency of a real-time PCR assay.

Assays were conducted for evaluating the calibration performances of each of the four plasmid systems, considering three crucial parameters of the standard curves, that is, dynamic range, correlation coefficient (R^2), and amplification efficiency related to slope. As for the dynamic range, according to our previous experience (data not shown) we chose to adopt the plasmid serial dilution 1:6 with five concentration points, starting from 500 000 copy numbers. In addition, R^2 and slope values were acceptable (**Figure 4**).

The suitability of the plasmid calibration system was assessed in an ad hoc experiment, where the reaction efficiencies obtained with plasmid versus genomic DNA have been compared. Amplifications of the soybean and maize endogenes were conducted on three decreasing concentration points of plasmidic and genomic DNA in a real-time PCR, at the same reaction

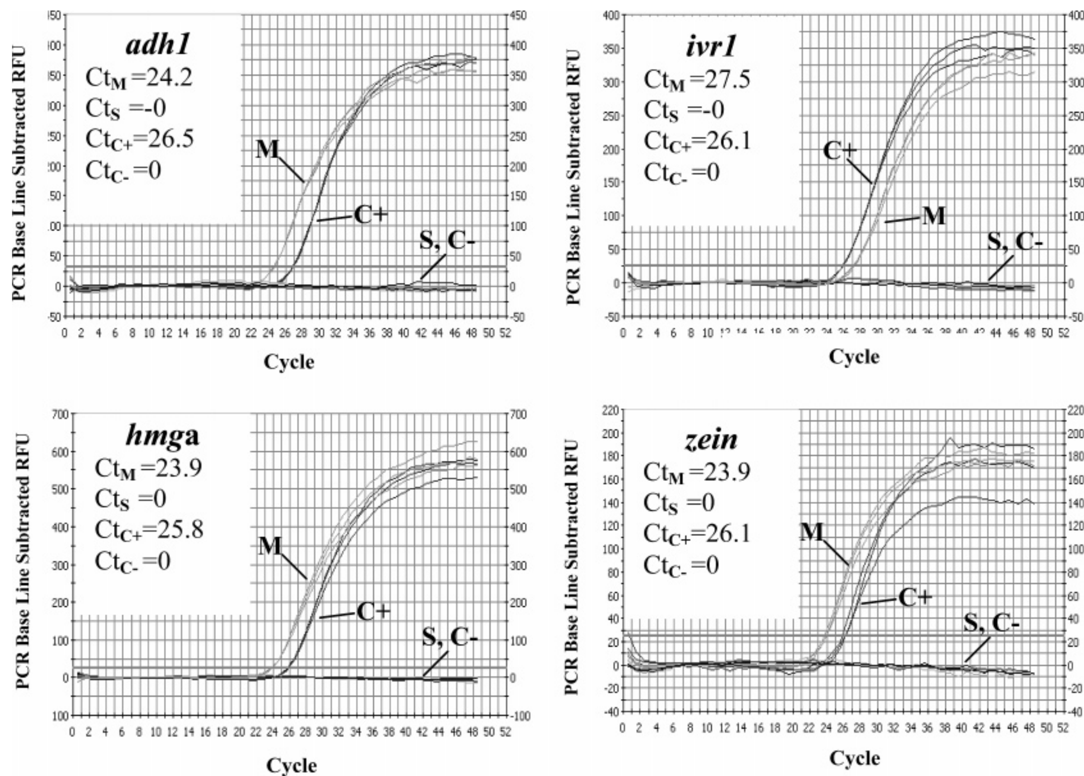
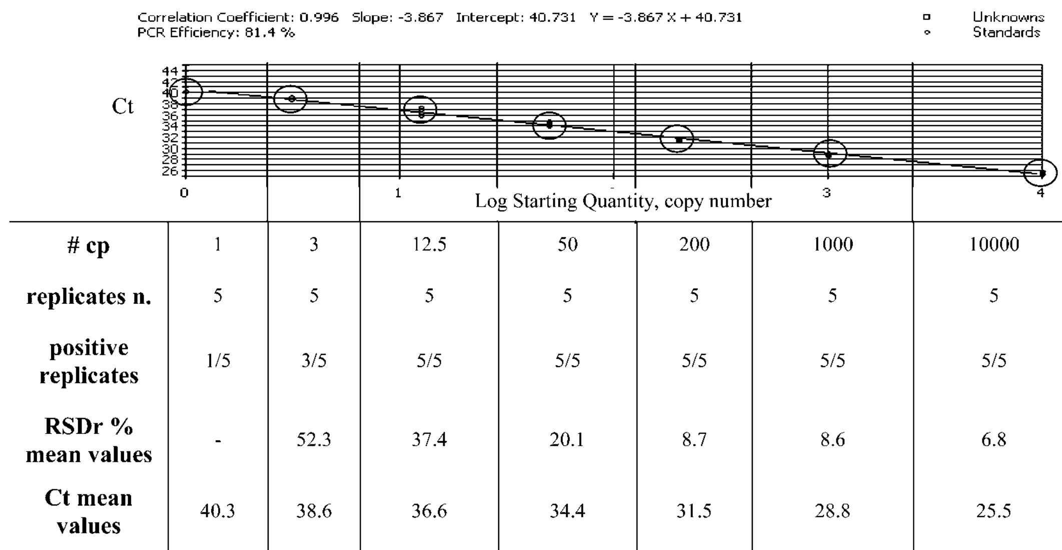


Figure 2. Real-time PCR setup. Real-time PCR amplification plots are shown for *adh1*, *ivr1*, *hmg*a, and *zein* genes on 100 ng of maize genomic DNA and on 50 ng of certified standard Fluka 5% RR genomic DNA. M, expected soybean-free maize crush; S, soybean standard Fluka 5% flour; C+, plasmid containing the amplified sequence used as positive control; C–, nuclease-free water; Ct_M, Ct_S, Ct_{C+}, Ct_{C-}, values of the threshold cycles (Ct) obtained, respectively, for maize, soybean, and positive and negative controls.



# cp	1	3	12.5	50	200	1000	10000
replicates n.	5	5	5	5	5	5	5
positive replicates	1/5	3/5	5/5	5/5	5/5	5/5	5/5
RSDr % mean values	-	52.3	37.4	20.1	8.7	8.6	6.8
Ct mean values	40.3	38.6	36.6	34.4	31.5	28.8	25.5

Figure 3. Precision assay on a multiple-target plasmid in circular configuration. The standard curve was obtained during a real-time PCR assay with decreasing copy numbers (10000–1) of pGEM-T easy plasmid calibrators for the soybean lectin 1 gene. RSDr measures are the mean values obtained in two real-time experiments in five replicates for the plate.

conditions, in triplicate. **Table 3** reports the results of this assay. The PCR efficiencies calculated from the slope values are comparable between the two systems and fit the ranges recommended by ENGL method performance requirements (*36*).

DNA plasmids as standard curve calibrators have recently been proposed as valuable alternatives to genomic DNA of certified standards in GMO quantification assays based on real-time PCR. They show high performance, a potential to assess a wide range of transgenic events, long-term stability, and simple and low-cost production (*18*−*20*, *23*, *37*). Single- and multiple-target plasmids containing more than one target sequence on the same molecule have been compared extensively in previous studies, the latter proving to be the most promising ones (*18*). This is the rationale for exploiting the dual plasmid system containing a hybrid amplicon in the present study.

Quantification of the Soybean Intermixing with Maize. Four methods based on standard calibration plasmids were exploited to quantify soybean occurrence in the three levels (0.1, 0.5, and 1%) of soybean−maize mix samples specifically prepared. The aim of this assay was to identify the method that provides the best accuracy and precision performances in quantification.

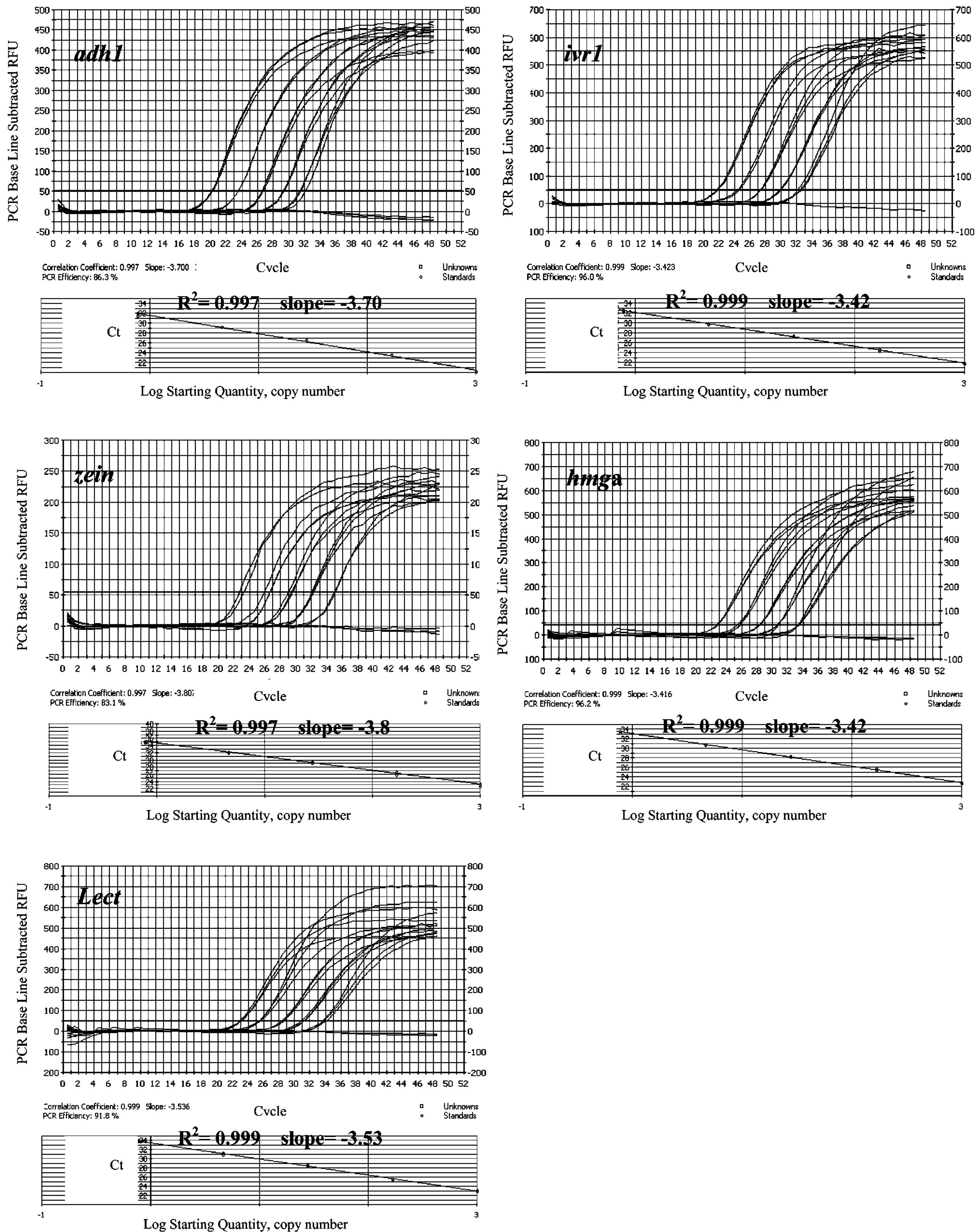


Figure 4. Evaluation of the calibration performances of the four plasmid systems: choice of real-time PCR amplification plots and standard curves with the relative correlation coefficient (R^2) and slope values, obtained for the *adh1*, *ivr1*, *hmg*a, *zein,* and *lect1* target sequences carried from the pGEM-T plasmids.

Three distinct DNA extracts of each mix level were quantified in three independent real-time PCR runs, in triplicate, for each plasmid calibration system, in order to obtain at least eight quantification measures. According to the EU recommendation (*32*), the most suitable unit for expressing a GM content is "the percentage of GM-DNA copy number in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes". In our model system, however, we wanted to take into account the ratio between two different species. For this reason, we considered the difference of the genomic weights

Table 3. PCR Efficiencies of the Plasmidic and Genomic DNA Calibration Systems[a]

gene	DNA	slope	PCR efficiency (%)
adh	plasmidic	–3.315	100.3
	genomic	–3.400	96.8
hmga	plasmidic	–3.419	96.1
	genomic	–3.596	89.7
ivr	plasmidic	–3.412	96.4
	genomic	–3.031	113.7
zein	plasmidic	–3.165	107.0
	genomic	–3.408	96.5
lect	plasmidic	–3.252	103.0
	genomic	–3.205	105.1

[a] The PCR efficiencies are calculated from the slope values according to the following formula: PCR efficiency = [10(–1/slope)] – 1.

Table 4. Quantification Results of the Soybean Intermix with Maize[a]

plasmid system	mean %	RSDr %	CI		t
	Soybean–Maize Mix 0.1%				
pGEM(*adh1–lect1*)	0.094	17	0.083	0.105	0.2
pGEM(*hmga–lect1)*	0.029	39	0.021	0.037	2.8*
pGEM(*ivr–lect1*)	0.23	32	0.18	0.28	3.7*
pGEM(*zein–lect1*)	0.091	6.5	0.087	0.095	0.4
	Soybean–Maize Mix 0.5%				
pGEM*(adh1–lect1*)	0.41	12	0.38	0.44	1.7
pGEM(*hmga–lect1)*	0.11	28	0.088	0.13	7.6*
pGEM(*ivr1–lect1*)	1.0	30	0.79	1.22	4.3*
pGEM(*zein–lect1*)	0.34	20	0.29	0.38	3.0*
	Soybean–Maize Mix 1%				
pGEM*(adh1–lect1)*	1.1	17	0.95	1.2	0.63
pGEM(*hmga–lect1)*	0.32	23	0.27	0.37	6.6*
pGEM(*ivr1–lect1*)	2.4	38	1.7	3.0	4.1*
pGEM(*zein–lect1*)	0.84	13	0.76	0.91	1.5

[a] Mean quantification values (mean %), relative standard deviations (RSDr %), confidence intervals (CI) at 95%, and *t* values at 95% were obtained during the analysis of the three (0.1%, 0.5% and 1%) soybean-maize mix levels, with the four different plasmid methods (*adh11–lec*, *hmga–lec*, *ivr–lec*, *zein–lec*). The tabulated *t* value for 7 degrees of freedom at 95% is 1.90. *, calculated *t* value > tabulated *t* value; i.e., experimental values significantly different from expected values.

between soybean and maize. Consequently, once the copy number value of the target endogenous genes was estimated, the soybean–maize ratio was calculated and expressed as a percentage, according to the following formula: [soybean gene copy number/(maize gene copy number × 2.4)] × 100. We point out that in the formula, the difference in the maize and soybean haploid genome size, respectively, 2.73 and 1.13 pg (according to the Plant C-value Database of the Royal Botanic Garden, Kew, U.K.), was overcome by considering the corrective ratio (2.73/1.13 = 2.4) between the two haploid genome sizes. The percent soybean values (mean value of eight measures) calculated for each of the three mixes represent the "test results" of our analysis and are reported in **Table 4** (mean percent) together with the results of statistical analysis. The precision (the measure of repeatability) of the method was checked through the evaluation of the relative standard deviations (coefficients of variation, RSDr %). Among the four plasmids, the *adh1–lect1* and *zein–lect1* systems provided the best performance, giving values remarkably lower than 25%, for each soybean–maize mix level analyzed RSDr as recommended by ENGL method performance requirements (*36*). To the contrary, RSDr values consistently higher than 25% were obtained for the *hmga–lect1* and *ivr1–lect1* systems.

Table 5. One-Way Analysis of Variance (ANOVA)[a]

S/M %	plasmid	mean %	*F* value	*P* value
0.1	pGEM(*adh1–lect1*)	0.094	40.16	2.9 E–10
	pGEM(*hmga–lect1)*	0.029		
	pGEM(*ivr–lect1*)	0.23		
	pGEM(*zein–lect1*)	0.091		
0.5	pGEM(*adh1–lect1*)	0.41	46.52	5.3 E–11
	pGEM(*hmga–lect1)*	0.11		
	pGEM(*ivr–lect1*)	1.005		
	pGEM(*zein–lect1*)	0.34		
1	pGEM(*adh1–lect1*)	1.1	28.15	1.3 E–8
	pGEM(*hmga–lect1)*	0.32		
	pGEM(*ivr–lect1*)	2.4		
	pGEM(*zein–lect1*)	0.84		

[a] The mean percentage values (mean %) quantified with the four calibration plasmid systems at the three soybean–maize mix levels (S/M %) were compared. *F* value = calculated Fischer value; *P* value = probability to assess no difference among quantification measures.

In addition, the accuracy (the closeness of agreement between a "test result" and the "accepted reference value") of the method was analyzed through the confidence intervals (CI) at 95%, where expected values (0.1, 0.5, and 1%) should be included within the estimated ranges. Moreover, accuracy was also estimated by means of Student's *t* test, which compares the actual difference between the mean percentage obtained and the expected reference values in relation to the variation in the data. For this test, assessment of the reference standard error is required. This is a factor linked to the sample preparation procedure, which, in the case of Fluka GM certified reference material (CRM), is provided by the company as "expanded uncertainty". For the RRS CRM 0.1, 0.5, and 1%, these values are, respectively, 0.05, 0.1, and 0.2. They were arbitrarily applied as standard errors for the three soybean–maize mix levels (0.1, 0.5, and 1%). In the presence of an unknown mix level uncertainty linked to a "home-made" sample preparation, as in our case, this practical solution can be considered as a realistic compromise in view of performing Student's *t* test with the highest strictness.

The most accurate quantification results (**Table 4**) were obtained with the *adh1–lect1* and *zein–lect1* plasmid systems for each of the three mix levels analyzed. In fact, the expected values (0.1, 0.5, and 1%) turned out to be included in the CI or were very near. Moreover, Student's *t* test confirmed excellent accuracy performance results for the 0.1 and 1% mix levels. For the 0.5% mix level, however, *t* values were less suitable, possibly as a consequence of a higher experimental error during sample preparation.

Besides, *hmga–lect1* and *ivr1–lect1* systems, respectively, over- and underestimated the expected percentage values (**Table 4**). This result is constant at all three mix levels, where the values obtained are, respectively, one-third and twice the expected value. Accordingly, the respective expected reference values are not included in the confidence intervals, and calculated *t* values are significantly higher than tabulated ones.

The quantified mean percentage values (mean %) obtained with the four different plasmid calibrators for each soybean–maize mix level were compared with the analysis of variance (ANOVA) in view of verifying the significance of these differences. As shown in **Table 5**, the test was significant because the probability that the differences occurred by change (*P* value) was infinitesimal, thus proving the nonequivalence of the four plasmids as standard calibrators. Moreover, to identify which mean % values differ from another, a pairwise

Table 6. Least Significant Difference (LSD) Test at 95% Probability Level[a]

S/M %	plasmid	mean %	means % absolute differences 2	3	4	LSD
0.1	(1) pGEM(*adh1–lect1*)	0.094	0.065*	0.14*	0.0031	0.027
	(2) pGEM(*hmga–lect1*)	0.029		0.204*	0.14*	
	(3) pGEM(*ivr–lect1*)	0.23			0.062*	
	(4) pGEM(*zein–lect1*)	0.091				
0.5	(1) pGEM(*adh1–lect1*)	0.41	0.3005*	0.59*	0.073	0.114
	(2) pGEM(*hmga–lect1*)	0.11		0.89	0.67*	
	(3) pGEM(*ivr–lect1*)	1.005			0.23*	
	(4) pGEM(*zein–lect1*)	0.34				
1	(1) pGEM(*adh1–lect1*)	1.1	0.75*	1.3*	0.24	0.336
	(2) pGEM(*hmga–lect1*)	0.32		2.04*	1.5*	
	(3) pGEM(*ivr–lect1*)	2.4			0.51*	
	(4) pGEM(*zein–lect1*)	0.84				

[a] The mean percentage values (mean %) quantified with the four different calibration plamids at the three soybean–maize mixing levels (S/M %) were pairwise compared. *, significantly different percentage means (mean %): values of absolute difference between two percentage means higher than the LSD have to be considered significantly different.

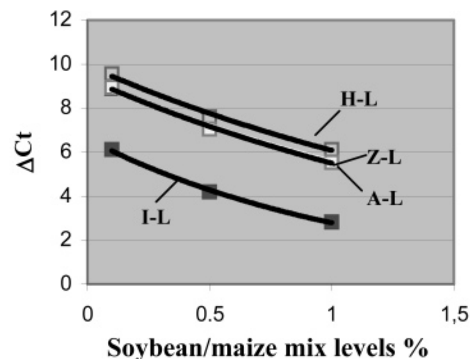
comparison was performed with the least significant difference (LSD) test (**Table 6**). As expected, the only insignificant differences observed were for the *adh1*−*lect1* and *zein*−*lect1* systems at all three soybean−maize mix levels (0.1, 0.5, and 1%) because the absolute differences of their percentage means were always lower than the LSD ones.

Evaluation of the Calibration Systems. Statistical analysis proved the reliability of *adh1*−*lect1* and *zein*−*lect1* plasmids as calibration systems for quantifying soybean mixing with maize meal, assuring precision and accuracy. Moreover, these two systems can be considered to be equally suitable because no significant differences between their quantification measures were discovered with the LSD test (**Table 6**). To the contrary, imprecise and inaccurate results were obtained with *ivr1*−*lect1* and *hmga*−*lect1* systems, which gave, respectively, over- and underestimations of the expected values, with high coefficient of variations (RSDr %) (**Table 4**).

In view of better verifying such widely different quantitative results, for each of the calibration systems, we analyzed the ΔCt values between soybean and maize endogenous gene Cts at the same threshold line. **Figure 5** illustrates the four regression curves obtained, where the Ct mean values are correlated to the mix levels. According to the performance already described, the *adh1*−*lect1* and *zein*−*lect1* regression curves were almost overlapping, whereas respectively higher and lower curves (constantly higher and lower Ct values) were obtained for the *hmga1*−*lect1* and *ivr1*−*lect1* systems.

Our results confirm the relevance of the proper endogenous gene/genes to use as reference for the quantification assays in real-time PCR. We consider this aspect particularly important when multiple-target systems are applied. For these constructs, the reference gene adequacy and the most suitable standard reaction conditions for each gene are two requirements that have to be compatible.

Calculation of the Percentage of the GM Soybean on the Whole Maize Sample. In our method, once the soybean intermix with maize meal and the percentages of the GM soybean are known and quantified, it is possible to determine the GM soybean rate in the whole maize sample. In our case study, for preparing the samples simulating the genetically modified soybean intermix with maize meal, we used the 5%



		regression curves	R^2
adh1-lect1	**(A-L)**	$y= 9{,}2989^{e-0{,}5135x}$	0,996
ivr1-lect1	**(I-L)**	$y= 6{,}5938^{e-0{,}8626x}$	0,997
hmga-lect1	**(H-L)**	$y= 9{,}9167^{e-0{,}4903x}$	0,994
zein-lect1	**(Z-L)**	$y= 9{,}3417^{e-0{,}5295x}$	0,997

Figure 5. Analysis of ΔCt values. Regression curves describing the relationship between the soybean–maize mix levels (0.1, 0.5, and 1%) and the respective ΔCt value (maize reference gene Ct − soybean reference gene Ct) obtained with the four calibration plasmid systems. The *adh11–lect1* and *zein–lect1* regression curves were approximately overlapping, whereas differences could be appreciated for *adh1–lect1/zein–lect1* ΔCt and *hmga–lect1* ΔCt (ΔΔCt ∼ −0.6 for each test sample) and for *adh11–lect1/zein–lect1* ΔCt and *ivr1–lect1* ΔCt (ΔΔCt ∼ 2.8 for each test sample).

Roundup Ready soybean (RRS) CRM produced and certified by IRMM (Geel, Belgium) and provided by Fluka. Thus, the GM soybean rate is known. The calculation of the transgenic RR component values on the whole samples for each of the three expected soybean−maize mix levels can be obtained as follows: (i) for the 0.1% level as $[(0.05 \times 0.001) \times 100] =$ 0.005%; (ii) for the 0.5% level as $[(0.05 \times 0.005) \times 100] =$ 0.025%; (iii) for the 1% level as $[(0.05 \times 0.01) \times 100] =$ 0.05%.

Finally, according to the values (mean %) reported in **Table 4**, we can estimate the observed % RRS soybean intermix with maize for each plasmid system at each of the soybean−maize mix levels. As an example, for the *adh1*−*lec1* system, for the 0.1% soybean mix, the % RRS obtained is 0.0047% $[(0.05 \times 0.00094) \times 100]$.

It is worth stressing that this method has to be considered as an additional, complementary assay of the official detection analysis of GMOs required by law (*14*, *15*). Current regulations, in fact, claim to trace in a food or feed sample the presence of each GM ingredient, individually considered. Our assay, conversely, aims to detect an unexpected intermix occurrence of a certain species with different components and—once the intermix is detected—to quantify the GM rate in the whole sample.

We are aware that the question we examine and the analytical solution we propose may raise some issues related to the calculation of the ratio between two different species. Among them is the complexity derived from the specific quali-quantitative chemical composition (dry matter, crude protein, fiber, fat, mineral, etc.) that constitutes the total mass of each species. This cannot be estimated exclusively by an assay based on the DNA analysis. Our system, however, seems to be an appropriate approach for managing the problem of the unexpected intermix with a molecular analysis.

In addition, it may be pointed out that, alternatively to our method, the GM percentage of soybean intermix in a maize meal could also be directly calculated as the ratio between the soybean transgene and the maize endogenous gene. In this case, however, the unbalanced amounts between the two components—GM intermixing soybean and maize—would require the building of standard curves with a remarkably wide dynamic range to include both quantities of the high maize endogene and the tiny soybean transgene. This may affect the accuracy of the quantification performance. Thus, with the aim of assessing small amounts, even traces, of soybean intermix in the maize meal, we believe that quantifying the soybean RR percentage in the contaminating soybean would be the better technical choice. Our strategy, moreover, follows the EU recommendation on GMO detection that requires a GM event to be related to the target taxon (*32*).

In conclusion, the method we developed proved to be a reliable analytical assay for determining the unexpected occurrence of GM soybean in different simulated intermix levels with maize meal.

The use of duplo target plasmids as calibrator standards forms the crucial part of the overall analysis and proved to be a powerful tool for real-time PCR analysis. A relevant aspect of this study is also the comparison of four different maize endogenous genes, respectively coding for the alcohol dehydrogenase 1, the invertase 1, the high-mobility group protein a, and the zein. We hope our results will contribute to research aimed at finding the most suitable reference gene.

The method we propose offers additional applications that go beyond the current analytical techniques for detecting GMOs, within the framework of a feed regulation that imposes precise rules on composition, traceability, and labeling (*38*). Once suitable endogenous genes are selected for detecting a certain species employed in a fodder formulation, in fact, ad hoc multiple-target plasmids allow precise quantification of its presence. This would be particularly useful for ascertaining the source of the protein component in a feed when less expensive alternative protein sources might be employed as substitutes for soybean (*3*). As a consequence, the actual value of the product can be determined. Additional research can be developed to design the best plasmids to employ in cases where intermixing involves other relevant feed components, including pea, wheat, barley, sunflower, and flax, whether used as single components or in combination.

For all of these reasons, the proposed method appears to be a valuable tool for checking the complex trade system with the aim of identifying and better controlling the most critical steps of the overall chain, where various unexpected contaminations could take place.

ABBREVIATIONS USED

adh1, alcohol dehydrogenase 1; *hmg*a, high-mobility group protein a; *ivr1*, invertase 1; *lect1*, lectin 1; CTAB, cetyltrimethylammonium bromide; EDTA, ethylenediaminetetraacetic acid; TE, 100 mM Tris-Cl, 10 mM EDTA.

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