Anal Bioanal Chem (2013) 405:6831–6844
DOI 10.1007/s00216-013-7125-5

RESEARCH PAPER

Development and validation of a multiplex real-time PCR method to simultaneously detect 47 targets for the identification of genetically modified organisms

Geoffrey Cottenet · Carine Blancpain · Véronique Sonnard · Poh Fong Chuah

Received: 24 April 2013 / Revised: 29 May 2013 / Accepted: 5 June 2013 / Published online: 7 July 2013

Abstract Considering the increase of the total cultivated land area dedicated to genetically modified organisms (GMO), the consumers' perception toward GMO and the need to comply with various local GMO legislations, efficient and accurate analytical methods are needed for their detection and identification. Considered as the gold standard for GMO analysis, the real-time polymerase chain reaction (RTi-PCR) technology was optimised to produce a high-throughput GMO screening method. Based on simultaneous 24 multiplex RTi-PCR running on a ready-to-use 384-well plate, this new procedure allows the detection and identification of 47 targets on seven samples in duplicate. To comply with GMO analytical quality requirements, a negative and a positive control were analysed in parallel. In addition, an internal positive control was also included in each reaction well for the detection of potential PCR inhibition. Tested on non-GM materials, on different GM events and on proficiency test samples, the method offered high specificity and sensitivity with an absolute limit of detection between 1 and 16 copies depending on the target. Easy to use, fast and cost efficient, this multiplex approach fits the purpose of GMO testing laboratories.

Keywords Genetically modified organisms · Screening · Identification · Multiplex · Real-time PCR

G. Cottenet (✉) · C. Blancpain · V. Sonnard
Food Safety & Quality, Nestec Ltd., Nestlé Research Center, Vers-chez-les-Blanc, Route du Jorat 57, Lausanne 26, Vaud 1000, Switzerland
e-mail: geoffrey.cottenet@rdls.nestle.com

P. F. Chuah
Nestlé Quality Assurance Center, 29 Quality Road, Jurong West, Singapore 618802, Singapore

Abbreviations

CRM	Certified reference material
GMO	Genetically modified organisms
IPC	Internal positive control
LOD	Limit of detection
MGB	Minor groove binder
NTC	No template control
PCR	Polymerase chain reaction
RTi-PCR	Real-time PCR

Introduction

With limited crop resources and climate disorders, the total cultivated land area dedicated to GMO has been rising for several years, with a leading commercial use of genetically modified (GM) soybean, GM cotton, GM maize and GM rapeseed [1]. According to a prospective study of the European Joint Research Centre published in 2009, the projected number of commercial GM events could increase from 30 to 120 over 6 years. Up to 17 GM soybean, 24 GM maize, 8 GM rapeseed, 27 GM cotton and 15 GM rice events would be available in 2015 [2]. Interestingly, the number of other GM crops (such as sugar beet, papaya, tomato, eggplant) could increase from 10 to 23 GM events during the same period and is currently in the development pipeline in developing countries [3].

To comply with various local GMO legislations and to respect consumer preference, effective and accurate analytical methods are needed for GMO detection and their quantification. To ensure product authenticity and traceability of GM material, many methods have been developed, such as two-dimensional electrophoresis [4] and isoelectric focusing

[5], protein capillary electrophoresis [6], HPLC [7], and ELISA [8]. However, to overcome limitations related to heat-treated or processed products, the use of DNA-based methods and especially polymerase chain reaction (PCR) techniques is preferred for both raw ingredients and processed food [9, 10]. Furthermore, PCR and real-time PCR (RTi-PCR) are internationally recognised and recommended for GMO analyses [11, 12]. While conventional PCR methods need to handle post-PCR products for gel electrophoresis or enzymatic digestion, RTi-PCR does not need post-PCR manipulations which significantly reduce the risk of laboratory contamination.

Taking into account the GMO rise, the number of necessary target sequences for molecular identification will increase accordingly. Consequently, description of multiplex detection and identification PCR methods has been rising during these last 5 years, allowing a reduction of the analytical time and cost and conserving precious sample material. On the one hand, the screening approach using GM markers such as promoters (p-35S, p-FMV…), terminators (t-NOS, t-E9…) or transgenes (pat, CP4epsps…) has often been described [13, 14], but it lacks the identification step, which is necessary for a complete GMO analysis. On the other hand, a specific identification method has been described [15], but the lack of GM markers could miss the detection of new GM events or unauthorised ones.

To adapt the analytical approach with the growing GMO environment and to cover a wider range of GM targets, the development of a new GMO multiscreening method was undertaken. Still considered as the golden standard for GMO analysis, RTi-PCR was selected using minor groove binder (MGB) TaqMan® probes to improve the sensitivity and the specificity of the assays [16, 17]. To increase the sample throughput and optimise the screening capability, multiplex RTi-PCR was adapted on a 384-well plate format, allowing the simultaneous detection of up to 47 targets on seven samples in duplicate. Consequently, a complete fingerprint of their GM content is thus obtained with reduced time and cost. In addition, it includes an internal positive control (IPC) recommended for the detection of potential PCR inhibition and complies with analytical GMO quality requirements described in ISO 24276:2006 [18].

Materials and methods

Reference materials

Certified reference materials (CRMs) of Bt11, Bt176, MON810, MON863, NK603, GA21, TC1507, DAS59122, 98140, 3272, MIR604, GTS-40-3-2 (RRS), 356043, 305423, and EH92-527-1 GM events were purchased from the Institute of Reference Materials and Measurements (IRMM, Geel, Belgium), and T25, MIR162, MON88017, MON89034, MON89788, A2704-12, A5547-127, MON87701, LL62, GT73, Topas 19/2, T45, MON1445, MON531, MON15985-7, GHB614 and LLCotton25 GM events were purchased from the American Oil Chemist's Society (Urbana, USA). KMD1 and IR72/Xa21 GM rice powders were kindly provided by Zhejiang University (Hangzhou, China), whereas CBH351 (Starlink) GM maize powder was kindly provided by formerly Mid-West Seed Services, Inc. (Brookings, SD, USA). Since no Bt63 GM rice reference was available, a Bt63 plasmid was purchased from Eurofins GeneScan (Freiburg, Germany). Non-GM materials (potato and seeds from maize, soya, rice, wheat, tomato, cotton, rapeseed and mustard) as well as cocoa-based products and instant coffee powders were purchased from local markets. GeMMA proficiency test samples were acquired from the Food and Environment Research Agency (FAPAS, York, UK). Certified reference animal DNA (beef, pig, horse, sheep, goat and chicken) was purchased from Coring System Diagnostix GmbH (Gernsheim, Germany).

DNA extraction and preparation

DNA from each sample or reference was extracted in duplicate. Ground seeds or homogenised samples, 100 mg, were incubated in 1 mL of CTAB lysis buffer (Applichem GmbH, Darmstadt, Germany), 400 μg/mL protease (QIAgen, Hilden, Germany) and 200 μg/mL RNAse A (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) at 65 °C for 1 h. After maceration, the samples were centrifuged at 15,000×*g* for 2 min. The supernatant was transferred into a new tube, mixed with an equal volume of chloroform and centrifuged again at 15,000×*g* for 2 min. The supernatant was added to five volumes of PB binding buffer (QIAgen), mixed and loaded onto a QIAquick column (QIAgen) over a vacuum manifold. The column was washed with 1.5 mL of PE buffer (QIAgen), and the membrane was dried at 15,000×*g* for 2 min and placed in a new centrifuge tube. The retained DNA was eluted by centrifugation for 1 min at 15,000×*g* with 50 μL of elution buffer (EB) (QIAgen) after 2 min of incubation. When the eluted DNA was coloured and thus suspected to contain PCR inhibitors (such as phenolic compounds contained in cocoa or coffee-based products), the extracted DNA was further purified by gel filtration using Sephacryl resin (MicroSpin S-300 HR columns, GE Healthcare, Glattbrugg, Switzerland) according to the supplier's instructions. Following the measurement of the DNA concentration using a nanophotometer (Implen GmbH, Munich, Germany), DNA extracts were finally diluted at 40-ng/μL in EB buffer.

CRMs available as lyophilised DNA were reconstituted at 40 ng/μL with EB buffer. Extracted DNA or reconstituted DNA was stored at −20 °C until further use.

Selection of target sequences for GMO detection

To allow an efficient detection and identification of GM material, three different categories of targets were selected.

1. Plant endogenous sequences were chosen to specifically detect soya, maize, rice, wheat, rapeseed, potato, cotton and tomato, which provide information on the host species of the transgenic material and also indicates potential plant cross-contamination in raw material. A generic plant target was also added to detect vegetable DNA and especially residual plant genetic material in processed products.
2. Considered as key indicators of GM material, the frequently used Cauliflower Mosaic Virus 35S promoter (p-35S), Figwort Mosaic Virus 35S promoter (p-FMV), nopaline synthase terminator from *Agrobacterium tumefaciens* (t-NOS), 5-enolpyruvylshikimate-3-phosphate synthase gene from *A. tumefaciens* strain CP4 (CP4epsps) and phosphinothricin *N*-acetyltransferase genes from *Streptomyces hygroscopicus* (Bar) and from *Streptomyces viridochromogenes* (Pat) were selected. To broaden the screening capability of the method, the ribulose-1,5-bisphosphate carboxylase terminator E9 from *Pisum sativum* (t-E9) introduced in recent transgenic constructs was added. Used as selectable antibiotic marker in some GM events such as some GM rice, GM potato and GM tomato events, the hygromycin phosphotransferase and neomycin phosphotransferase genes from *Escherichia coli* (Hph and NptII, respectively) were targeted. Recommended by a recent European decision to further detect insect-resistant GM rice [19], a novel Cry1Ab/c assay was designed on a consensus DNA fragment of *cry1Ab* and *cry1Ac* genes from *Bacillus thuringiensis*.
3. Specific assays were designed to identify 28 GM events, including 16 GM maize, 9 GM soya and 3 GM rice events. Although event-specific assays shall be preferred to construct-specific ones, single nucleotide polymorphisms were shown to occur more likely in the endogenous host plant sequence than in transgenic sequence [20]. To avoid such nucleotide variations, construct-specific assays were privileged. Since the full transgenic cassette from each GM event is generally not publicly available, an overall PCR preferably spanning specific transgenic elements introduced in the targeted GM event (such as the association of a specific transgene and its terminator) was carried out (data not shown) and the amplicon sequenced (GATC, Konstanz, Germany). The primers and the probe were then designed on this specific genetic association only present in the targeted GM event and on the specific oligonucleotide linker used to build and clone it.

Finally, an IPC was used to evaluate the absence of PCR inhibition, especially in the case of a negative result.

Oligonucleotide primers and probes

Primers and MGB TaqMan® probes (Table 1) were designed using the Primer Express® 3.0 Software (Life Technologies, Carlsbad, CA, USA) and produced amplicons below 150 bp, as recommended for analysis of the processed sample [21]. TaqMan® probes were 5′ labelled with 6-carboxyfluorescein (FAM) or VIC fluorophores and 3′ labelled with a nonfluorescent quencher (NFQ). All oligonucleotides were ordered as customised assays at Life Technologies.

An IPC from Life Technologies was used to check for potential PCR inhibitions. Sequences of the IPC primers and its NED-labelled probe were kept proprietary.

The ready-to-use GMO 384-well plate

Based on a 384-well plate, each assay was lyophilised columnwise by Life Technologies at a final concentration corresponding to 900-nM primers and 250-nM probe for each target per RTi-PCR. Each well contained a FAM-, a VIC- and a NED-labelled target, with the exception of the 24th column which only contained a FAM- and a NED-labelled target (Table 2). FAM and VIC fluorophores enabled the detection of plant and GM markers, and the identification of GM events, while the NED fluorophore was linked to the IPC probe. Row-wise, the plate enabled the analysis of seven samples in duplicate (one DNA extract per row), while the first and last rows were dedicated to the analysis of a no template control (NTC) and a positive control, respectively. Ordered at Eurofins MWG Operon (Zurich, Switzerland), the positive control consisted of pEX-A plasmids containing the different amplicons at 2,000 copies/μL.

Real-time PCR

RTi-PCR runs were performed using an ABI PRISM 7900 Sequence Detection System (Life Technologies). For each RTi-PCR, 10 μL of an amplification mix consisting of 1 μL of sample DNA at 40 ng/μL, 5 μL of 2× TaqMan® Environmental Master Mix 2.0 (Life Technologies), 0.21 μL of 5× Exo IPC DNA (Life Technologies) and 3.79 μL of water was prepared. Each sample amplification mix was distributed row-wise, filling the 24 wells of the 384-well plate.

Following a dissolution step of the lyophilised oligonucleotides for 5 min at 30 °C and an activation of the hot-start DNA Taq polymerase for 10 min at 95 °C, the specific thermocycling consisted of 45 cycles of a denaturation step of 15 s at 95 °C and an annealing/elongation step of 1 min at 60 °C. Although qualitative, experimental conditions and

Table 1 List of forward (F), reverse (R) primers and TaqMan® MGB probes (P) used in this study

Assays	Oligonucleotides	Sequences (5′–3′)	Targeted sequences[a]	Amplicon size (bp)	References
Soya	F	AACCGGTAGCGTTGCCAG	*lectin* gene	81	This study
	R	AGCCCATCTGCAAGCCTTT	GenBank accession number K00821		
	P	FAM-CTTCCTTCAACTTCACC-NFQ			
Maize	F	GGGCTTGCCAGCTTGATG	*zein* gene	60	This study
	R	CGGTAAGGCCAACAGTTGCT	GenBank accession number X07535		
	P	VIC-CGTGTCCGTCCCTG-NFQ			
Rice	F	GCGGCAAGCCCCTTCTT	*prolamin* gene	59	This study
	R	TGCCAGACTTGGTTGTTTCTCA	GenBank accession number AY896773		
	P	FAM-TCAGCTGCGTTTCAA-NFQ			
Wheat	F	GTCCATTGCTTGTAGAAGACCGTTA	*phosphoenolpyruvate carboxylase* gene	121	This study
	R	TCAAGGCAAGTCGATTTCAAGA	GenBank accession number AJ007705		
	P	VIC-CCTTACCTAACAAAGCCT-NFQ			
Rapeseed	F	CATGGTTCAATTTGGTTTATATACGG	*acetyl-CoA-carboxylase* gene	98	This study
	R	AACATCAGCCTGTCCAAAAGAAA	GenBank accession number X77576		
	P	FAM-CTGAGGACTCTTAATTAT-NFQ			
Potato	F	CTGCCTCCGTCAAGATTTGGTCACT	*β-fructosidase* gene	146	Retrieved from [34]
	R	CTCTTCCCTTTCTTGATGG	GenBank accession number DQ478950		
	P	VIC-ACTTGTAATTCATCAAGCCAT-NFQ			
Cotton	F	CCAAAGGAGGTGCCTGTTCA	*stearoyl-acyl-carier protein desaturase* gene	107	Optimised from [35]
	R	TTGAGGTGAGTCAGAATGTTGTTC	GenBank accession number AJ132636		
	P	FAM-TCACCCACTCCATGCC-NFQ			
Tomato	F	CTGCCTCCGTCAAGATTTGGTCACT	*β-fructosidase* gene	143	Retrieved from [34]
	R	CTCTTCCCTTTCTTGATGG	GenBank accession number Z12027		
	P	VIC-ACTTGTAATCTTCTTTATTTCGT-NFQ			
p-35S	F	GACAGTGGTCCCAAAGATGGA	p-35S	80	This study
	R	TGCTTTGAAGACGTGGTTGGAA	GenBank accession number V00141		
	P	FAM-CCCACGAGGAGCATC-NFQ			
p-FMV	F	CAAAGTAAACTACTGTTCCAGCACATG	p-FMV	71	This study
	R	AGTCTTCGGTGGATGTCTTTTTCT	GenBank accession number X06166		
	P	VIC-ATCATGGTCAGTAAGTTT-NFQ			
t-NOS	F	CCCGCAATTATACATTTAATACGCGATAG	t-NOS	76	This study
	R	CACCGCGCGCGATAATTTAT	GenBank accession number U12540		
	P	FAM-TTTGCGCGCTATATTT-NFQ			
t-E9	F	TTTGTTGTGCTTGTAATTTACTGTGTT	t-E9	75	This study
	R	TTCTCCATCCATTTCCATTTCA	GenBank accession number X00806		
	P	VIC-TTTATTCGGTTTTCGCTATC-NFQ			
Bar	F	GCACCATCGTCAACCACTACA	*bar* gene	76	This study

Table 1 (continued)

Assays	Oligonucleotides	Sequences (5′–3′)	Targeted sequences[a]	Amplicon size (bp)	References
	R	GTCCACTCCTGCGGTTCCT	GenBank accession number AF218816		
	P	FAM-CGGTCAACTTCCGTACCG-NFQ			
CP4EPSPS	F	CCGGCGACAAGTCGATCTC	*cp4epsps1* gene	72	This study
	R	CGGTGATGCGCGTTTCA	GenBank accession number AY125353		
	P	VIC-CCACCGGTCCTTCATG-NFQ			
Pat	F	GCATGAGGCTTTGGGATACACA	*pat* gene	69	This study
	R	TGCCATCCACCATGCTTGT	GenBank accession number AY562541		
	P	FAM-ATCCAGCTGCGCGCAAT-NFQ			
Hph	F	GCGCAGGCTCTCGATGA	*hph* gene	62	This study
	R	ACGAGGTGCCGGACTTC	GenBank accession number AY818364		
	P	VIC-CTCGGCCCAAAGCAT-NFQ			
NptII	F	CTCCTGCCGAGAAAGTATCCA	*nptII* gene	66	This study
	R	TAGCCGGATCAAGCGTATGC	GenBank accession number AM235741		
	P	FAM-ATGGCTGATGCAATGC-NFQ			
Cry1Ab/c	F	CGGTTACACTCCCATCGACAT	Consensus fragment from *cry1Ab* & *cry1Ac* genes	114	This study
	R	CCAAAGATACCCCAGATGATGTC			
	P	VIC-CAGCGAGTTCGTGCC-NFQ			
Bt176	F	GCGGCCGCACTCGTT	Junction between *p-mCDPK*:: *cry1Ab* sequences	94	This study
	R	GGGTTGCTCAGGCAGTTGTAG			
	P	FAM-CCGGATCCAACAAT-NFQ			
CBH351	F	CTATTACTTCAGCCATAACAAAA GAACTCT	Junction between *cab22L*:: *cry9c* sequences	82	This study
	R	GTCGGTCATCTGCAGGTAGTCA			
	P	VIC-CTTCTTATTAAACCAAAACC-NFQ			
T25	F	GTGTGGAATTGTGAGCGGATAA	Junction between β-*lactamase*:: *p-35S* sequences	82	Optimised from [36]
	R	TGAATCTTTGACTCCATGGGAAT			
	P	FAM-CACAGGAAACAGCTATG-NFQ			
MON810	F	ACCAAGCGGCCATGGA	Junction between *mHSP70intron*:: *cry1Ab* sequences	57	This study
	R	GGCAGTTGTACGGGATGCA			
	P	VIC-AACAACCCAAACATCA-NFQ			
Bt11	F	CAAGCCGCGGATCCTCTA	Junction between *ADH1intron*:: *cry1Ab* sequences	58	This study
	R	TGCATTCGTTGATGTTTGGG			
	P	FAM-AGTCGACCATGGACAAC-NFQ			
MON863	F	GGAGAGGACACGCTGACAA	Junction between *p-35S*::*cab* sequences	65	This study
	R	TGTGTGGAAGATGGTTCTAGGAT			
	P	VIC-CTAGCTTGGCTGCAGGTA-NFQ			
NK603	F	AGCGCGCAAACTAGGATAAATT	Junction between *t-NOS*::*p-35S* sequences	65	This study
	R	CCTGCAGAAGCTATCCCCG			
	P	FAM-CGGTGTCATCTATGTTAC-NFQ			
GA21	F	CAACGTCAGCAACGGCG	Junction between $ctp_{RuBisCo}$:: *mepsps* sequences	110	This study
	R	TGGACCCCGGCAGCTT			

Table 1 (continued)

Assays	Oligonucleotides	Sequences (5′–3′)	Targeted sequences[a]	Amplicon size (bp)	References
	P	VIC-CAGCCCATCAAGGAG-NFQ			
TC1507	F	TGTACATTGACAGGTTTGAGTTGATTC	Junction between *cry1Fa2*::*ORF25* sequences	65	This study
	R	GCAGGTCGACGGATCCTTAC			
	P	FAM-AGTTACTGCCACACTCG-NFQ			
DAS59122	F	AGGATCCACACGACACCATGT	Junction between *p-UBI*::*cry34Ab1* sequences	64	This study
	R	TGGCCGGTCTTGTTGTTCA			
	P	VIC-CCCGCGAGGTGCA-NFQ			
MIR604	F	CCCGTGAACTAGATCTGAGCTCTAG	Junction between *cry3A*::*t-NOS* sequences	94	This study
	R	CCGGCAACAGGATTCAATCTTA			
	P	FAM-AATTTCCCCGATCGTTC-NFQ			
MON88017	F	ATTTGCGGCCGCGTTAA	Junction between *t-NOS*::*p-35S* sequences	67	This study
	R	CCGGATATTACCCTTTGTTGAAA			
	P	VIC-AAGCTTCTGCAGGTCC-NFQ			
MON89034	F	GCCTCGTGCGGAGCTTT	Junction between *ract1*::*cry1Ab* sequences	65	This study
	R	CGTTGATGTTTGGGTTGTTGTC			
	P	FAM-AGGTAGAAGTGATCAACC-NFQ			
98140	F	CACCCTGTTGTTTGGTGTTACTTCT	Junction between *p-UBI*::*gat4621* sequences	86	This study
	R	GCGTTGATAGGCTTAACCTCAATAG			
	P	VIC-ATCCACACGACACCAT-NFQ			
MIR162	F	CCCTGTTGTTTGGTGTTACTTCTG	Junction between *p-UBI*::*vip3Aa20* sequences	71	This study
	R	AGCTTGGTGTTGTTCTTGTTCATG			
	P	FAM-TCGACTCTAGAGGATCCA-NFQ			
SYN3272	F	GACGAGCTGTGATAGGTAACGAAA	Junction between *amy797E*::*pepc9* sequences	72	This study
	R	TCGATGACTGACTACTCCACTTTGT			
	P	VIC-AGAGCTCTAGATCTGTTCTG-NFQ			
RRS	F	GGCGCGAAGATCGAACTC	Junction between *cp4epsps*::*t-NOS* sequences	68	This study
	R	ATCCGGTACCGAGCTCGAA			
	P	FAM-CCGATACGAAGGCTG-NFQ			
A2704-12	F	GCAAAAAAGCGGTTAGCTCCTT	Junction between *bla*::*lacZ* sequences	61	Optimised from [37]
	R	CAGGCTGCGCAACTGTTG			
	P	VIC-CCTCCGATCGCCCTT-NFQ			
356043	F	TCATAGGTATCCTCTGCGTTAATCG	Junction between *p-TMV*::*gat4601* sequences	67	This study
	R	AGTCGACCCGGGATCCA			
	P	FAM-TTCACCTCTATCATGGTGTC-NFQ			
305423	F	CCCAACATTGCTTATTCACACAAC	Junction between *p-KTi3*::*fad2-1* sequences	87	This study
	R	GACCACACTCGTGAGCAATCA			
	P	VIC-ATAGCCCCCCAAGCG-NFQ			
MON89788	F	GTTCTTTTTTTTGCAGATTTGTTGAC	Junction between *p-Tsf1*::*ctp* sequences	70	This study
	R	ACACCATTGCAGATTCTGCTAACT			
	P	FAM-AGAGATCTACCATGGCGC-NFQ			

Table 1 (continued)

Assays	Oligonucleotides	Sequences (5′–3′)	Targeted sequences[a]	Amplicon size (bp)	References
MON87701	F	CCTTCCTGACCTTACCGATTCC	Junction between *Rbcs4-ctp*:: *cry1Ac* sequences	74	This study
	R	CGTTGATGTTTGGGTTGTTGTC			
	P	VIC-TGGTCGCGTCAACTG-NFQ			
BPS-CV127-9	F	TCCCATGCATTCCAAGCA	*ahas* fragment unique recombination	80	This study
	R	TTTTCATTTCTTTTTCCAGCATGAG			
	P	FAM-AACGACAACTCATCATC-NFQ			
FG72	F	AAGCCCATCAGGCCCATT	Junction between *hppd*::*ctp* sequences	84	This study
	R	CAGAAGCCTCGGCAACGT			
	P	VIC-TATAGATCTGCCATGCACC-NFQ			
A5547-127	F	TGCCGCAAAAAAGGGAATAA	Junction between *bla*::*lacZ*:: *p-35S* sequences	89	Optimised from [38]
	R	TGAATCTTTGACTCCATGGGAAT			
	P	FAM-CCAGGGTTTTCCCAGTCA-NFQ			
Bt63	F	GACTGCTGGAGTGATTATCGACAGA	Junction between *cry1Ac*::*t-NOS* sequences	83	Optimised from [39]
	R	AGCTCGGTACCTCGACTTATTCAG			
	P	VIC-TCGAGTTCATTCCAGTTAC-NFQ			
KMD1	F	TGTCGATGCTCACCCTGTTG	Junction between *mUBIintron*:: *cry1Ab* sequences	86	This study
	R	CATTCGTTGATGTTTGGGTTGT			
	P	FAM-TGCAGGTCGACTCTAGA-NFQ			
IR72/Xa21	F	ATCGTGTTGTGTGTACCATGCA	*Xa21* gene from *Oryza longistaminata*	151	This study
	R	AAATTCTGAAAGAACACACGCAAA	GenBank accession number U72723		
	P	VIC-CTCCTTTAAAACAAATAATG-NFQ			
Plant	F	TGGATTGAGCCTTGGTATGGAA	*tRNA-Leu* chloroplastic gene	≈90	Optimised from [30]
	R	GGATTTGGCTCAGGATTGCC	GenBank accession number GQ861354		
	P	FAM-ATTCCAGGGTTTCTCTGAAT-NFQ			
IPC		Proprietary from Applied Biosystems (Life Technologies)			

[a] When not described, gene abbreviations are identical to those used in CERA GMO database [27]

assay characteristics are depicted according to the MIQE guidelines [22].

All detector signals were analysed with the SDS 2.4 software (Life Technologies) using an automatic baseline and a manual threshold of 0.2. The IPC signal (NED labelled) was, however, analysed individually with a manual baseline between 5 and 30 and a manual threshold of 0.1. A positive amplification was considered when a C_T value below 45 was obtained.

Specificity and sensitivity trials

To evaluate the specificity of the assays, high percentages (≥1 % (*m*/*m*)) of GM materials and plant materials were tested in duplicate. Since the limit of detection (LOD) is the amount of analyte at which the analytical method detects the presence of the analyte at least 95 % of the time, the LOD is reached when a maximum of one replicate out of 20 is negative. To determine the LOD of the different assays, a minimum of 20 replicates of low percentages (≤0.1 %) were tested. To obtain these low concentrations of GM materials, DNA extracted from highly contaminated CRMs was serially diluted into their non-GM counterpart DNA. Similarly, plant DNA was diluted into animal CRM DNA (Coring System Diagnostix GmbH). LOD in copy number (LOD_{copies}) was calculated by dividing the target DNA mass (in picogram) by the 1C value from the host plant genome [23]. As tolerated, an additional

Table 2 Schematic template of the ready-to-use 384-well plate. The assays are lyophilised in the corresponding 24 columns, whereas the 16 rows (from A to P) allow the analysis of seven samples in duplicate as well as a negative and a positive control, as described in the "Materials and methods" section

		Fluorophores	Targets																										
		FAM	Soya	Rice	Rapeseed	Cotton	p-35S	t-NOS	Bar	Pat	NptII	Bt176	T25	Bt11	NK603	TC1507	MIR604	MON89034	MIR162	RRS	356043	MON89788	CV127	A5547-127	KMD1	Plant
		VIC	Maize	Wheat	Potato	Tomato	p-FMV	t-E9	CP4epsps	Hph	cry1Ab/c	CBH351	MON810	MON863	GA21	DAS59122	MON88017	98140	3272	A2704-12	305423	MON87701	FG72	Bt63	Xa21	
		NED	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC	IPC
Samples	Rows		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
NTC negative control	A	FAM VIC NED																								
Sample 1a	B	FAM VIC NED																								
Sample 1b	C	FAM VIC NED																								
Sample 2a	D	FAM VIC NED																								
Sample 2b	E	FAM VIC NED																								
Sample 3a	F	FAM VIC NED																								
Sample 3b	G	FAM VIC NED																								
Sample 4a	H	FAM VIC NED																								
Sample 4b	I	FAM VIC NED																								
Sample 5a	J	FAM VIC NED																								
Sample 5b	K	FAM VIC NED																								
Sample 6a	L	FAM VIC NED																								
Sample 6b	M	FAM VIC NED																								
Sample 7a	N	FAM VIC NED																								
Sample 7b	O	FAM VIC NED																								
Positive control	P	FAM VIC NED																								

conversion factor of 0.5 was applied for heterozygous GM maize events to take into account the biological variability from the parents [24], even though this factor of variability ranges between 0.4 and 0.6 depending on hybrid male/female composition.

Results and discussion

Specificity

To determine the specificity of the method, plant materials and GM events with a high GM content (≥1 %) were tested in duplicate. To assess the reliability of the RTi-PCR runs, a negative NTC and a positive control were analysed in each run. All the 47 assays successfully amplified on the positive control, while no amplification curves were observed with NTC.

The generic plant assay successfully amplified on all the plant species tested (Table 3) and did not lead to any signals on animal DNA (beef, pig, horse, sheep, goat and chicken) . The assays targeting soya, maize, rice, wheat, rapeseed, potato, cotton and tomato were specific to their respective plant species only, genetically modified or not. As closely related species of rapeseed and known to regularly lead to rapeseed cross-amplification and misidentification [25], *Sinapis alba* and *Brassica nigra* mustards were also tested and did not lead to any rapeseed amplification. Therefore, the specificity of the targeted *acetyl-CoA carboxylase* gene was confirmed, as already reported [25, 26].

The screening marker assays, namely p-35S, p-FMV, t-NOS, t-E9, CP4epsps, Bar, Pat, Hph, NptII and Cry1Ab/c, successfully amplified the expected GM events containing these genetic elements (Table 3). While p-35S, p-FMV, t-NOS, t-E9, CP4epsps, Bar, Pat and NptII are well-known transgenic elements of GM constructs, the *hph* gene was introduced in KMD1 and IR72/Xa21 GM rice events as selective markers and was correctly detected. In addition to KMD1, our novel Cry1Ab/c assay successfully amplified on Bt176, Bt11, MON810, MON89034, MON87701, Bt63, MON15985 and MON531 GM events, containing the insect-resistant *cry1Ab* or *cry1Ac* genes (Table 3). No cross-reactivity was observed on any other *cry* genes such as *cry9c* and *cry3Bb1* contained in CBH351 and MON863 GM maize events. However, since *cry1Ab* gene sequence has been truncated and highly modified to optimise its expression in Bt176 GM maize [27], its amplification was less efficient on Bt176 and led to higher C_T values compared to the other GM events (data not shown). Amongst the ten GM marker assays available, CP4epsps assay was designed on *cp4epsps1* DNA sequence [14], which successfully amplified on the GM events containing this transgene sequence, namely NK603, MON88017 and RRS, and did not lead to any signal when tested on MON89788, MON1445, and GT73 containing the *cp4epsps2* DNA sequence (Table 3). A new set of primers and probe would need to be designed to amplify both *cp4epsps1* and *cp4epsps2* DNA sequences. Globally, based on the theoretical transgenic construct of the tested GM events, no false-positive or false-negative signals were observed for these GM marker assays, indicating a reliable behaviour from the screening capabilities of the method.

The GM event assays were tested against all the GM events available and their non-GM-counterparts. Mainly based on construct-specific designs, the corresponding assays only amplified on their targeted events (Table 3). However, GA21 assay cross-amplified on GHB614 GM cotton and FG72 GM soya. Designed on the association of the maize chloroplastic transit peptide from the *RuBisCo* gene and the maize *epsps* gene in GA21 GM maize, the same construct was also introduced in GHB614 GM cotton and FG72 GM soya, leading to GA21 cross-amplification. Hopefully, these three GM events can easily be discriminated by their endogenous species (maize, cotton or soya). Although no other false-positive signals were obtained, late amplifications (C_T values >37) of MON810 GM maize and RRS GM soya were randomly observed in several CRMs. Since these cross-contaminations were already reported by the IRMM CRM supplier and known to be intrinsically linked to the CRMs' purity and their adventitious cross-contamination [28], they were not reported in the present study.

With the exception of GA21 assay cross-reactivity previously observed, the results obtained with the described method perfectly matched the expected analytical profile (plant, GM marker and GM event), which confirms the specificity of the different assays and of the whole method.

Sensitivity

To determine the sensitivity of the different assays, plant materials and GM events with a low GM content (≤0.1 %) were analysed. Since the limit of detection is the amount of analyte at which the analytical method detects the presence of the analyte at least 95 % of the time, a minimum of 20 replicates were tested for each target. Aligned with the European guidelines [29], the majority of the assays reached a LOD ≤0.045 % (Fig. 1). With the exception of wheat which led to a LOD of 0.1 %, the endogenous plant assays were able to detect their corresponding species between 0.001 and 0.02 %. Targeting a multicopy chloroplastic gene, the generic plant assay allowed a very sensitive detection of plant material (LOD of 0.001 %), known to be suitable for the detection of very low levels of plant genetic material [30], which could be very useful for the detection of residual plant DNA in highly processed products, such as starch or lecithin.

Table 3 Screening patterns obtained on reference materials for specificity testing. An expected and an analytical positive amplification is indicated with a grey highlight and with an “X,” respectively

	Plant assays									GM-markers assays										GM-events assays																											
Tested samples	Plant	Maize	Soya	Rice	Wheat	Rapeseed	Potato	Cotton	Tomato	p-35S	p-FMV	t-NOS	t-E9	Bar	CP4epsps	Pat	Hph	NptII	cry1Ab/c	Bt176	CBH351	T25	MON810	Bt11	MON863	NK603	GA21	TC1507	DAS59122	MIR604	MON88017	MON89034	98140	MIR162	3272	RRS	A2704-12	356043	305423	MON89788	MON87701	CV127	FG72	A5547-127	Bt63	KMD1	Xa21
Non GM maize	X	X																																													
Bt176	X	X								X				X					X	X																											
CBH351	X	X								X		X		X							X																										
T25	X	X								X						X						X																									
MON810	X	X								X									X				X																								
Bt11	X	X								X		X				X			X					X																							
MON863	X	X								X		X						X							X																						
NK603	X	X								X		X			X											X																					
GA21	X	X										X															X																				
TC1507	X	X								X						X												X																			
DAS59122	X	X								X						X													X																		
MIR604	X	X										X																		X																	
MON88017	X	X								X		X			X																X																
MON89034	X	X								X	X	X							X													X															
98140	X	X								X																							X														
MIR162	X	X									X																							X													
SYN3272	X	X									X																								X												
Non GM soya	X		X																																												
RRS	X		X							X		X			X																					X											
A2704-12	X		X							X						X																					X										
356043	X		X							X																												X									
305423	X		X																																				X								
MON89788	X		X								X		X																											X							
MON87701	X		X																X																						X						
BPS-CV127-9	X		X																																							X					
FG72	X		X									X															X																X				
A5547-127	X		X							X						X																												X			
Basmati rice	X			X																																											
Bt63	X			X								X							X																										X		
KMD1	X			X						X		X					X	X	X																											X	
IR72/Xa21	X			X								X					X	X																													X
LL62	X			X						X						X																															
Non GM rapeseed	X					X																																									
RT73 / GT73	X					X					X		X																																		
Topas 19/2	X					X				X						X		X																													
T45	X					X				X						X																															
Non GM potato	X						X																																								
EH92-527-1	X						X					X						X																													
Non GM cotton	X							X																																							
MON15985	X							X		X		X						X	X																												
LLCotton25	X							X		X		X		X																																	
GHB614	X							X																			X																				
MON1445	X							X		X	X	X	X					X																													
MON531	X							X		X		X						X	X																												
Tomato	X								X																																						
Mustard	X																																														
Wheat flour	X				X																																										
Rye flour	X																																														
Barley flour	X																																														
Animal CRM																																															

The LOD of GM maize targets were all enclosed between 0.02 and 0.045 %, except for T25. The LOD of GM soya and GM rice events ranged between 0.005 and 0.03 %. Considering the genetic structure of these GM crops, the heterozygous trait of GM maize events led to higher LOD, whereas the homozygous structure of GM soya and GM rice events enabled to detect slightly lower GM levels.

In addition, since RTi-PCR is a molecular method detecting DNA molecules (or copies), the absolute sensitivity (LOD_{copies}) was estimated. All assays allowed a very sensitive detection, reaching in some cases the theoretical PCR limit of one copy (Fig. 1). Considering that absolute LOD_{copies} obtained by RTi-PCR are regularly close to ten copies [13, 31], the described method has a similar range of sensitivity.

The competitiveness of the multiplex reactions and its impact on the sensitivity of the assays were also evaluated by analysing strongly asymmetric samples containing a low content of a target in a large excess of the competing one assayed in the same well. Considered as the most frequent

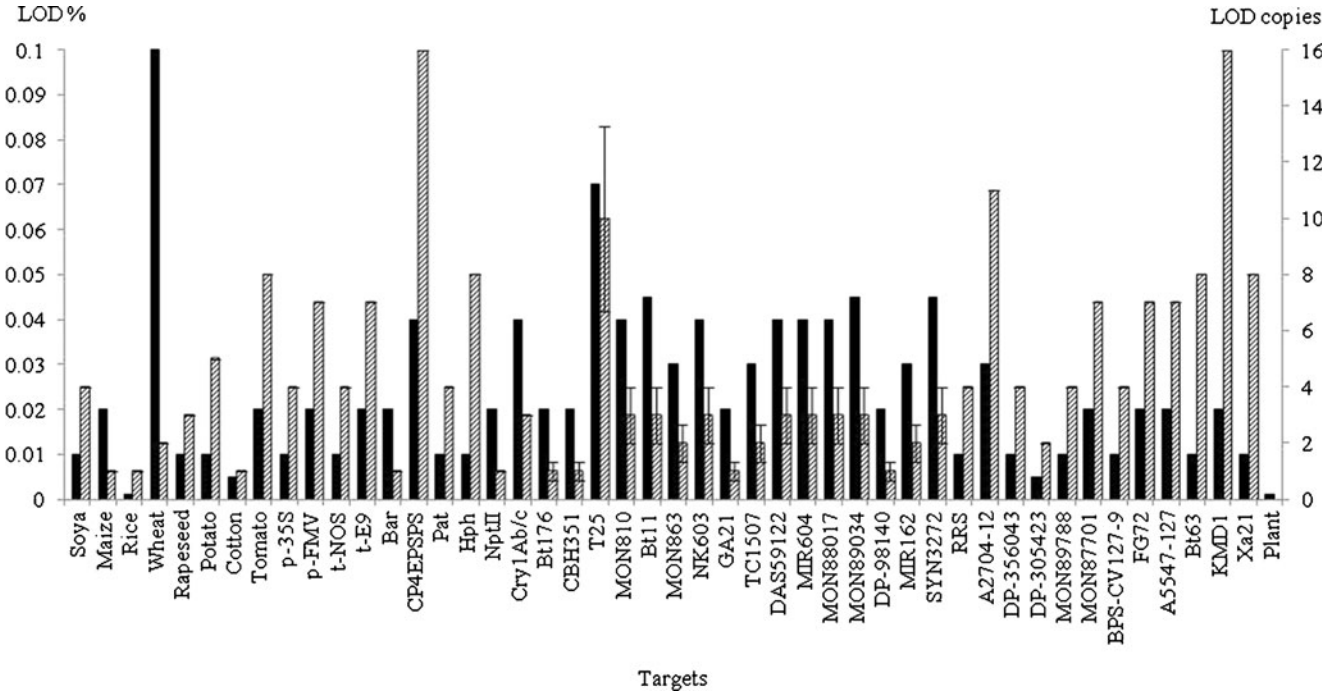

Fig. 1 LOD (in percent) (*black bars*) and LOD_{copies} (*streaked bars*) of the 47 RTi-PCR assays. Variability of LOD_{copies} for heterozygous GM maize events coming from biological variability [24] is indicated by the *error bars*

targets, p-35S, p-FMV, t-NOS, t-E9, RRS, MON810, NK603, MON863, Bt11, GA21, and maize and soya DNA were individually diluted to their LOD in their respective competing DNA (p-FMV, p-35S, t-E9, t-NOS, A2704-12, T25, GA21, Bt11, MON863, NK603, soya and maize, respectively) and analysed. All the targets tested at their LOD in an excess amount of competing background were successfully detected (Table 4). Furthermore, even in the presence of a high content of FAM and/or VIC targets, the IPC always showed constant C_T values which confirm the robustness of the multiplex reactions in asymmetric target scenarios.

Evaluation of PCR inhibition

Known as the main drawback of PCR methodologies, food compounds such as the high level of polyphenols contained in red berries, coffee, chocolate and tea [32] can inhibit PCRs [33]. To address this phenomenon, the extracted DNA underwent an additional purification treatment by gel filtration to remove potential inhibitors. In addition, TaqMan® Environmental Master Mix 2.0 was used as it has been specifically developed for improved performance in the presence of PCR inhibitors. To evaluate the impact of these

Table 4 Performance of several assays when diluting their target DNA to their LOD in highly concentrated competing target

Plate column	Diluted target		Competing target		Number of positive reactions of the diluted target
	Target	Concentration	Target	Concentration	
1	Soya	0.01 %	Maize	99.99 %	7/7
1	Maize	0.02 %	Soya	99.98 %	7/7
5	p-35S	0.01 %	p-FMV	99.99 %	7/7
5	p-FMV	0.02 %	p-35S	9.98 %	7/7
6	t-NOS	0.01 %	t-E9	99.99 %	7/7
6	t-E9	0.02 %	t-NOS	9.98 %	7/7
12	MON810	0.04 %	T25	99.96 %	7/7
13	Bt11	0.04 %	MON863	9.96 %	7/7
13	MON863	0.03 %	Bt11	4.97 %	7/7
14	NK603	0.04 %	GA21	99.96 %	7/7
14	GA21	0.02 %	NK603	4.98 %	7/7
19	RRS	0.01 %	A2704-12	99.99 %	7/7

Table 5 Average IPC C_T values (n=24) obtained on NTC, on three instant coffee powders and on seven cocoa-based samples analysed in duplicates (a and b). DNA extracts were or were not purified by gel filtration

Matrix	Sample	Replicate	C_T values on DNA extracts	
			Non-purified	Purified
NTC	–	–	37.3±0.7	37.4±1.0
Instant coffee powders	1	a	37.4±0.9	37.3±0.7
		b	37.3±0.6	37.2±0.8
	2	a	37.6±0.6	37.5±1.1
		b	37.2±0.7	37.5±0.9
	3	a	37.5±0.7	37.4±0.9
		b	37.5±0.9	37.5±0.9
Cocoa-based products	1	a	37.4±1.6	37.2±1.4
		b	37.2±1.5	37.1±1.4
	2	a	37.4±1.6	37.2±1.6
		b	37.3±1.4	37.1±1.4
	3	a	37.4±1.3	37.3±1.7
		b	37.1±1.5	36.9±1.5
	4	a	37.3±1.3	37.1±1.6
		b	37.4±1.8	37.0±1.2
	5	a	37.2±1.4	37.0±1.9
		b	37.1±1.5	37.1±2.0
	6	a	37.4±1.2	37.2±1.6
		b	37.3±1.8	37.2±1.5
	7	a	37.4±1.7	37.0±1.6
		b	37.2±0.9	37.0±1.6

strategies, some coffee and cocoa-based products were tested. Seven cereal flours containing up to 10 % of cocoa and three different instant coffee powders were tested. The average of the 24 IPC C_T values obtained from each sample replicate was compared to the average of 24 IPC C_T values obtained on the NTC using Student's test at 95 % confidence (Table 5). The IPC C_T averages were not statistically different, whether the DNA extracts were purified by gel filtration against PCR inhibitors or not. Although these samples are known to contain inhibitory phenolic compounds, the results did not show any inhibition without purification of the DNA extracts. The TaqMan® Environmental Master Mix 2.0 was thus robust enough to reliably amplify these non-purified and coloured DNA extracts. However, without this purification treatment, a higher background noise was observed in the raw RTi-PCR data which could interfere with C_T values. Therefore, to avoid any false-positive or false-negative results linked to this background fluorescence, the purification by gel filtration of coloured DNA extracts is recommended. This strategy allowed ensuring reliable results on challenging food matrices, known to inhibit the PCR efficiency and lead to false-negative results.

Amongst 30 runs, the IPC C_T values never varied more than 2.5 %; therefore, this value was considered as the maximum tolerated variation before considering an inhibition.

Applicability of the multiplex real-time PCR on proficiency test samples

To evaluate the reliability and the routine behaviour of the method, proficiency test samples (p test) were tested. These p

Table 6 Screening patterns obtained on p test samples. An expected and an analytical positive amplification are indicated with a grey highlight and with an "X," respectively. Targets which were not evaluated by FAPAS are streaked, while those which did not lead to any consensus are indicated with parentheses

Tested Samples	Plant assays									GM-markers assays									
	Plant	Maize	Soya	Rice	Wheat	Rapeseed	Potato	Cotton	Tomato	p-35S	p-FMV	t-NOS	t-E9	Bar	CP4epsps	Pat	Hph	NptII	cry1Ab/c
GeMMU04	X	X	X		X					X		X			(X)	X			X
GeMMU10	X	X	X		X					X		X			X				( )
GeMMU11A	X	X	X		X					(X)		X			(X)				(X)
GeMMU11B	X	X	X		X					(X)		(X)			(X)				(X)
GeMMU11C	X	X	X		X					X		X			X				(X)
GeMMU12	X	X	X		X					X		X			X				
GeMMU14	X	X	X		X					X		X			X				X
GeMMU17A	X	X			X							X							
GeMMU17B	X	X			X														
GeMMU17C	X				X														
GeMMU20A	X				X														
GeMMU20B	X		X		X					X		X			X				
GeMMU20C	X	X			X														
GeMMP03	X	X	X		X					X		X			( )			X	X
GeMMP05	X	X	X		X					X		X			X				( )
GeMMP09	X	X	X		X					X		X			X				X
GeMMP11	X	X	X		X					X		X			X				

Tested Samples	GM-events assays																											
	Bt176	CBH351	T25	MON810	Bt11	MON863	NK603	GA21	TC1507	DAS59122	MIR604	MON88017	MON89034	98140	MIR162	3272	RRS	A2704-12	356043	305423	MON89788	MON87701	CV127	FG72	A5547-127	Bt63	KMD1	Xa21
GeMMU04				( )	X			X									( )											
GeMMU10				( )				(X)			X						X											
GeMMU11A				(X)				X			X						(X)											
GeMMU11B				(X)													(X)											
GeMMU11C				(X)			X										(X)											
GeMMU12							X	(X)			X						( )											
GeMMU14				X				X									X											
GeMMU17A								X																				
GeMMU17B																												
GeMMU17C																												
GeMMU20A																												
GeMMU20B																	X											
GeMMU20C																												
GeMMP03				X		X											( )											
GeMMP05				( )			X										X											
GeMMP09				X								X					(X)											
GeMMP11								X									X											

test samples were mimicking real world samples composed of maize, soya and wheat ingredients and were either unprocessed (GeMMU samples) or processed flours (GeMMP samples). Although these *p* test schemes did not evaluate all the GM events targeted by the GMO 384-well plate, they allowed testing the detection performance on the most commons ones such as RRS, MON810 and NK603. Amongst the 17 individual samples tested, four of them were non-GM-materials and were correctly identified as such (Table 6). On the other hand, the GM events contained in the contaminated samples were successfully identified, as well as their corresponding GM markers. In addition to these correct identifications, late amplifications (C_T value >37) of RRS GM soya and MON810 GM maize were randomly detected. Reported as adventitious contaminations of the main sample matrix in FAPAS *p* test reports, these cross-contaminations did not lead to any consensus results by FAPAS and were not taken into account for the method evaluation.

Used as indicators to evaluate the analytical performance of a method or a laboratory, the analyses of these *p* test samples were all satisfactory and in agreement with the final FAPAS reports. This successful evaluation indicates that the described method is adapted for a routine usage in a GMO testing laboratory.

Conclusion

As a summary, the utmost optimisation of the multiplex RTi-PCR developed in this method offers a broad, simple and cost-efficient strategy in GMO analysis. In addition to the detection of potential plant cross-contamination, the described prespotted 384-well plate allows the simultaneous screening of seven routine samples to obtain their global transgenic fingerprint and content. The 47 assays would theoretically enable the screening of around 95 % of the worldwide known GMO described in public databases. Fast, specific, sensitive and straightforward, this method fits for purpose of GMO testing laboratories, complying with the analytical requirements described in ISO 24276:2006 [18].

Acknowledgments We would like to thank Frederic Aymes, Celeste Chia, Hui Zhen Ho and Yuying Zhong from Nestlé Quality Assurance Center, Singapore, for their valuable contribution, as well as Matthias Kiehne from Life Technologies for his support on this project.

References

1. James C (2011) Global status of commercialized biotech/GM crops: 2011. ISAAA Briefs 2011:43
2. Stein AJ, Rodriguez-Cerezo E (2009) The global pipeline of new GM crops: implications of asynchronous approval for international trade. JRC Sci Tech Rep. doi:10.2791/12087
3. Ruane J (2006) An FAO e-mail conference on GMOs in the pipeline in developing countries: the moderator's summary. FAO. http://www.fao.org/biotech/biotech-forum/. Accessed 23 January 2013
4. Ruebelt MC, Lipp M, Reynolds TL, Astwood JD, Engel KH, Jany KD (2006) Application of two-dimensional gel electrophoresis to interrogate alterations in the proteome of genetically modified crops. 2. Assessing analytical validation. J Agric Food Chem 54:2162–2168
5. Kim Y-H, Choi SJ, Lee H-A, Moon TW (2006) Quantitation of CP4 5-enolpyruvylshikimate-3-phosphate synthase in soybean by two-dimensional gel electrophoresis. J Microbiol Biotechn 6:25–31
6. Latoszek A, García-Ruiz C, Marina ML, De La Mata FJ, Gómez R, Rasines B, Cifuentes A, Pobozy E, Trojanowicz M (2011) Modification of resolution in capillary electrophoresis for protein profiling in identification of genetic modification in foods. Croat Chem Acta 84:375–382
7. López MCG, Garcia-Cañas V, Alegre MLM (2009) Reversed-phase high-performance liquid chromatography-electrospray mass spectrometry profiling of transgenic and non-transgenic maize for cultivar characterization. J Chromatogr A 1216:7222–7228
8. Xu W, Huang K, Liang Z, Deng A, Yuan Y, Guo F, Luo Y (2009) Application of stepwise ammonium sulfate precipitation as cleanup tool for an enzyme-linked immunosorbent assay of glyphosate oxidoreductase in genetically modified rape of gt73. J Food Biochem 33:630–648
9. Elenis DS, Kalogianni DP, Glynou K, Ioannou PC, Christopoulos TK (2008) Advances in molecular techniques for the detection and quantification of genetically modified organisms. Anal Bioanal Chem 392:347–354
10. Marmiroli N, Maestri E, Gullì M, Malcevschi A, Peano C, Bordoni R, De Bellis G (2008) Methods for detection of GMOs in food and feed. Anal Bioanal Chem 392:369–384
11. International Organization for Standardization (2005) ISO 21569:2005. Foodstuffs—methods of analysis for the detection of genetically modified organisms and derived products—qualitative nucleic acid based methods. International Organization for Standardization, Geneva, Switzerland
12. nternational Organization for Standardization (2005) ISO 21570:2005. Foodstuffs—methods of analysis for the detection of genetically modified organisms and derived products—quantitative nucleic acid based methods. International Organization for Standardization, Geneva, Switzerland
13. Dörries H-H, Remus I, Grönewald A, Grönewald C, Berghof-Jäger K (2010) Development of a qualitative, multiplex real-time PCR kit for screening of genetically modified organisms (GMOs). Anal Bioanal Chem 396:2043–2054
14. Guo J, Chen L, Liu X, Gao Y, Zhang D, Yang L (2012) A multiplex degenerate PCR analytical approach targeting to eight genes for screening GMOs. Food Chem 132:1566–1573
15. Querci M, Foti N, Bogni A, Kluga L, Broll H, Van den Eede G (2009) Real-Time PCR-based ready-to-use multi-target analytical system for GMO detection. Food Anal Method 2:325–336
16. Kutyavin IV, Afonina IA, Mills A, Gorn VV, Lukhtanov EA, Belousov ES, Singer MJ, Walburger DK, Lokhov SG, Gall AA, Dempcy R, Reed MW, Meyer RB, Hedgpeth J (2000) 3′-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res 28:655–661
17. Gasparic MB, Tengs T, La Paz JL, Holst-Jensen A, Pla M, Esteve T, Zel J, Gruden K (2010) Comparison of nine different real-time PCR chemistries for qualitative and quantitative applications in GMO detection. Anal Bioanal Chem 396:2023–2029
18. International Organization for Standardization (2006) ISO 24276:2006. Foodstuffs—methods of analysis for the detection of genetically modified organisms and derived products—general requirements and definitions. International Organization for Standardization, Geneva, Switzerland

19. European Commission (2011) EC decision (2011/884/EU) on emergency measures regarding unauthorised genetically modified rice in rice products originating from China and repealing Decision 2008/289/EC. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:343:0140:0148:EN:PDF. Accessed 28 March 2012
20. Ghedira R, Papazov N, Vuylsteke M, Ruttink T, Taverniers I, De Loose M (2009) Assessment of primer/template mismatch effects on real-time PCR amplification of target taxa for GMO quantification. J Agric Food Chem 57:9370–9377
21. Gryson N (2010) Effect of food processing on plant DNA degradation and PCR-based GMO analysis: a review. Anal Bioanal Chem 396:2003–2022
22. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622
23. Arumuganathan K, Earle ED (1991) Nuclear content of some important plant species. Plant Mol Biol Rep 9:208–218
24. Joint Research Center, European Union Reference Laboratory for GM Food & Feed (EURL), Ispra, Italy (2011) Technical guidance document from the European Union Reference Laboratory for genetically modified food and feed on the implementation of commission regulation (EU) no 619/2011. http://gmo-crl.jrc.ec.europa.eu/doc/Technical%20Guidance%20from%20EURL%20on%20LLP.pdf. Accessed 14 May 2013
25. Wu G, Zhang L, Wu Y, Cao Y, Lu C (2010) Comparison of five endogenous reference genes for specific PCR detection and quantification of *Brassica napus*. J Agric Food Chem 58:2812–2817
26. Schmidt A-M, Rott ME (2006) Real-time polymerase chain reaction (PCR) quantitative detection of *Brassica napus* using a locked nucleic acid TaqMan probe. J Agric Food Chem 54:1158–1165
27. CERA (2013) CERA GM crop database, ILSI Research Foundation, Washington DC, USA. http://cera-gmc.org/index.php?action=gm_crop_database&mode=ShowProd&data=176. Accessed 28 March 2012
28. Institute for Reference Materials and Measurements (IRMM), Geel, Belgium (2007) Additional information related to the raw material used by IRMM for the production of GMO CRMs. http://irmm.jrc.ec.europa.eu/reference_materials_catalogue/user_support/Documents/FAQ%20GMO_attachment.pdf. Accessed 24 April 2012
29. Joint Research Center, European Union Reference Laboratory for GM Food & Feed (EURL), Ispra, Italy (2008) Definition of minimum performance requirements for analytical methods of GMO testing. http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm. Accessed 19 December 2012
30. Laube I, Hird H, Brodmann P, Ullmann S, Schöne-Michling M, Chisholm J, Broll H (2010) Development of primer and probe sets for the detection of plant species in honey. Food Chem 118:979–986
31. Bahrdt C, Krech AB, Wurz A, Wulff D (2010) Validation of a newly developed hexaplex real-time PCR assay for screening for presence of GMOs in food, feed and seed. Anal Bioanal Chem 396:2103–2112
32. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L (2004) Polyphenols: food sources and bioavailability. Am J Clin Nutr 79:727–747
33. Demeke T, Jenkins GR (2010) Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. Anal Bioanal Chem 396:1977–1990
34. Chaouachi M, El Malki R, Berard A, Romaniuk M, Laval V, Brunel D, Bertheau Y (2008) Development of a real-time PCR method for the differential detection and quantification of four Solanaceae in GMO analysis: potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melogena*), and pepper (*Capsicum anuum*). J Agric Food Chem 56:1818–1828
35. Yang L, Chen J, Huang C, Liu Y, Jia S, Pan L, Zhang D (2005) Validation of a cotton-specific gene, Sad1, used as an endogenous reference gene in qualitative and real-time PCR quantitative PCR detection of transgenic cottons. Plant Cell Rep 24:237–245
36. Collonier C, Schattner A, Berthier G, Boyer F, Coué-Philippe G, Diolez A, Duplan MN, Fernandez S, Kebdani N, Kobilinski A, Romaniuk M, De Beuckeleer 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
37. Takabatake R, Akiyama H, Sakata K, Onishi M, Koiwa T, Futo S, Minegishi Y, Teshima R, Mano J, Furui S, Kitta K (2011) Development and evaluation of event-specific quantitative PCR method for genetically modified soybean A2704-12. Food Hyg Saf Sci 52:100–107
38. Tengs T, Kristoffersen AB, Zhang H, Berdal KG, Lovoll M, Holst-Jensen A (2010) Non-prejudiced detection and characterization of genetic modifications. Food Anal Methods 3:120–128
39. Mäde D, Degner C, Grohmann L (2006) Detection of genetically modified rice: a construct-specific real-time PCR method based on DNA sequences from transgenic Bt rice. Eur Food Res Technol 224:271–278