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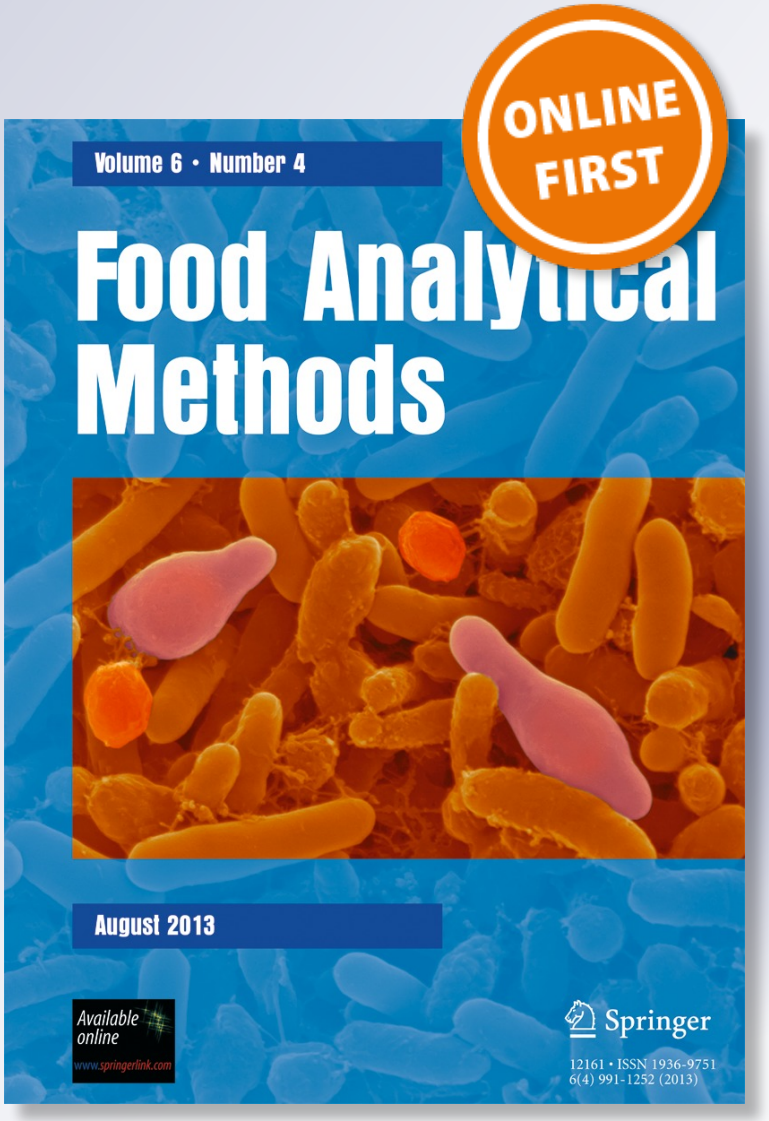

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Molecular Identification of Four Genetically Modified Maize (Bt11, Bt176, Mon810 and T25) by Duplex Quantitative Real-Time PCR

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Abstract In response to the increasing number of genetically modified (GM) events released on the market, control laboratories explore various strategies to simplify and reduce the number of tests needed to characterise the content in genetically modified organism (GMO) of a given sample. Lastly, multiplexing is considered as one of the possible ways to decrease the time and cost of analysis. Here, we report the development of four duplex polymerase chain reaction (PCR) tests for the identification and the quantification of four maize transformation events from which commercial lines have been authorised in Europe namely, Bt11 and Bt176 (Syngenta, DE, USA), Mon810 MaisGard™ (Monsanto, MO, USA) and T25 Liberty Link™ (Bayer CropScience, Monheim, Germany). The duplex PCR tests combine a maize-specific PCR test hybridising in the *Adh1* locus with an event-specific detection system designed on a junction fragment for each of these four GM maize. Real-time PCR tests, suitable to comply with the European regulation, were designed by using Taqman® chemistry.

Keywords Maize · GMO · Event-specific · Quantification · Duplex · QRT-PCR

Introduction

New types of plant food products including genetically modified (GM) ingredients or meat from animals fed with food containing genetically modified organism (GMO) are found in the markets. Since the impact of GMOs on human health and natural environment is still a subject of debates and controversy, many countries in the world decided to provide freedom of choice to their consumers by mandatory labeling of plant foods and feed products containing more than defined thresholds of GM material derived from authorised GMO. The traceability regulations and the thresholds for labeling vary from a country to another (Sylvia et al. 2012). In the European Union, all products containing or derived from authorised GMO used for human nutrition have to be labeled except in the case of a fortuitous or technically unavoidable presence of less than 0.9 % of authorised GMO per ingredient (European Commission Regulations N° 1829/2003). To comply with the regulation, detection methods are worldwide developed either by the biotech companies producing the GM events or by research laboratories, and databases were created (Dong et al. 2008). PCR is considered as the reference method of choice, widely used for its high sensitivity and large scope covering seed, plants food and feed matrices. While screening methods are very often based on the detection of quite ubiquitous sequences such as recurrent genes, regulatory sequences and construct-specific sequence identification and quantification are based on event-specific sequences localised on the edge-fragments (Fernandez et al. 2005; Pansiot et al. 2011). As a consequence, the number of PCR analyses to be carried out is increasing in parallel with the number of GMO to be detected. The cost and duration of analyses are thus rapidly growing for control laboratories. Multiplexing of PCR tests which allows the amplification of several target sequences at the same time is one of the potential answers to these issues. Moreover, the usual way to quantify GMO relies on the use of reference material and calibration curves, but the accuracy of such quantification scheme depends on the quality of DNA extracts (inhibitors content) and other bias. The deltaCt method

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decreases these biases but requires PCR tests to be done in exact same conditions. Multiplex PCR, amplifying several target sequences in the same tube, is adapted to the application of this method and thus provides means to overcome some of the technical issues faced by detection laboratories. Despite of the use of alternative methods for GMO quantification based on the high-throughput systems or platforms for the detection of multiple targets, for example, microarrays, MIPC, PCR combined with capillary gel electrophoresis (fingerprinting) and SNPlexing (Chaouachi et al. 2008; Hamels et al. 2009; Nadal et al. 2009; Guo et al. 2011), their application are often more expensive, prone to error, difficult to standardise and validate, and require extensive, sophisticated work and equipment. For this, quantitative real-time PCR (QRT-PCR) is until now the most used and validated method in all GMO control and routine laboratories all over the world (Mano et al. 2012). The current unequivocal way to identify and quantify GMO is based on event-specific regions located on the 5′- or 3′-edge fragments of the integrated sequences (Tengel et al. 2001; Rizzi et al. 2001; Matsuoka et al. 2002). Such regions have already been sequenced and used to develop validated detection tests in various GMO (http://gmo-crl.jrc.ec.europa.eu/). In terms of multiplexing, several detection methods have been published allowing the simultaneous detection of a high number of GM events in the same amplification tube (Germini et al. 2004; Ronning et al. 2003; Heide et al. 2007). To guaranty an optimal amplification of all the targets, multiplex PCR tests need to be optimised by using appropriate experimental designs. In addition to the detection and quantification of GMO, the assessment of the proportion of GMO per ingredient implies the quantification of the ingredient. It is usually performed by amplifying species-specific sequences located in reference genes. Many of these genes have been identified for various species (Takabatake et al. 2013). The percentage of GMO per ingredient is then estimated by the ratio between the amounts of GMO and of the reference gene. Recently, a duplex screening method targeting namely P35S/Tnos and Pnos/T35S has been developed with QRT-PCR using different multiplexing Kits (Pansiot et al. 2011). Development of a multiplex PCR often starts by the addition in the same tube singleplex tests with acceptable efficiencies and low detection limits. Then, PCR reaction conditions (concentration of primers, dNTP, $MgCl_2$ and Taq DNA polymerase; thermal cycling conditions) are optimised in multiplex PCR (Henegariu et al. 1997). Germini et al. (2004), for example, started with careful check of primer and primer–dimer formation when setting up a multiplex aiming the simultaneous amplification of five targets. In this way, they managed to develop a pentaplex efficient for food and feed products testing. It is not only primers' sequences that are important but also their concentration. For example, Matsuoka et al. showed an increase in sensitivity when decreasing the concentration of one primer pair. Other factors such as length of the

Table 1 Sequences of the primers and probes

Mon810	Forward primer: Mon810-fo	5′-CTTCGAAGGACGAAGGACT-3′
	Reverse primer: Mon810-re	5′-CCTTCCTTTTCCACTATCT-3′
	Probe: Mon810-pr	5′-TAACATCCTTTGCCATTGCCCAGCTA-3′
Amplicon length, 90 bp		
T25	Forward primer: T25-fo	5′-ATGATACTCCTTCCACCGCCG-3′
	Reverse primer: T25-re	5′-TAGACAAGCGTGTCGTGCTCCAC -3′
	Probe: T25-pr	5′-CAGTCATTGAGTCGTTCCGCCATTGTCGCAA-3′
Amplicon length, 152 bp		
Bt176	Forward primer: Bt176-fo	5′-GAACTGGCATGACGTGGGTT-3′
	Reverse primer: Bt176-re	5′-GAAGGGAGAAACGGTCGGCC-3′
	Probe: Bt176-pr	5′-GGTCCTGCCCGTCACCGAGATCTGATGT-3′
Amplicon length, 151 bp		
Bt11	Forward primer: Bt11-fo	5′-GTCATGATAATAATGGTTTCTTA-3′
	Reverse primer: Bt11-re	5′-ATACTAGAGGCTAACACCTACAGATTT-3′
	Probe: Bt11-pr	5′-ATGTATCCGCTCATGGAGGGATTCT-3′
Amplicon length, 170 bp		
Adh1	Forward primer: Adh-F3	5′-CGTCGTTTCCCATCTCTTCCTCCT-3′
	Reverse primer: Adh-R4	5′-CCACTCCGAGACCCTCAGTC-3′
	Probe: Adh-pr	5′-AATCAGGGCTCATTTTCTCGCTCCTCA-3′
Amplicon length, 134 bp		

amplified fragment and efficiency of amplification of each target are critical points for multiplex PCRs. Adjustment of PCR conditions is especially required when setting up multiplex for GMO detection. In fact, specific prerequisites exist for GMO detection. European Commission (EC) requirements for GMO product labeling are strict, so it is important that these multiplex PCR methods are sensitive and that they can detect GMO content as low as 0.1 % (based on haploid genome copy number unit for measurement and expression of GMO content; Commission Recommendation 2004/787/EC). It is noticeable that, in many papers (Hernandez et al. 2005; Forte et al. 2005; Matsuoka et al. 2000; Yang et al. 2005), the limit of detection (LOD) tested for the multiplex PCRs is low enough to fit the EC requirement. The LOD is tested on DNA mixtures containing all GMO targets in the multiplex. However, these experiments are often done on samples containing the same amount of each target, usually ranging from 5 % to 0.05 %. Hernandez et al. (2003a) tested their duplex on amounts of DNA very different for each target, 0.1 % of one and 99.9 % of the other one. In many cases, the influence of target concentration on the PCR efficiency or on the LOD is not assessed. Thus, multiplex PCRs conducted in routine laboratories could be used on complex mixes with variable GMO amounts and compositions.

In this paper, we report the development of four duplex qualitative and quantitative PCR tests, using *Adh1* maize reference gene, for the detection and the quantification of four maize events authorised for human nutrition in the European Union namely: Bt11 and Bt176 (Syngenta, DE, USA, Mon810 MaisGard™ (Monsanto, MO, USA) and T25 Liberty Link™ (Bayer CropScience, DE).

Materials and Methods

Plant Material

Heterozygote seeds from the four GM cultivars were provided by the different companies owning them, as described in Fernandez et al. (2005): Mon810 by Monsanto, T25 by Aventis (now Bayer CropScience), Bt11 and Bt176 by Syngenta. Plants were grown in the greenhouse. Just before flowering, the leaves were carefully harvested and stored separately at −20 °C in sealed plastic bags. Wild-type DNA from the non-GMO near isogenic Bt176 corn cultivar Pactol (Syngenta) was used as negative control DNA during all experiments. Certified Mon810 corn (IRMM-413) and Bt11 corn (IRMM-412) were purchased from Fluka (Buchs, Switzerland). The transgenic material used was composed of CBH351 and NK603 maize, MS1 and RF2 rapeseed, Tg7TF tomato and RR soybean.

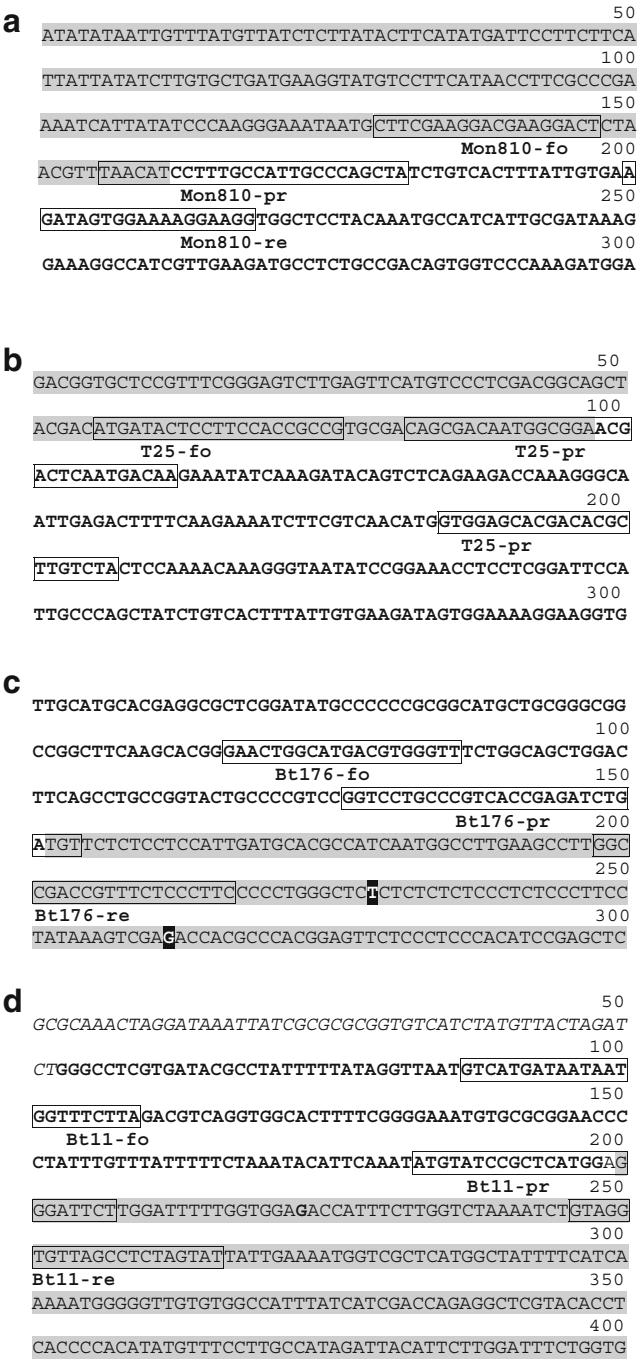

Fig 1 **a** Position of the primers and probe on Mon810 5′-edge fragment (Acc. N: AF434709). *Grey background*: maize genomic DNA, *bold letters*: P35S; **b** position of the primers and probe on T25 5′-edge fragment. *Grey background*: maize genomic DNA, *bold letters*: truncated P35S [29]; **c** Position of the primers and probe on Bt176 3′-edge fragment (Acc. N: AJ878607). *Grey background*: maize genomic DNA, *bold letters*: bar gene. The polymorphisms detected after sequencing are shown with *bold black letters*; **d** position of the primers and probe on Bt11 event's 3′-edge fragment (Acc. N: AY123624). *Grey background*: maize genomic DNA, *bold letters*: pUC, *italic letters*: *nos* terminator. All PCR-amplified junction sequences were cloned and sequenced

DNA Extraction and Quantification

Genomic DNA was isolated from 1 g of frozen leaves per sample according to a CTAB-based protocol (Chaouachi et al. 2013) followed by an additional step of purification using the Qiaquick® DNA purification kit (Qiagen, Hilden, Germany). DNA concentration was estimated with Bio-1D® v. 99.06 software (Bio-profil, Vilber Lourmat Biotechnology, France) by comparing the ethidium bromide fluorescence of the extracted samples to a concentration range of 0 to 30 ng of phage λ DNA (MBI Fermentas, Vilnius, Lithuania).

PCR Primers and TaqMan® Probes

Primers and probes were designed using Oligo6® and PrimerExpress® software. Freeze-dried primers were purchased from Genset (Evry, France). Freeze-dried TaqMan® probes were provided by Eurogentec (Seraing, Belgium). GM probes were 5′-YakimaYellow and 3′-DarkQuencher-labelled, and Adh1 probe was 5′-FAM and 3′-DarkQuencher-labelled. Primers and probes were resuspended in DNA free cell culture-grade water (Biological Industries, Beit Haemek, Israel) and TE buffer (pH 8), respectively, and stored at −20 °C until use.

Design of the Primers and Probes

Amplification of maize reference gene was performed using the primers and probe developed by Hernandez et al. (2004) and validated by the EU-RL (http://gmo-crl.jrc.ec.europa.eu/). For the four maize events, published sequences of the inserts (Collonier et al. 2005; Holck et al. 2002; Hernandez et al. 2003b) were used to design a pair of event-specific primers and a probe on one of the edge-fragments of each insert, taking into account their necessary compatibility with the chosen *Adh1* set. The sequence of the primers and probes and the expected amplicon length for each event are given in Table 1. All the amplicons were sequenced and aligned with public sequences. The results showed perfect identities except for the event Bt176 junction with two polymorphisms in the positions 228 (T/C) and 261 (G/T). As described in Fig. 1a to d, the probe overlaps the junction fragment in between the two primers which hybridise on the 5′ end of the insert for Mon810 and T25 events, and on the 3′ end of the insert for Bt176 and Bt11 events.

Optimisation of the PCR Conditions

A multifactorial plan designed for qualitative PCR allowed us to identify the best amplification conditions for each Adh1/GM event duplex. The variables tested were magnesium chloride concentration, annealing temperature and primers concentrations. A factor was changed when a minimum increase of 10 % in the concentration of the tested amplicon could be achieved. After several plans, the best PCR conditions for the three duplex tests Adh1/T25, Adh1/Mon810 and Adh1/Bt176 appeared to be—60 ° C, 2.5 mM $MgCl_2$, 1 μM of each primer. For the duplex Adh1/Bt11, optimised PCR conditions were—56 ° C, 2.5 mM $MgCl_2$ and 3 μM of each primer. The difference in primers concentration for Bt11 can be explained by the nature of the sequence where Bt11 construct is inserted in the plant

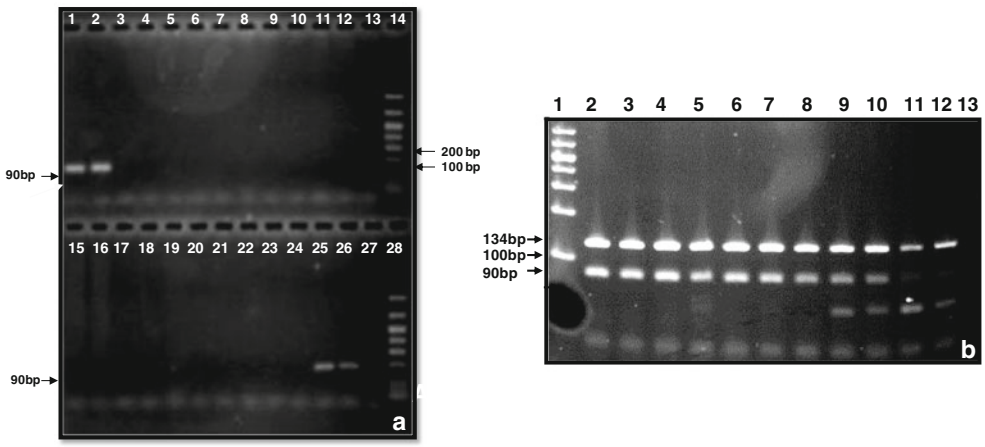

Fig 2 **a** Example of qualitative PCR amplification testing the specificity of Mon810 primer set. *Lanes 1* and *2*: Mon810, *lane 3*: non GM maize (variety MO17), *lane 4*: Bt176, *lane 5*: Bt11, *lane 6*: T25, *lane 7*: CBH351, *lane 8*: NK603; *lane 9*: MS1; *lane 10*: RF2; *lane 11*: Tg7TF; *lane 12*: RRS; *lane 13*: no template control; *lane 14*: BioMarker® low ladder; *lane 15*: barley (*Hordeum vulgare)*; *lane 16*: common wheat (*Triticum aestivum*); *lane 17*: rapeseed (*Brassica napus*); *lane 18*: soybean (*Glycine max*); *lane 19*: tomato (*Solanum lycopersicum*); *lane 20*: sugarbeet (*Beta vulgaris*); *lane 21*: potato (*Solanum tuberosum*); *lane 22*: sorghum (*Sorghum vulgare*); *lane 23*: durum wheat (*Triticum durum*); *lane 24*: sunflower (*Helianthus annuus*); *lanes 25* and *26*: Mon810; *lane 27*: NTC; *lane 28*: BioMarker® low ladder. **b:** Sensitivity of Mon810 event-specific qualitative detection. *lane 1*, NTC (no template control); *lanes 2–12*: 0.4 to 40,000 haploid genome copies of Mon810 event maize contents, respectively; *lane 1*, 1,000 DNA marker and *lane* 13: NTC

genome. Indeed, the forward primer of Bt11 specific test hybridises in a type of DNA sequence highly present in the maize genome (large blocks of heterochromatin composed of 180 bp repeats called Knobs (Ananiev et al. 1998).

Qualitative PCR Amplification

Qualitative PCR amplification was first carried out under the following standard conditions—1× PCR buffer (100 mM Tris–HCl pH 8.3; 500 mM KCl), 1.5 mM to 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM to 3 μM of each primer, 1 U Amplitaq Gold (ABI) and 50 ng DNA. Water was added to a final volume of 25 μl. PCR conditions were tested according to a multifactorial plan aimed at optimising the concentration of the different amplicons. Concentration of the amplicons was estimated by measuring the intensity of the bands on the gels using Bio 1-D® image analysis software. Primers used are presented in Table 1. The PCR profile consisted in a first heating step at 94 °C for 5 min, followed by 40 cycles of the three following steps 30 s at 94 °C, 30 s at annealing temperature (defined after optimisation for each Adh1/GM event duplex) and 30 s at 72 °C. It ended by a final extension step of 10 min at 72 °C.

Amplicons Sequencing

PCR products were amplified using a 9700 thermocycler (ABI) with the following cycling conditions: initial denaturation (5 min at 94 °C); denaturation (30 s at 94 °C), annealing (30 sec at 60 °C) and extension (30 s at 72 °C) for 40 cycles and last extension step (10 min at 72 °C). After purification using the Qiaquick PCR purification kit, the amplicons were prepared for sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing Kit. They were sequenced on both genomic DNA strands on an ABI 3730XL sequencer (Applied Biosystems).

Quantitative Real-Time PCR Optimisation

Real-time PCR amplification was performed using the TaqMan® Universal PCR Master Mix (ABI) and the annealing temperature optimised in qualitative PCR for each duplex test. Standard concentrations of 300 nM of each primer and 200 nM of probe were used for the three duplex tests Adh1/T25, Adh1/Mon810 and Adh1/Bt176. According to the observations made in qualitative PCR, these concentrations were multiplied by three for the duplex Adh1/Bt11 to get an optimised amplification.

QRT-PCR Amplification

Real-time PCR experiments were carried out on an ABI Prism® 7900 SDS thermocycler (ABI) using the sequence Detector software V1.7.a. PCR reactions were performed in a 25-μL volume using the TaqMan® Universal PCR Master Mix (ABI). The reaction mix contained a final concentration of 0.01 U/μL uracil-*N*-glycosylase (AmpErase UNG, ABI), 3.5 mM of $MgCl_2$, 200 μM dATP, dCTP, dGTP and 400 μM dUTP, 0.025 U/μL AmpliTaq Gold (ABI), 300 to 900 nM of each primer, 200 to 600 nM of each probe and 5 μL of sample. The PCR programme consisted in a first decontamination step of 2 min at 50 °C (UNG activation) followed by a 10 min DNA denaturation step at 95 °C and a series of 45 cycles including 30 s at 95 °C and 1 min at 60 °C. Baseline was automatically determined. Two independent assays were realised, and each PCR test was done in triplicate. DNA concentrations expressed in haploid genome equivalent copies (5 pg for two copies) for the standard curves were calculated according to the maize genome size described by Arumuganathan and Earle (1991).

Table 2 Amplification data used to determine the absolute LOD and LOQ of Adh1/Mon810 QRT-PCR detection system

Initial template copies	Signal rate (no. positive signals)	Mean Ct-values	SD of observed Ct-values
Maize event Bt176			
2,000	6/6	24.71	0.017
200	6/6	28.17	0.033
20	6/6	32.42	0.139
2	6/6	37.97	0.363
0.2	1/6	42.85	–
Maize event Mon810			
2,000	6/6	24.46	0.014
200	6/6	28.31	0.029
20	6/6	32.23	0.145
2	6/6	37.88	0.401
0.2	1/6	43.70	–
Maize event T25			
2,000	6/6	24.82	0.019
200	6/6	28.28	0.039
20	6/6	32.51	0.198
2	6/6	37.76	0.367
0.2	1/6	43.55	–
Maize event Bt11			
2,000	6/6	24.28	0.019
200	6/6	28.37	0.039
20	6/6	32.29	0.156
2	6/6	37.67	0.387
0.2	1/6	42.98	–

Analyses were done on a series of dilutions of 40,000 to 0.4 Mon810 haploid genome copies. The LODa was reached when at least one target DNA amplification out of six PCR reactions (two independent dilutions with three replicates per dilution) was not successful. The LOQa was reached when the value of the standard deviation (SD) of the Ct-values could jeopardise a reliable quantification

Results and Discussion

Development of the Qualitative Duplex PCR Tests: Performance Criteria

Study of the Specificity

The routine analysis of multiplex PCR products may lead to false-positive results if an artifact of similar size as a given target sequence is amplified. This limitation can be overcome by simultaneous detection of two PCR and validation by amplicon sequencing. Specificity of the detection tests were carefully evaluated by qualitative PCR on non-GM maize lines (MO17) and other transgenic lines (depending on test: Mon 810, T25, Bt176, Bt11, CBH351 and NK603 maize, MS1 and RF2 rapeseed, Tg7TF tomato, RR soybean). As illustrated for Mon810 in Fig. 2a, the primers gave the expected amplicons when the four GM maize were used as template, and no amplification signal was detected with the non-transformed maize DNA and the other GM lines tested. Sequencing showed a 100 % sequence similarity between the amplified products and the sequence of the targeted junction fragments of the four events.

Sensitivity of the Duplex PCR Tests: Determination of Limit of Detection (LOD)

Sensitivity of the qualitative duplex PCR was assessed by using series of dilutions from 0.4 to 40,000 haploid genome copies of each event. We were able to detect each GM event in all six parallels down to four haploid copies, while only one of the parallels was positive when we used an estimated average of 0.4 haploid copies in the PCR (Fig. 2b). From these results, we estimate the absolute LOD to be located between 0.4 and 4 copies of each insert-edge fragment.

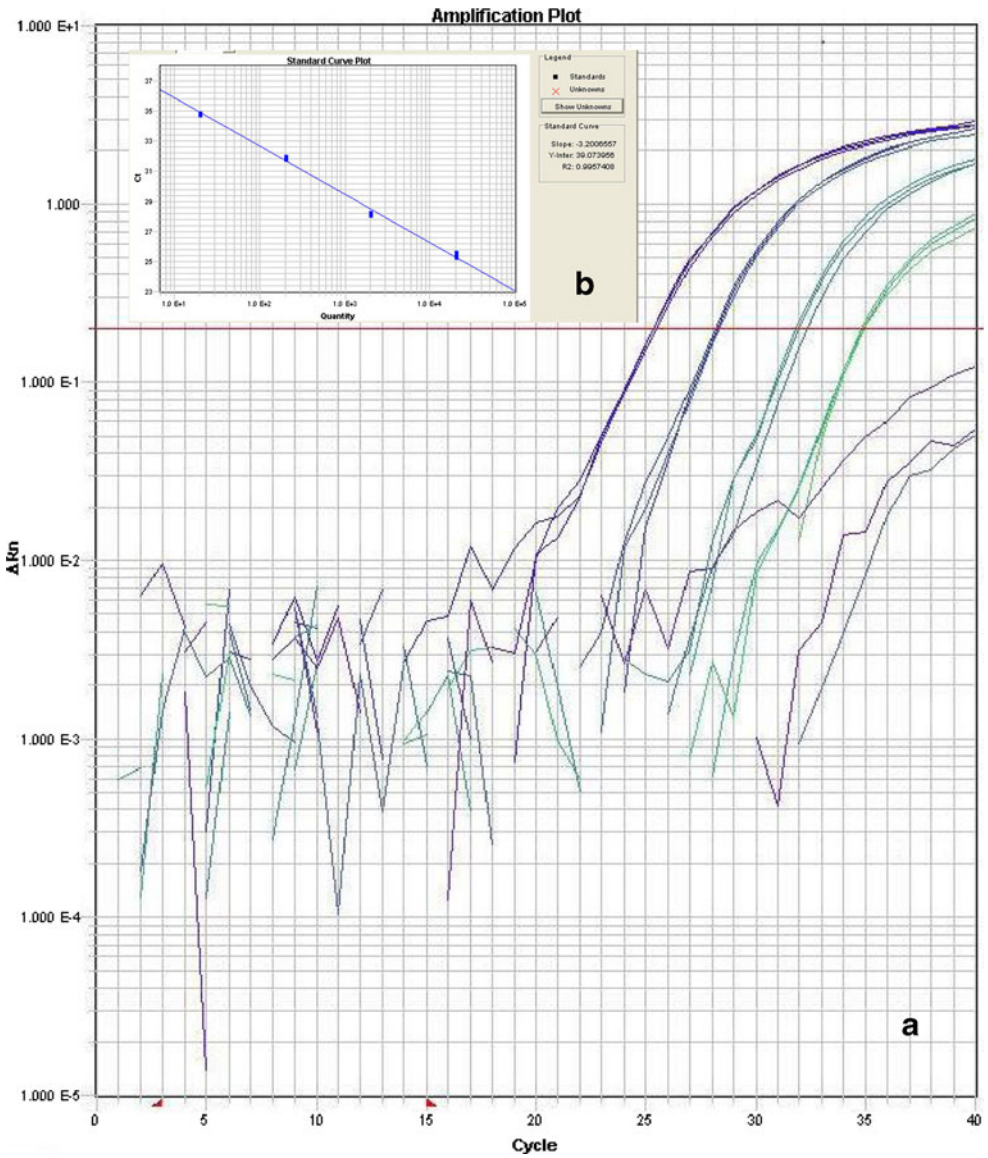

Fig 3 Example of amplification plot and standard curve for maize Mon810 using duplex real-time PCR assay targeting the maize endogenous reference gene (*Adh1*). **a** Amplification plots generated by tenfold serial dilution of maize Mon810 DNA ranging from 2,000 HGC to 0.2 copies with the adhf and adhr primer pair and Adhpr probe. Assays were performed in triplicate. **b** Calibration curves generated from the amplification data given in **a**. No amplification observed (Ct=40) with the concentration two copies, confirming that the absolute LOD is less than two copies

Development of the Quantitative Duplex PCR Tests: Performance Criteria

Determination of the PCR Efficiencies

The efficiency of the QRT-PCR tests, calculated from the slopes of the standard curves obtained with series of dilutions from 0.4 to 40,000 haploid genome copies of each event, was rather high for the four GM systems, ranging from 90 % to 96 %. The mean square regression coefficient of the calibration curves (R^2) ranged from 0.975 to 0.993 with five conducted runs, and a correlation seems more accurate between copy numbers and fluorescent values.

Determination of the Absolute and Relative Limits of Detection and Quantification (LOD_a, LOD_r and LOQ)

The limit of detection of a DNA sequence corresponds to the lowest DNA content that can be detected with reasonable statistical certainty (at least 95 % probability of detection of the sequence if it is present in the sample) (ISO5725 1994, 1998). In this study, the LOD has been considered as the smallest genome copy number for which all the target DNA amplifications out of six PCR reactions (two independent dilutions with three replicates per dilution) were successful. The sensitivity of each detection test was assessed by determining the absolute and relative limits of detection and quantification. The limit of quantification of a DNA sequence can be defined as the lowest amount or concentration of target DNA which can be statistically quantified with an acceptable level of precision and accuracy. In this study, the absolute and relative limit of quantification was determined using the same series of dilution used for the determination of the limits of detection. In order to determine the absolute LOD and LOQ of the 5′-assay, a dilution series containing from 2,000 to 0.2 haploid genome copies of each event were analysed in three parallel real-time PCR analyses. As expected, the ability to detect the GM lines decreased with decreasing copy numbers. We were able to detect each GM event in all three parallels down to two haploid copies. When using an estimated average of 0.2 haploid copies in the PCR, all the parallels were negative (Table 2). From these results, we estimated the absolute LOD to be below two haploid copies for each of the four systems tested (Fig. 3). To obtain reliable quantification results under ideal conditions, approximately 20 initial haploid copies were required for all four systems (Table 2). Thus, we concluded that their LOQa was approximately of 20 initial haploid genome copies. These LOD and LOQ were estimated in pure GM maize samples. In real food and feed samples, the presence of a large background of non-target DNA may modify these estimations. In order to evaluate the performances of our detection test in more real conditions, the relative limits of detection and quantification were determined using series of dilutions from 2 to 0.001 % of event DNA in presence of non GM maize ballast DNA. The final number of copies used was 20,000 HGC. We were able to detect each event in all six parallels down to four haploid copies in the PCR (Table 3). For each detection test, the method can reach the LODr of 0.01 % targeted. This meant that the lowest tested level is less than four haploid genome copies on the basis of maize genomic DNA of 2.725 pg per haploid genome (Arumuganathan and Earle 1991). Since reliable quantification results were again obtained with approximately 40 initial haploid copies (Table 3), we concluded that the LOQr of each detection test was approximately 0.1 % GMO, which is nine times less than the legal European threshold for labelling.

Table 3 Amplification data used to determine the relative LOD and LOQ of Adh1/Mon810 QRT-PCR detection method

Initial template copies (with ballast)	Signal rate (no. positive signals)	Mean Ct-values	SD of observed Ct-values
Maize event Bt176			
2 %	6/6	31.41	0.085
1 %	6/6	32.37	0.105
0.1 %	6/6	35.43	0.285
0.01 %	6/6	41.38	0.467
0.001 %	0/6	–	–
Maize event Mon810			
2 %	6/6	31.56	0.103
1 %	6/6	32.87	0.123
0.1 %	6/6	35.76	0.298
0.01 %	6/6	41.25	0.502
0.001 %	0/6	–	–
Maize event T25			
2 %	6/6	31.76	0.111
1 %	6/6	32.39	0.176
0.1 %	6/6	35.55	0.288
0.01 %	6/6	41.29	0.519
0.001 %	0/6	–	–
Maize event Bt11			
2 %	6/6	31.28	0.112
1 %	6/6	32.18	0.181
0.1 %	6/6	35.14	0.290
0.01 %	6/6	41.33	0.537
0.001 %	0/6	–	–

Analyses were done on a series of dilutions of 2 % to 0.001 % of Mon810 DNA in 100 ng (approximately 40,000 maize haploid genome copies) of non GM maize DNA. The LODr was reached when at least one target DNA amplification out of 6 PCR reactions (2 independent dilutions with 3 replicates per dilution) was not successful. The LOQr was reached when the value of the standard deviation (SD) of the Ct-values could jeopardise a reliable quantification

Table 4 Evaluation of the accuracy and precision of the four maize duplex QRT-PCR detection systems

Expected GMO % (100 ng total DNA)	Corresponding expected copies	Measured GM copies			Measured GMO %		
		Mean	%RSDr	SD	Mean	SD	Bias
Maize event Bt176							
6 %	1,200	1,280	15.3	196	6.05	0.96	0.83
5 %	1,000	1,007	19.06	192	5.13	1.32	2.6
4 %	800	941	6.3	60	4.68	0.37	17
2 %	400	535	4.6	25	2.44	0.20	22
1 %	200	236	16.9	40	1.14	0.20	14
0.5 %	100	111	21.6	24	0.55	0.12	10
0.1 %	20	13	4.6	0.6	0.07	0.01	−30
0.05 %	10	2	10	0.2	0.1	0.01	100
0.01 %	2	0.7	5.7	0.04	0.03	0.002	200
Maize event Bt11							
6 %	1,200	1,263	14.09	178	6.14	0.83	2.33
5 %	1,000	1,013	18.8	191	5.10	1.41	2
4 %	800	938	6.5	61	4.34	0.41	8.5
2 %	400	529	5.2	28	2.23	0.26	11.5
1 %	200	242	12.08	31	1.10	0.23	10
0.5 %	100	119	23.5	28	0.45	0.19	−10
0.1 %	20	29	6.9	2	0.09	0.02	−10
0.05 %	10	4	4.5	0.18	0.03	0.01	260
0.01 %	2	0.9	4.4	0.04	0.06	0.003	300
Maize event Mon810							
6 %	1,200	1,235	15.38	190	6.19	0.88	3.16
5 %	1,000	1,009	17.44	176	5.23	1.50	4.6
4 %	800	879	7.84	69	4.44	0.29	11
2 %	400	482	4.97	24	2.29	0.19	14.5
1 %	200	213	6.10	13	1.19	0.18	19
0.5 %	100	123	17.07	21	0.58	0.21	16
0.1 %	20	16	18.75	3	0.08	0.02	−20
0.05 %	10	9	21.1	1.9	0.04	0.03	−20
0.01 %	2	1	30	0.3	0.02	0.003	100
Maize event T25							
6 %	1,200	1,290	12.09	156	6.29	0.90	4.83
5 %	1,000	1,024	14.06	144	5.38	1.30	7.6
4 %	800	891	7.40	66	4.39	0.30	9.75
2 %	400	489	4.08	20	2.20	0.27	10
1 %	200	219	5.47	12	1.15	0.29	15
0.5 %	100	133	16.54	22	0.54	0.14	8
0.1 %	20	26	19.23	5	0.07	0.03	−30
0.05 %	10	7	27.14	1.9	0.04	0.01	−20
0.01 %	2	3	23.33	0.7	0.03	0.002	200

Analyses were done on a range of DNA solutions with known percentages of DNA in non GMO ballast DNA. The measured percentage of GMO in the sample was given by the ratio between the measured number of Bt176 genome copies and the measured number of maize genome copies, both estimated through the same duplex RT-PCR amplification of edge-junction fragments and of *Adh1* maize reference gene

RSDr repeatability relative standard deviation, *SD* standard deviation

Determination of Accuracy and Precision

Accuracy and precision were estimated using a range of DNA solutions with known percentages of GM DNA (6 %, 4 %, 2 %, 1 %, 0.1 %, 0.01 % and 0.001 %) in presence of non GM DNA. The expected and measured GM target copies were compared. To practically compare the expected and measured percentages of GMO content in the sample, the ratio between measured GM target copy number and measured maize copy number, estimated through duplex QRT-PCR amplifications of the target gene and of the maize reference gene *Adh1*, was calculated. Accuracy was measured as bias (percent) of the experimental value from the theoretical value. Precision was evaluated by relative standard deviation (RSD). Accuracy is evaluated by determining trueness and precision. Trueness is usually estimated by the difference between the experimental average value (here the measured GMO % of the sample) and the reference value (here the expected GMO % of the sample) (ISO5725 1994, 1998). The precision corresponded to the standard deviation of the measure. A high standard deviation value indicates a rather poor precision. As illustrated in Table 4, all detection tests allow an accurate and precise measure of the amount of GM DNA from 6 % to 0.1 % of GMO in the sample. The RSDs at the level of 0.9 %, 3 % and 5 % which are the thresholds of unintentional mixing level in EU, Korea and Japan, respectively, ranged from 7 % to 25 % within the same range as in most publications published earlier on GM soybean and maize detection systems.

Validation of the QRT-Duplex Methods Using Processed Food

The use of the maize QRT-PCR duplex methods to analyse biscuits made from 80 % commercial wheat flour and 20 % laboratory-prepared maize flour containing known concentrations (0.1–2 % *w/w*) of maize was tested. The developed methods performed using the maize primer pairs, amplified bands corresponding to endogenous *Adh1* and the recombinant target. The duplex QRT-PCR failed to amplify the recombinant DNA sequence only in biscuits made from soybean flour containing 0.1 % RR (*w/w*). This result fits well with the limit of detection (0.2 ng) calculated previously, considering the small amount of GMO-derived DNA in this sample (0.1 ng GM maize DNA in 500 ng total DNA from a 20:80 maize/wheat mixture in which the maize flour contained 0.1 % *w/w* GM maize).

Conclusion

Demand has never been greater for revolutionary technologies that deliver fast, inexpensive and accurate genome information. This latter has facilitated to the scientific community the access to gene sequences via the databanks and has contributed to the development of more molecular food control. This challenge has catalysed the implementation of plenty of new methodologies such as those used for GMO quantification in plant foods for human nutrition. Our results, in agreement with those obtained on several other commercialised GMO, showed that a precise description of the inserts is required for the accurate detection of transgenic cultivars. Due to the specificity of the established systems, we believe that this method is a new contribution to the labeling system for GMOs and also suitable for event-specific qualitative and quantitative detection for maize events containing this specie as a main ingredient in plant foods and feed products. Finally, amplicons of the five GM lines were cloned and may be used as controls and internal reference material in our laboratory.

Conflict of Interest All the authors have no financial relationship with the organisation that sponsored the research. The Conflict of Interest statements must list each author separately by name:

Dr. Maher Chaouachi declares that he has no conflict of interest. Mr. Mohamed Salem Zellama declares that he has no conflict of interest. Ms. Nesrine Nabi declares that he has no conflict of interest. Mr. Ahmed Ben Hafsa declares that he has no conflict of interest. Pr. Khaled Said declares that he has no conflict of interest. This article does not contain any studies with human or animal subjects.

References

Ananiev EV, Phillips RL, Rines HW (1998) A knob-associated tandem repeat in maize capable of forming fold-back DNA segments: are chromosome knobs megatransposons? Proc Natl Acad Sci 95:10785–10790

Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9:208–218

Chaouachi M, Alaya A, Ali IB, Hafsa AB, Nabi N, Bérard A, Romaniuk M, Skhiri F, Saïd K (2013) Development of real-time PCR method for the detection and the quantification of a new endogenous reference gene in sugar beet "*Beta vulgaris* L.": GMO application. Plant Cell Rep 32:117–28

Chaouachi M, Chupeau G, Berard A, McKhann H, Romaniuk M, Giancola S, Laval V, Bertheau Y, Brunel D (2008) A high-throughput multiplex method adapted for GMO detection. J Agric Food Chem 56:11596–11606

Collonnier C, Schattner A, Berthier G, Boyer F, Coué-Philippe G, De Beuckeler M, de Loose M, Windels P, Bertheau Y (2005) Characterization and event specific-detection by quantitative real time PCR of T25 maize insert. J AOAC Int 88:536–546

Dong W, Yang L, Shen K, Kim B, Kleter GA, Marvin HJ, Guo R, Liang W, Zhang D (2008) GMDD: a database of GMO detection methods. BMC Bioinforma 9:260

European Commission Regulations N° (1829/2003) of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. Off J Eur Union 2003a L268:1–23

Fernandez S, Charles-Delobel C, Geldreich A, Berthier G, Boyer F, Collonnier C, Coué-Philippe G, Diolez A, Duplan MN, Kebdani

N, Romaniuk M, Feinberg M, Bertheau Y (2005) Quantification of the 35S promoter in DNA extracts from genetically modified organisms using real-time polymerase chain reaction and specificity assessment on various genetically modified organisms, part I: operating procedure. J AOAC Int 88:547–557

Forte VT, Di Pinto A, Martino C, Tantillo GM, Grasso G, Schena FP (2005) A general multiplex-PCR assay for the general detection of genetically modified soya and maize. Food Control 16:535–539

Germini A, Zanetti A, Salati C, Rossi S, Forré S, Schmid S, Marchelli R, Fogher C (2004) Development of a seven-target multiplex PCR for the simultaneous detection of transgenic soybean and maize in feeds and foods. J Agric Food Chem 52:3275–3280

Guo JL, Yang L, Chen L, Morisset D, Li X, Pan L, Zhang D (2011) MPIC: a high-throughput analytical method for multiple DNA targets. Anal Chem 83:1579–1586

Hamels ST, Glouden K, Gillard M, Debode F, Foti N, Sneyers M, Nuez TE, Pla M, Berben G, Moens W, Bertheau Y, Audéon C, Van den Eede G, Remacle J (2009) A PCR-microarray method for the screening of genetically modified organisms. Eur Food Res Technol 228:531–541

Heide BR, Heir E, Holck A (2007) Detection of eight GMO maize events by qualitative, multiplex PCR and fluorescence capillary gel electrophoresis. Eur Food Res Technol 227:527–535

Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH (1997) Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques 23:504–511

Hernandez M, Duplan M-N, Berthier G, Vaitilingom M, Hauser W, Freyer R, Pla M, Bertheau Y (2004) Development and comparison of four real-time polymerase chain reaction systems for specific detection and quantification of *Zea mays* L. J AgricFood Chem 52:4632–4637

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

Hernandez M, Rodriguez-Lazaro D, Esteve T, Prat S, Pla M (2003b) Development of melting temperature-based SYBR Green I polymerase chain reaction methods for multiplex genetically modified organism detection. Anal Biochem 323:164–170

Hernandez M, Rodriguez-Lazaro D, Zhang D, Esteve T, Pla M, Prat S (2005) Interlaboratory transfer of a PCR multiplex method for simultaneous detection of four genetically modified maize lines: Bt11, MON810, T25, and GA21. J Agric Food Chem 53:3333–3337

Holck A, Vaïtilingom M, Didierjean L, Rudi K (2002) 5′-Nuclease PCR for quantitative event-specific detection of the genetically modified Mon810 MaisGard maize. Eur Food Res Technol 214:449–453

ISO 5725 (1994) Accuracy (trueness and precision) of measurement methods and results—part 2: basic method for the determination of repeatability and reproducibility of a standard measurement method. http://www.iso.ch/iso/en/ISOOnline.openerpage

ISO 5725 (1998) Accuracy (trueness and precision) of measurement methods and results—part 1: general principles and definitions. International Standard Organization, http://www.iso.ch/iso/en/ISOOnline.openerpage

Mano J, Harada M, Takabatake R, Furui S, Kitta K, Nakamura K, Akiyama H, Teshima R, Noritake H, Hatano S, Futo S, Minegishi Y, Iizuka T (2012) Comprehensive GMO detection using real-time PCR array: single-laboratory validation. J AOAC Int 95:508–16

Matsuoka T, Kawashima Y, Akiyama H, Miura H, Goda Y, Kusakabe Y, Isshiki K, Toyoda M, Hino A (2000) A method of detecting recombinant DNAs from four lines of genetically modified maize. J Food Hyg Social Japan 41:137–143

Matsuoka T, Kuribara H, Takubo K, Akiyama H, Miura H, Goda Y, Kusakabe Y, Isshiki K, Toyoda M, Hino A (2002) Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). J Agric Food Chem 50:2100–2109

Nadal A, Esteve T, Pla M (2009) Multiplex polymerase chain reaction-capillary gel electrophoresis: a promising tool for GMO screening-assay for simultaneous detection of five genetically modified cotton events and species. J AOAC Int 92:765–772

Pansiot J, Chaouachi M, Cavellini L, Romaniuk M, Ayadi M, Bertheau Y, Laval V (2011) Development of two screening duplex PCR assays for genetically modified organism quantification using multiplex real-time PCR master mixes. Eur Food Res Technol 232:327–334

Rizzi A, Agosti F, Daffonchio D, Sorlini C (2001) Detection of genetically modified Bt-maize in cooked food products by PCR. Ital J of Food Sci 3:265–273

Ronning S, Vaïtilingom M, Berdal K, Holst-Jensen A (2003) Event specific real-time quantitative PCR for genetically modified Bt11 maize (*Zea mays*). Eur Food Res Technol 216:347–354

Sylvia R, Broeders M, Sigrid C, De Keersmaecker J, Roosens NHC (2012) How to deal with the upcoming challenges in GMO detection in food and feed. J Biomed Biotechnol. doi:10.1155/2012/402418

Takabatake R, Onishi M, Koiwa T, Futo S, Minegishi Y, Akiyama H, Teshima R, Kurashima T, Mano J, Furui S, Kitta K (2013) Development and interlaboratory validation of quantitative polymerase chain reaction method for screening analysis of genetically modified soybeans. Biol Pharm Bull 36:131–134

Tengel C, Schüβler P, Setzke E, Balles J, Sprenger-Hauβels M (2001) PCR-based detection of genetically modified soybean and maize in raw and highly processed foodstuffs. Biotechniques 31:426–429

Yang L, Pan A, Zhang K, Guo J, Yin C, Chen J, Huang C, Zhang D (2005) Identification and quantification of three genetically modified insect resistant cotton lines using conventional and TaqMan real-time polymerase chain reaction methods. J Agric Food Chem 53:6222–6229