Anal Bioanal Chem (2014) 406:1397–1410
DOI 10.1007/s00216-013-7562-1

RESEARCH PAPER

A high-throughput method for GMO multi-detection using a microfluidic dynamic array

Fábio Cristiano Angonesi Brod · Jeroen P. van Dijk · Marleen M. Voorhuijzen · Andréia Zilio Dinon · Luis Henrique S. Guimarães · Ingrid M. J. Scholtens · Ana Carolina Maisonnave Arisi · Esther J. Kok

Received: 10 October 2013 / Revised: 29 November 2013 / Accepted: 6 December 2013 / Published online: 20 December 2013

Abstract The ever-increasing production of genetically modified crops generates a demand for high-throughput DNA-based methods for the enforcement of genetically modified organisms (GMO) labelling requirements. The application of standard real-time PCR will become increasingly costly with the growth of the number of GMOs that is potentially present in an individual sample. The present work presents the results of an innovative approach in genetically modified crops analysis by DNA based methods, which is the use of a microfluidic dynamic array as a high throughput multi-detection system. In order to evaluate the system, six test samples with an increasing degree of complexity were prepared, preamplified and subsequently analysed in the Fluidigm system. Twenty-eight assays targeting different DNA elements, GM events and species-specific reference genes were used in the experiment. The large majority of the assays tested presented expected results. The power of low level detection was assessed and elements present at concentrations as low as 0.06 % were successfully detected. The approach proposed in this work presents the Fluidigm system as a suitable and promising platform for GMO multi-detection.

Keywords High throughput · GMO analysis · Preamplification · Fluidigm system

Electronic supplementary material The online version of this article (doi:10.1007/s00216-013-7562-1) contains supplementary material, which is available to authorized users.

F. C. A. Brod · J. P. van Dijk (✉) · M. M. Voorhuijzen · I. M. J. Scholtens · E. J. Kok (✉)
RIKILT Wageningen UR, P.O. Box 230, 6700 AE Wageningen, The Netherlands
e-mail: jeroen.vandijk@wur.nl
e-mail: esther.kok@wur.nl

A. Z. Dinon · A. C. M. Arisi
Department of Food Science and Technology, Federal University of Santa Catarina, Rod. Admar Gonzaga, 1346, Florianópolis, SC 880034-001, Brazil

L. H. S. Guimarães
Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, São Paulo University, Avenida Bandeirantes 3900, Monte Alegre, Ribeirão Preto, SP 14040-901, Brazil

Introduction

A continuous increase in the production of genetically modified (GM) crops has been observed in the last decades. In 2012, 170.3 million hectares were planted with GM crops in 28 countries, corresponding to a 100-fold increase since 1996, when the first data for GM crops production were published [1]. Herbicide-tolerant soybean remains the most cultivated GM crop, followed by maize, cotton and canola. At the same time, a diversity of new GM organisms (GMO) can be observed that are moving towards the market.

The process of GMO development has led to legislations worldwide to regulate and/or track the presence of GMOs in feed and foodstuffs resulting in an ongoing demand for methods for detection, identification and quantification. The development of accurate, sensitive, reproducible and, especially, high-throughput detection methods has become a very important aspect in the field of GMO research. Screening methods can be used for discriminating between authorized and non-authorized organisms, safe and potentially unsafe material or, even, certification of purity [2–4]. Quantitative testing, on the other hand, is commonly used for testing for regulatory purposes, e.g. when a legal level of compliance must be addressed [2]. Among a number of analytical techniques that can be used for GMO testing, the most commonly accepted and used is quantitative real-time PCR (q-PCR), which can be considered as the "gold standard" technique nowadays.

Due to the increasing number and complexity of GM crops, the traditional single-target strategy for GMO testing has

become time consuming and expensive [5, 6]. To fulfil the increasing need for GMO analysis, the detection of multiple targets has become a necessity. A number of multi-target methods have been published. Regarding q-PCR-based methods, major efforts can be highlighted such as the early works by Permingeat [7, 8] and Hernandez [9] where multiplex q-PCR allowing the detection of several GMOs is presented. Another strategy is the "ready-to-use multi-target analytical system" based on TaqMan q-PCR detection [10] allowing the simultaneous identification of 39 GM events in seven plant species. Mano et al. [11] developed a multi-target approach that provides semi-quantitative information and a spread sheet application for interpreting the results and indicating a possible unapproved GMO contamination. Microarray-based approaches also played an important role in GMO multi-detection. Several so-called DNA chips presenting different degrees of multiplexing have been developed [12–16]. A method demonstrating the use of ten different padlock probes for GMO detection was described [17, 18] and the recently described novel alternative method named NAIMA [19, 20] is based on multiplex quantitative DNA-based target amplification coupled to microarray detection. Other methods such as multiplex PCR coupled to capillary gel electrophoresis [21, 22] or transgenic DNA fingerprinting methodology using restriction enzyme digestion, adaptor ligation and nested PCR [6] were also developed and used for detection and identification of multiple GM events. These platforms, however, are laborious and time- as well as reagent-consuming approaches. New technologies, such as the microfluidic dynamic arrays present a high-throughput combined with the low time- and reagent-consuming features [23–26].

In recent years, the detection and identification of unauthorised GMOs has increasingly received attention in many countries around the world due to several incidents with unapproved GMOs in food and feed supply chains [27]. The detection and identification of specific elements such as promoters, terminators or coding sequences that are present in GM events can be used for identification of unknown GMs (UGMs) [28], mainly if using the matrix approach [10, 16, 29]. The matrix approach consists of applying combinations of screening, construct and event-specific methods and comparing the results with tabulated data on presence/absence in individual authorized events, where patterns that do not match are indicative of presence of UGM [2, 4].

It is well accepted that regular q-PCR, i.e. the q-PCR performed in standard real-time PCR instruments with 96 wells format and reaction volumes ranging from 10 to 50 μL, is a low-throughput technique with a limiting number of analysable targets. In this sense, the Fluidigm system from BioMark is an innovative platform with a wide range of possibilities of sample throughput by the use of the dynamic arrays. These microfluidic matrices enable the performance of many combinatorial assays on a set of reagents whilst realizing a significant economy in reagent consumption, pipetting steps and labour [25, 30]. The detailed functioning principle of this system was described by Spurgeon [25]. In short, the microfluidic chip consists of a nanoscale network of fluid lines, valves and chambers under pressure control. The samples and assays are individually pipetted into the corresponding inlets and immediately loaded and mixed in the chip. Afterwards, the chip is ready for thermal cycling, which is carried out as a regular PCR cycling step. The chip is imaged at the end of each cycle. At the end of the thermal cycling, the analysis software generates amplification curves for each of the reaction chambers in the chip. The high-throughput power claimed by the system relies on the flexible chip setup. Available chips such as the 48.48 (48 samples × 48 assays), 192.24 (192 samples × 24 assays) or the 96.96 (96 samples × 96 assays) perform, respectively, 2,304, 4,608 and 9,216 reactions in a single run. Because of such a high number of reactions, the low volume where reactions take place (8 nl) and the possible presence of low level targets a preamplification step is recommended in order to ensure an adequate amount of input DNA [23]. Preamplification will not only increase the sensitivity of the system but also expand the number of analyzable target genes [31].

The microfluidic dynamic array technology allowed the development of a number of novel applications such as protein crystallization [32], gene expression analysis [33], single cell gene expression analysis [34] and digital PCR [35]. So far, the only papers describing the use of microfluidic dynamic arrays for GMO analysis are based on the digital PCR approach [36, 37]. However, the main interesting features of microfluidic dynamic arrays such as reagent, time economy and the high throughput power have not yet been applied for GMO detection.

The present study investigates the capacity of the Fluidigm system as a high-throughput screening tool for multi-detection of GMOs. In order to show multiplex features of the Fluidigm system, samples containing several GM events at different concentrations were tested. The applicability of the system to detect and identify authorised as well as unauthorised GMOs in food and feed supply chains will be discussed.

Material and methods

Plant materials

Certified Reference Materials (CRMs) of GM maize lines Bt11 (ERM-BF412f), Bt176 (ERM-BF411f), GA21 (ERM-BF414f), MIR604 (ERM-BF423d), TC1507 (ERM-BF418d), MON810 (ERM-BF413f) and Event3272 (ERM-BF420c); GM soybean lines RRS event 40-3-2 (ERM-BF410f), DP305423 (ERM-BF426d) and DP356043 (ERM-BF425d); and GM sugar beet line H7-1 (ERM-BF419b) were purchased

from European Reference Materials, ERM (Belgium). CRMs of GM maize lines MON88017 (AOCS 0406-D), MON89034 (AOCS 0906-E) and GM canola line RT73 (AOCS 0304-B) were purchased from American Oil Chemists' Society, AOCS (USA). Also, non-GM lines of maize (ERM-BF411a), soybean (ERM-BF410a), sugar beet (ERM-BF419a) and canola (AOCS 0304-A) were purchased from the respective CRM companies.

DNA isolation

Three different DNA isolation methods were used, depending on the reference material.

For soybean reference materials, the DNeasy Plant Mini Kit (Qiagen) was used according to the manufacturer's recommendations with one minor modification: incubation with buffer AP1 and RNase A was carried out for 1 h at 65 °C instead of 10 min at 60 °C.

For maize and sugar beet reference materials, the DNeasy Plant Mini Kit (Qiagen) was used according to the manufacturer's recommendations with the following modifications: a lysis step was carried out using cetyltrimethylammonium bromide (CTAB) buffer (20 g/L CTAB, 1.4 M NaCl, 0.1 % Tris, 20 mM EDTA, pH 8.0) and RNase A (4 μL; 100 mg/mL) instead of the supplied buffer AP1. After incubation for 15 min at 65 °C with 200 rpm in a shaker incubator, 20 μL of Proteinase K (20 μg/μL) was added to each sample with a following incubation 15 min at 65 °C at 200 rpm [38].

For canola reference material, the CTAB-adapted method proposed in [39] was used for the DNA isolation of five samples of 100 mg of ground canola seeds. After isolation, the DNA was pooled in a microcentrifuge tube and mixed with ¼ volumes of 3 M sodium acetate (pH 5.2) and two volumes of 96 % ethanol. Samples were mixed by inversion and subsequently centrifuged at 14,000 rpm for 20 min. The supernatant was discarded and the resulting pellet was cleaned-up using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's recommendations.

DNA quantification

The Nanodrop ND1000 (Thermo Scientific) was used to evaluate DNA quality by analysing the absorption ratios at 260/280 and 260/230 nm and to measure the DNA concentrations. Subsequently, quantitative PCRs targeting the endogenous reference gene of each species were carried out, and by comparing the Ct values of the DNA samples with the Ct values of samples with a known DNA concentration determined during in-house validation, the actual concentration could be determined more precisely. PCR amplifications were carried out in an optical 96-well reaction plate in a final volume of 25 μl containing 1× Diagenode Mastermix (Diagenode, Belgium), 50 ng of template DNA and a primer/probe combination. Concentrations of primers and probes are presented in Table 1. Reactions were performed in a MyiQ or CFX96 Real Time System (both BioRad) under the following cycling conditions: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Samples were analysed in duplicate.

Preparation of test samples

DNA mixtures containing a number of different GM varieties at different mass fractions were prepared by mixing the extracted genomic DNA of GM and non-GM varieties. The exact composition of the mixtures, named samples A to F, is presented in Table 2. In order to avoid cross-contamination during the preparation of the test samples, all pipetting procedures were carried out in the QIAgility robot (Qiagen). After preparation, q-PCR was used to test the mixtures for unintended cross-contamination and presence of low level targets. Reaction conditions were the same as mentioned above.

Preamplification

Twenty-eight assays targeting different GM elements, GM events and species-specific references (Table 1) were selected to be included in the system. All event-specific and species-specific assays were validated in European ring trials. For the element specific assays Cry1Ab and nptII, no target was present in any of the six test samples.

Preamplifications were carried out in quadruplicate for each test sample using the TaqMan PreAmp Master Mix (Applied Biosystems) according to the manufacturer's instructions with minor modifications: after 14 preamp cycles, products from the reactions were diluted 1:5 in TE buffer instead of 1:20 as recommended in the manufacturer's protocol. One hundred nanograms of template DNA was used in each preamplification reaction.

After preamplifications, all 24 samples (six test samples × four preamplifications) were tested for the presence of unintended cross-contamination and for the presence of low level targets. Preamplification factors were determined by comparing Ct values obtained before and after preamplification. The preamplification factors were calculated using the formula $2^{(Ct_a - Ct_b)} \times 625$ where Ct_a is the Ct obtained from the non-preamplified sample, Ct_b is the Ct obtained from the preamplified sample and 625 is the correction factor, which corrects for the differences in target concentration caused by dilution steps in the procedure.

q-PCR conditions

q-PCR amplifications were carried out in an optical 96-well reaction plate in a final volume of 25 μL containing 1× Diagenode Mastermix (Diagenode, Belgium), template DNA

Table 1 List of primers and TaqMan probes used in the Fluidigm system experiment

Assay	ID	Concentration (nM)	Reference	Validation
P35S	P35S-1-5′	500	[41]	Collaborative trial validated [42]
	P35S-1-3′	500		
	P35S-Taq	100		
T-NOS	NOS ter 2-5′	500	[41]	Collaborative trial validated [42]
	NOS ter 2-3′	500		
	NOS-Taq	200		
cry1Ab	Cry1Abf-n1	400	[4]	In house
	Cry1Abr-n1	400		
	Cry1Abp-n1	200		
cry1F	Cry1Fr-n1	400	[4]	In house
	Cry1Fr-n2	400		
	Cry1Fp-n1	200		
Bar	bar 2-5′	400	[11]	In house
	bar 2-3′	400		
	BAR-1-Taq	200		
cp4EPSPS	epsps 1-5′	400	[43]	In house
	epsps 3-3′	400	[4]	
	epsps-probe	200		
cry3Bb	Cry3Bbf-n2	400	[4]	In house
	Cry3Bbr-n2	400		
	Cry3BbP-n3	200		
nptII	npt 1-5′	400	[43]	In house
	npt 1-3′	400		
	nptII-probe	200	[4]	
cry1A.105	Cry1A.105-F1	300	[38]	In house
	Cry1A.105-R1	300		
	Cry1A.105-P	150		
cry2Bb	Cry2Ab-F	300	[38]	In house
	Cry2Ab-R	300		
	Cry2Ab-P	150		
E3272	ES3272-F	400	EU-RL GMFF	Collaborative trial validated [44]
	ES3272-R	400		
	ES3272-P	200		
Bt11	Bt113JFor primer	750	[45]	Collaborative trial validated [44]
	Bt113JRev primer	750		
	Bt113JFT	250		
GA21	GA21 primer F	150	EU-RL GMFF	Collaborative trial validated [44]
	GA21 primer R	150		
	GA21 probe	50		
MON89034	MON89034 F	450	EU-RL GMFF	Collaborative trial validated [44]
	MON89034 R	450		
	MON89034 P	100		
MON810	Mail-F1	300	EU-RL GMFF	Collaborative trial validated [44]
	Mail-R1	300		
	Probe Mail-S2	180		
MIR604	MIR604 F	600	EU-RL GMFF	Collaborative trial validated [44]
	MIR604 R	300		
	MIR604 P	200		
MON88017	MON88017 AF	150	EU-RL GMFF	Collaborative trial validated [44]
	MON88017 AR	150		
	MON88017 AP	50		
TC1507	MaiY-F1	300	EU-RL GMFF	Collaborative trial validated [44]
	MaiY-R3	300		
	MaiY-S1	160		

Table 1 (continued)

Assay	ID	Concentration (nM)	Reference	Validation
Bt176	E176 2-5′	312	[41]	Collaborative trial validated [42]
	E176 2-3′	312		
	E176-Taq	125		
RRS event 40-3-2	RRS 01-5′	500	[41]	Collaborative trial validated [42]
	RRS 01-3′	500		
	RRS-Taq	200		
DP305423	DP305-f1	800	EU-RL GMFF	Collaborative trial validated [44]
	DP305-r5	500		
	DP305-p	220		
DP356043	DP356-f1	800	EU-RL GMFF	Collaborative trial validated [44]
	DP356-r1	500		
	DP356-p	220		
H7-1	ZRH7-R2	400	EU-RL GMFF	Collaborative trial validated [44]
	H7PLT1	400		
	ZRH7	100		
RT73	RT73 primer1	150	EU-RL GMFF	Collaborative trial validated [44]
	RT73 primer2	150		
	RT73 FAM probe	50		
HMG	ZM1-F	300	EU-RL GMFF	Collaborative trial validated [44]
	ZM1-R	300		
	Probe ZM	160		
LEC	Lec F	550	EU-RL GMFF	Collaborative trial validated [44]
	Lec R	550		
	Lec P	100		
GluA	GluA3-F	150	EU-RL GMFF	Collaborative trial validated [44]
	GluA3-R	150		
	GluD1	100		
CruA	MDB510 For	400	EU-RL GMFF	Collaborative trial validated [44]
	MDB511 Rev	400		
	TM003	200		

and a primer/probe combination according to the conditions presented in Table 1. Reactions were performed in a MyiQ or CFX96 Real Time System (both BioRad) under the following cycling conditions: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Samples were analysed in duplicate. PCR runs were evaluated using the automatic threshold settings in either the iQ5 Optical System Software version 2 or the CFX Manager Software 3.0 programs.

Fluidigm run

The Fluidigm 48.48 q-PCR run was performed according to the manufacturers' protocol. In short, all TaqMan assays were diluted in Assay Loading Reagent (Fluidigm PN 85000736) and ultrapure water to a 10× final concentration. Separately, DNA was supplemented with 2× TaqMan Universal PCR Master Mix (Applied Biosystems, PN 4304437) and 20× GE Sample Loading Reagent (Fluidigm, PN 85000746). The array chip was primed and loaded with the IFC controller MX (Fluidigm) and run on a Biomark system (Fluidigm). Cycling conditions were 2 min at 50 °C, 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Ct values were calculated using the Fluidigm Q-PCR Analysis Software 3.0.2. Preparation of the array and the Fluidigm run were performed in the facilities of BIOKE (Leiden, The Netherlands).

Per run, all samples were tested in duplicate resulting in 48 targets (six test samples × four preamplifications in duplicate). The assays RRS event 40-3-2, DP305423, DP356043, H7-1, RT73, Lec, GluA and CruA were analysed singular, while all other assays were analysed in duplicate. Added up, it results in 48 assays (eight singular assays plus 20 assays in duplicate). Assays performed singular count for eight reactions per run, while the number of reactions is 16 for the assays performed in duplicate.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical significance between preamplifications was evaluated by one-way analysis of variance (ANOVA)

Table 2 Composition of the test samples prepared for testing the Fluidigm system in the present study

Sample	Component	Source	Code	Final concentration (%[a])
A	4.89 % Bt11 maize	IRMM	ERM-BF412f	0.24
	0 % Bt176 maize	IRMM	ERM-BF411a	99.76
B	4.89 % Bt11 maize	IRMM	ERM-BF412f	0.11
	5 % Bt176 maize	IRMM	ERM-BF411f	0.11
	4.29 % GA21 maize	IRMM	ERM-BF414f	0.1
	9.85 % MIR 604 maize	IRMM	ERM-BF423d	0.22
	9.86 % TC1507 maize	IRMM	ERM-BF418d	0.22
	5 % MON810 maize	IRMM	ERM-BF413f	0.11
	100 % MON8817 maize	AOCS	AOCS-0406-D	2.22
	100 % MON89034 maize	AOCS	AOCS-0906-E	2.22
	9.80 % Event 3272 maize	IRMM	ERM-BF420c	0.22
	0 % Bt176 maize	IRMM	ERM-BF411a	94.47
C	4.89 % Bt11 maize	IRMM	ERM-BF412f	0.27
	5 % Bt176 maize	IRMM	ERM-BF411f	0.28
	4.29 % GA21 maize	IRMM	ERM-BF414f	0.24
	9.85 % MIR 604 maize	IRMM	ERM-BF423d	0.55
	9.86 % TC1507 maize	IRMM	ERM-BF418d	0.55
	5 % MON810 maize	IRMM	ERM-BF413f	0.28
	100 % MON8817 maize	AOCS	AOCS-0406-D	5.56
	100 % MON89034 maize	AOCS	AOCS-0906-E	5.56
	9.80 % Event 3272 maize	IRMM	ERM-BF420c	0.54
	10 % RRS event 40-3-2	IRMM	ERM-BF410g	0.1
	10 % DP305423 soybean	IRMM	ERM-BF426d	0.21
	10 % DP356043 soybean	IRMM	ERM-BF425d	0.21
	0 % Bt176 maize	IRMM	ERM-BF411a	36.17
	0 % RRS event 40-3-2	IRMM	ERM-BF410a	49.48
D	4.89 % Bt11 maize	IRMM	ERM-BF412f	0.05
	5 % Bt176 maize	IRMM	ERM-BF411f	0.06
	4.29 % GA21 maize	IRMM	ERM-BF414f	0.05
	9.85 % MIR 604 maize	IRMM	ERM-BF423d	0.11
	9.86 % TC1507 maize	IRMM	ERM-BF418d	0.11
	5 % MON810 maize	IRMM	ERM-BF413f	0.06
	100 % MON8817 maize	AOCS	AOCS-0406-D	1.11
	100 % MON89034 maize	AOCS	AOCS-0906-E	1.11
	9.80 % Event 3272 maize	IRMM	ERM-BF420c	0.11
	100 % H7-1 sugarbeet	IRMM	ERM-BF419b	0.1
	0 % Bt176 maize	IRMM	ERM-BF411a	87.23
	0 % H7-1 sugarbeet	IRMM	ERM-BF419a	9.9
E	4.89 % Bt11 maize	IRMM	ERM-BF412f	0.05
	5 % Bt176 maize	IRMM	ERM-BF411f	0.06
	4.29 % GA21 maize	IRMM	ERM-BF414f	0.05
	9.85 % MIR 604 maize	IRMM	ERM-BF423d	0.11
	9.86 % TC1507 maize	IRMM	ERM-BF418d	0.11
	5 % MON810 maize	IRMM	ERM-BF413f	0.06
	100 % MON8817 maize	AOCS	AOCS-0406-D	1.11
	100 % MON89034 maize	AOCS	AOCS-0906-E	1.11
	9.80 % Event 3272 maize	IRMM	ERM-BF420c	0.11
	100 % RT-73 canola	AOCS	AOCS-0304-B	0.1
	0 % Bt176 maize	IRMM	ERM-BF411a	87.23
	0 % RT-73 canola	AOCS	AOCS-0304-A	9.9

Table 2 (continued)

Sample	Component	Source	Code	Final concentration (%[a])
F	10 % RRS event 40-3-2	IRMM	ERM-BF410g	0.1
	10 % DP305423 soybean	IRMM	ERM-BF426d	0.21
	10 % DP356043 soybean	IRMM	ERM-BF425d	0.21
	100 % H7-1 sugarbeet	IRMM	ERM-BF419b	0.25
	100 % RT-73 canola	AOCS	AOCS-0304-B	0.25
	0 % RRS event 40-3-2	IRMM	ERM-BF410a	49.48
	0 % H7-1 sugarbeet	IRMM	ERM-BF419a	24.75
	0 % RT-73 canola	AOCS	AOCS-0304-A	24.75

[a]Relative to total mass

followed by the Tukey's multiple comparison test. A p value of $p<0.01$ was considered as statistically significant.

Results

Experimental design

Genomic DNA was extracted from the reference materials and immediately tested for cross-contamination by q-PCR using species-specific assays. No contamination was observed in either the DNA solutions or the extraction controls. The DNA solutions were then used for preparing the test samples (Table 2). After preparation, the samples were tested for the presence of low level targets: Bt11 in sample E, RRS event 40-3-2 in sample F, H7-1 in sample D and RT73 in sample E. All targets were detected in eight out of eight reactions per assay. The test samples were tested for cross-contamination as well and two minor contaminations were observed: in sample E, one out of two reactions was positive (Ct 37.4) for the presence of soybean and in sample F, one out of two reactions was positive (Ct 39.1) for the presence of maize. In order to increase the sensitivity of the system, preamplification reactions were carried out. The experimental design consisted of analysing, in duplicate, 4 independent preamplifications of six different samples (48 samples) with 28 assays targeting several species-, element- and event-specific sequences. Twenty out of these 28 assays were also duplicated in the Fluidigm system, resulting in a total of 48 assays. This set-up of 48 samples × 48 assays resulted in a total of 2,304 reactions, Fig. 1 illustrates the setup of the experiment.

Preamplification

After performing the preamplification reactions, the four preamplification replicates were tested for detection of the same low level targets and cross-contaminations as mentioned before plus Bt176 in samples B, C, D and E. In sample E, Bt11 was detected in eight out of eight reactions in three preamplifications and in seven out of eight in one preamplification. The same results were obtained for RRS event 40-3-2 detection in sample F. Eight out of eight positive reactions per sample in the four preamplifications were obtained for H7-1 in sample D, RT73 in sample E and Bt176 in samples B, C, D and E. A few false positive reactions were detected as well, soybean in sample E and maize in sample F in three preamplifications. However, both results were expected since the same false positives were detected in these samples before preamplification. In one preamplification, the presence of soybean and sugar beet was detected in samples B and A, respectively.

Fluidigm run

The majority of the assays, 23 out of 28, showed a high correlation between input material and output signals (Table 3). Twelve assays in fact showed perfect results with only true positives and true negatives. P35S was present and detected in all samples while Cry1A was neither present nor detected in any sample. For the other ten assays, both positive and negative samples were present. Eleven assays showed near-perfect results with minor incidences of false positives or negatives or both. In all 23 assays, a good correlation was observed between the amount of target and the obtained Ct value. Even targets with the same percentage in a different background resulted in similar Ct values, suggesting that there is no, or a very low, matrix effect.

Only minor incidents of false positive signals were observed in this study. In sample F, 1 out of 16 replications presented a false positive signal for cry3Bb1 (Ct 29.6) and in sample D one out of eight presented a positive signal for CruA (Ct 27.1). Two late signals were also observed in sample B, one out of eight reactions was positive for H7-1 (Ct 36.2) and one out of eight for CruA (Ct 38.2). A few other false positives were also detected, but they could be traced back to the contaminations observed previous to the Fluidigm run: in sample F, 1 out of 16 reactions was positive for the presence of MON88017 and in sample E three out of eight reactions were positive for the presence of soybean. Two potential

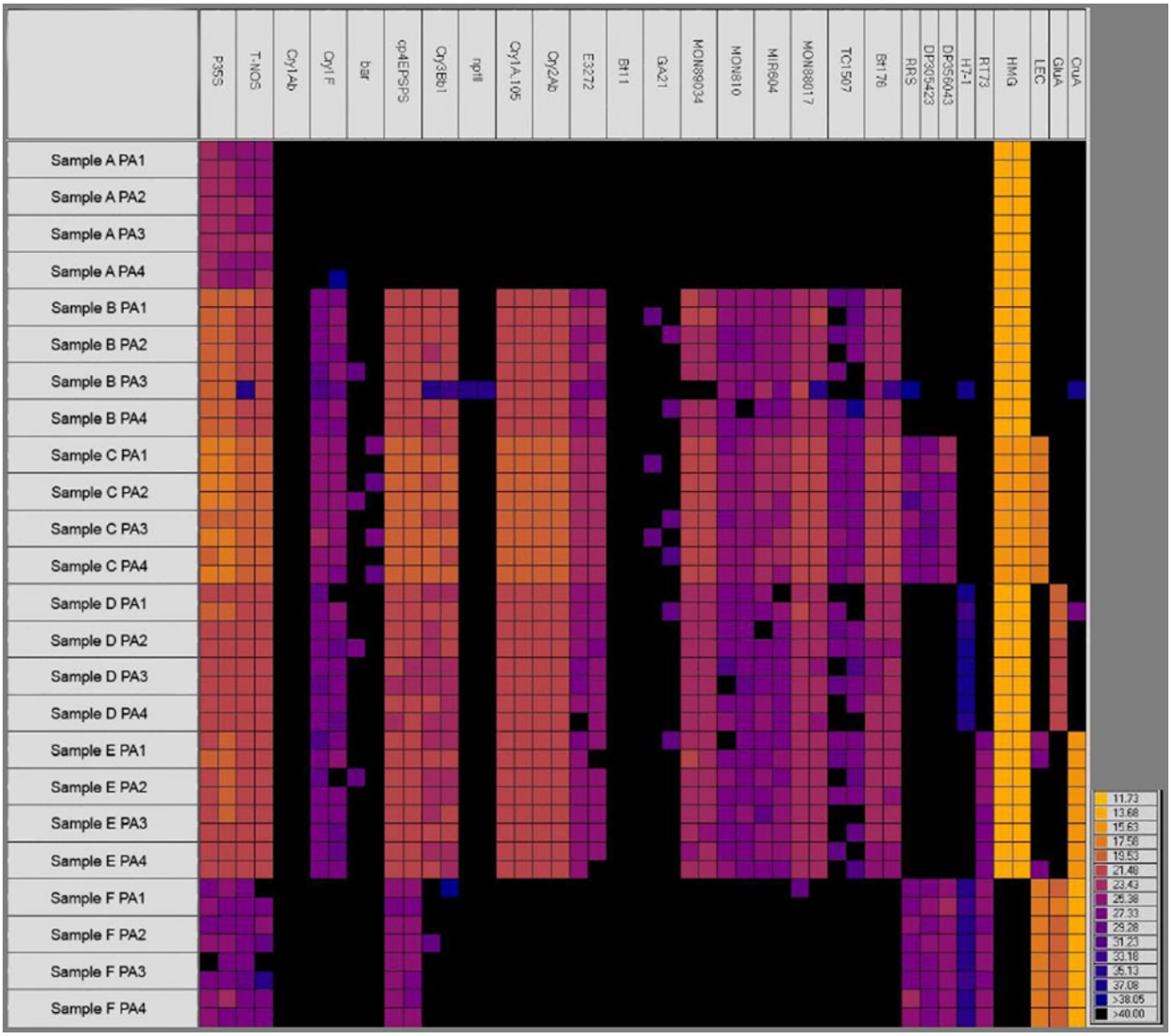

Fig. 1 The software generated "heat map" of the 48.48 dynamic array showing all the 2,304 reactions performed by the system. Each *square* represents one individual reaction chamber from the chip. The *colours* on the map correspond to a Ct value according to the colour-coded legend showed in the *right*

contaminations in samples A and B were not confirmed in the Fluidigm experiment: the presence of lectin (Lec assay) in soybean was not detected in sample B or the presence of sugar beet in sample A.

Five out of the 28 assays (GA21, TC1507, Bt11, H7-1 and bar) performed clearly less well than the other 23, with less true positive observations. In order to explain these unexpected results, the influence of preamplification and the used q-PCR mastermix were investigated. For assay GA21, a clear influence of preamplification was observed. The lowest preamplification factor of around 500 for GA21 was substantially lower than the factors of 3,000 to over 44,000 calculated for other assays (Electronic Supplementary Material, Table S1). Although the preamplification of GA21 was less efficient, still a correlation could be observed between the percentage of the target and the obtained Ct value. In case of Bt11 and H7-1, a clear influence of the used q-PCR mastermix could be determined (Table 4). In a regular q-PCR test, Bt11 did not show any amplification using the Fluidigm MM, but performed as expected using the Diagenode MM. H7-1 performed less efficient using the Fluidigm MM compared to the Diagenode MM. For the bar and TC1507 assay, neither the preamplification nor the influence of the q-PCR MM was found to be the cause of the unexpected results.

To assess the power of low level detection, the test samples were prepared in a way to contain several GM targets at low concentrations (Table 2). Five samples contained percentages between 0.11 and 0.06 % for a total of 13 different GM targets.

Table 3 Percentage of the each element, number of positive reactions/total of reactions and Ct values (mean ± SEM) for each assay

Assay	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F
P35S	0.24	4.99	12.6	2.5	2.5	0.1
	16/16	16/16	16/16	16/16	16/16	15/16
	24.2±0.12	20.1±0.05	18.8±0.04	21±0.09	20.9±0.05	26.5±0.22
T-NOS	0.24	5.09	12.83	2.54	2.54	0.1
	16/16	16/16	16/16	16/16	16/16	14[a]/16
	23.5±0.15	19.8±0.06	18.1±0.07	20.4±0.11	20.3±0.09	26.3±0.28
cry1Ab	–	–	–	–	–	–
	–	–	–	–	–	–
	–	–	–	–	–	–
cry1F	–	0.22	0.55	0.11	0.11	–
	–	16/16	16/16	15/16	15/16	–
	–	28.6±0.34	26.7±0.13	29±0.32	29.1±0.39	–
Bar	–	0.11	0.28	0.06	0.06	–
	–	1/16	5/16	1/16	1/16	–
	–	–	–	–	–	–
cp4EPSPS	–	2.22	5.66	1.21	1.21	0.6
	–	16/16	16/16	16/16	16/16	16/16
	–	20.4±0.08	19.1±0.04	21.3±0.09	21.1±0.06	25.2±0.18
cry3Bb1	–	2.22	5.56	1.11	1.11	–
	–	16[b]/16	16/16	16/16	16/16	1/16
	–	22±0.11	20.5±0.09	22.8±0.13	22.7±0.12	29.6
nptII	–	–	–	–	–	–
	–	2/16	–	–	–	–
	–	35.07±0.61	–	–	–	–
cry1A.105	–	2.22	5.56	1.11	1.11	–
	–	16/16	16/16	16/16	16/16	–
	–	20.6±0.06	19.2±0.04	21.4±0.08	21.3±0.07	–
cry2Bb	–	2.22	5.56	1.11	1.11	–
	–	16/16	16/16	16/16	16/16	–
	–	20.4±0.05	19.1±0.05	21.2±0.06	21.2±0.08	–
E3272	–	0.22	0.54	0.11	0.11	–
	–	16/16	16/16	15/16	14/16	–
	–	24.1±0.19	22.5±0.1	24.9±0.23	24.4±0.14	–
Bt11	0.24	0.11	0.27	0.05	0.05	–
	0/16	0/16	0/16	0/16	0/16	–
	–	–	–	–	–	–
GA21	–	0.1	0.24	0.05	0.05	–
	–	3/16	4/16	1/16	1/16	–
	–	–	–	–	–	–
MON89034	–	2.22	5.56	1.11	1.11	–
	–	14/16	16/16	16/16	16/16	–
	–	22.3±0.09	20.9±0.06	23.1±0.1	23.1±0.16	–
MON810	–	0.11	0.28	0.06	0.06	–
	–	16[a]/16	16/16	15/16	15/16	–
	–	25.7±0.2	24.2±0.19	26.5±0.29	26.1±0.17	–
MIR604	–	0.22	0.55	0.11	0.11	–
	–	16/16	16/16	14/16	16/16	–
	–	24±0.17	23±0.09	25.6±0.21	25.2±0.18	–
MON88017	–	2.22	5.56	1.11	1.11	–
	–	16[b]/16	16/16	16/16	16/16	1/16
	–	24.6±0.11	23.3±0.06	25.6±0.16	25.2±0.12	30.3

Table 3 (continued)

Assay	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F
TC1507	–	0.22	0.55	0.11	0.11	–
	–	10/16	16/16	9/16	10/16	–
	–	23.4±0.24	27.1±0.20	28.5±0.36	28.3±0.35	–
Bt176	–	0.11	0.28	0.06	0.06	–
	–	16/16	16/16	16/16	16/16	–
	–	22.5±0.08	21.1±0.05	23.6±0.17	23.3±0.18	–
RRS event 40-3-2	–	–	0.1	–	–	0.1
	–	–	8/8	–	–	8/8
	–	–	28.9±0.97	–	–	27.8±0.36
DP305423	–	–	0.21	–	–	0.21
	–	–	8/8	–	–	8/8
	–	–	26.5±0.29	–	–	25.7±0.21
DP356043	–	–	0.21	–	–	0.21
	–	–	8/8	–	–	8/8
	–	–	25.3±0.28	–	–	25.1±0.18
H7-1	–	–	–	0.1	–	0.25
	–	1/8	–	8[c]/8	–	8[c]/8
	–	36.2	–	–	–	–
RT73	–	–	–	–	0.1	0.25
	–	–	–	–	8/8	8/8
	–	–	–	–	27.5±0.22	26.6±0.28
HMG	100	100	50	90	90	–
	16/16	16/16	16/16	16/16	16/16	–
	13.9±0.03	14.2±0.03	15±0.03	14.4±0.05	13.6±0.04	–
LEC	–	–	50	–	–	50
	–	–	8/8	–	3/8	8/8
	–	–	18.2±0.04	–	26.9±0.6	17.9±0.03
GluA	–	–	–	10	–	25
	–	–	–	8/8	–	8/8
	–	–	–	21.1±0.29	–	19.7±0.13
CruA	–	–	–	–	10	25
	–	1/8	–	1/8	8/8	8/8
	–	38.2	–	27.1	16±0.07	14.9±0.04

[a]One Ct was a clear outlier and not considered for statistical analysis or calculations of average and SEM, even though the reaction was considered positive

[b]Two Cts were clear outliers and not considered for statistical analysis or calculations of average and SEM, even though the reactions were considered positive

[c]Reactions were considered positive, but the Cts were not considered for statistical analysis or calculations of average and SEM because of a clear mastermix effect

Results of the low level detection for different assays in different samples are presented in Fig. 2. All targets present at low concentrations were detected with the exception of the five assays reported in the previous paragraph. Targets with the lowest concentrations (0.06 %) MON810 and Bt176 in samples D and E were detected in 15 and 16 out of 16 reactions for both samples.

Statistical analysis

One-way ANOVA was performed in order to estimate the variance between the preamplifications. For the large majority of the observations (70 out of 76 preamplifications that were performed), no statistically significant difference was observed ($p>0.01$). Only six observations (p35S in sample E, cp4EPSPS in sample E, MON89034 in sample E and HMG in samples A, B and E) presented statistically significant differences ($p<0.01$) and were further submitted to a false discovery rate test which was performed in order to correct for false discovery rates. Tukey's multiple comparison test was then carried out and results are presented in Table 5. For the six observations tested, the highest significances were observed for the targets present at high percentages.

Linear relationships were observed between the amount of target in the samples and the Ct values obtained in the Fluidigm run for some assays (Electronic Supplementary Material, Table S2) suggesting that there is no, or a very low, matrix effect. The p35S assay, which showed the best correlation, presented an R^2 value of 0.962 and a slope of −3.551 representing an efficiency of 0.912 or 91.2 % (Fig. 3).

Table 4 Differences in Ct values generated by Real-time PCR analysis using Diagenode mastermix (DMM) and mastermix used in the Fluidigm System (FMM)

Sample	Target	MM	Ct mean	SEM
Bt176 maize 5 % (ERM-BF411f)	Bar	DMM	33.5	1.6
		FMM	29.9	0.3
Sample C (maize + soybean mix)[a]	Bar	DMM	37.0	1.7
		FMM	33.0	0.8
Sample C (maize + soybean mix)[b]	Bar	DMM	33.9	0.5
		FMM	32.9	0.6
Bt11 maize 4.89 % (ERM-BF412f)	Bt11	DMM	30.7	0.1
		FMM	ND	ND
Sample C (maize + soybean mix)[a]	Bt11	DMM	33.4	0.1
		FMM	ND	ND
Sample C (maize + soybean mix)[b]	Bt11	DMM	29.0	0.8
		FMM	ND	ND
TC1507 maize 9.86 % (ERM-BF418d)	TC1507	DMM	27.2	0.1
		FMM	28.6	0.6
Sample C (maize + soybean mix)[a]	TC1507	DMM	30.7	0.1
		FMM	31.7	0.0
Sample C (maize + soybean mix)[b]	TC1507	DMM	28.2	0.2
		FMM	29.2	0.4
H7-1 sugarbeet 100 % (ERM-BF419b)	H7-1	DMM	22.8	0.1
		FMM	28.8	0.3
Sample D (maize + sugarbeet mix)[a]	H7-1	DMM	31.5	0.2
		FMM	40.2	0.5
Sample D (maize + sugarbeet mix)[b]	H7-1	DMM	28.6	0.2
		FMM	33.5	0.8

[a] Non-preamplified sample

[b] Preamplified sample

Discussion

The ever-increasing number of GMOs available for trading and the requirements for labelling regulation make the development of reliable high throughput detection methods a necessity. Here is described for the first time a method for GMO multi-detection using the recently available Fluidigm system that allows the identification/detection of multiple targets (GM elements, GM events and species-specific references). In order to evaluate the system, six test samples with an increasing degree of complexity were prepared, preamplified and analysed using the Fluidigm system. In order to obtain a wide range of percentages to be tested, the samples were prepared to contain different levels of each GM variety and/or GM elements. Some samples contained percentages as low as 0.05 % for a GM variety. Elements were present at concentrations as low as 0.06 % in some samples (see "Experimental design").

The experimental design was able to provide information about the variation between the preamplifications. For the targets with high copy numbers, such as the HMG in samples A, B and E, the high statistical significance between preamplifications can be explained by the low variation within preamplifications. In these cases, with the increase in the

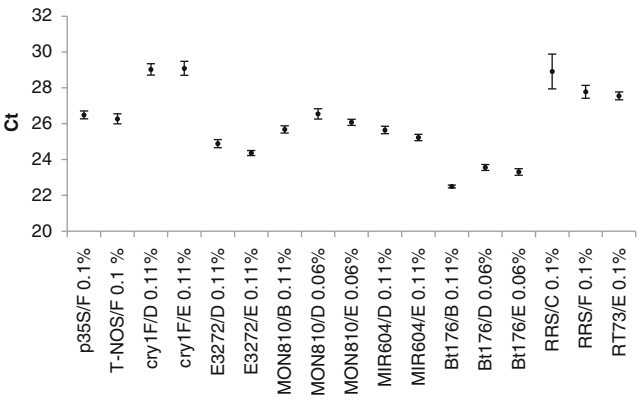

Fig. 2 Detection of the low level targets in the different test samples by the Fluidigm system. Plot represents Ct values±SEM (standard error of the mean). *x*-axis presents the assay/sample and percentage of the target in the sample

Table 5 Tukey's multiple comparison test performed in the six observations where significant differences were obtained by one-way ANOVA

	PA1	PA2	PA3	PA4	*p* value
MON89034 sample E	22.28a	23.00ab	23.44b	23.70b	0.001
HMG sample E	13.39a	13.60ab	13.73b	13.75b	0.001
p35S sample E	20.63a	20.84ab	20.97ab	21.90b	0.003
cp4EPSPS sample E	20.86a	21.15ab	21.08ab	21.41b	0.008
HMG sample B	14.09a	14.14a	14.24ab	14.37b	1.9E-04
HMG sample A	14.10c	13.78a	13.92b	13.97b	8.3E-07

Means with same letter do not differ statistically ($p>0.01$) by Tukey's test. Different letter in the same row represent statistically different means ($p>0.01$) by Tukey's test

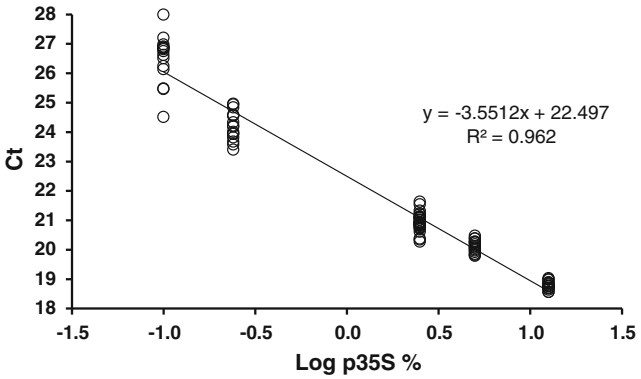

Fig. 3 Calibration curve for the assay p35S. The curve was generated using five concentrations of the promoter 35S, relative to its percentage in each sample. Each concentration in the graph represents a different sample with its own amount of the element, with the exception of point 2.5 %, which represents two different samples. For each concentration, 16 Ct values were plotted (with the exception of point 2.5 % where 32 Ct values were plotted)

accuracy of the detection, the in-plate variation became so small that even small differences between preamplifications became statistically significant, such as the 0.13 Ct difference between PA1 and 4 for HMG in sample A with a *p* value of 8.3E−07 (see "Statistical analysis").

Twenty-eight assays including GM elements, GM events and endogenous references as targets were selected to be included in the system. Twenty-three of the assays presented a large number of expected positive detections and only a few assays presented false negative reactions (see "Fluidigm run"). Such number, however, is not unusual in such a large number of reactions.

The preamplification approach used successfully in the present work (see "Preamplification") was already used in a number of applications [23, 31] with similar results, mainly for the low variation between preamplification replicates and the low in-plate variation. According to Gaudio et al. [31], variation observed in Ct values from preamplified samples was significantly lower than in non-preamplified samples. This result was also found by Devonshire et al. [23] when comparing RT-PCR using preamplified and non-preamplified RNA samples. The SEM values obtained for the preamplified samples subjected to amplification were low, mainly for targets at high percentages.

The five assays that presented unanticipated results (bar, GA21, TC1507, Bt11 and H7-1) were tested in order to exclude other possibilities than the direct influence of the Fluidigm system. Assay GA21 was clearly influenced by the preamplification efficiency. The amplification factor of 500 was clearly less than the 3,125-fold difference in volume between regular q-PCR and the Fluidigm system and hence most probably the cause of the stochastic effect presented by this assay. For the other assays, which presented preamplification factors higher than 2,000, an influence of the mastermix was tested. Assay H7-1 was influenced by the mastermix and the behaviour of the curves could be explained by this difference in efficiency between mastermixes used in q-PCR and in the Fluidigm. In the case of Bt11 assay, it was also drastically influenced by the mastermix used in the Fluidigm run, as proved by the later experiments (see "Fluidigm run"). Since the mastermixes used in the present work are commercial, no indication on the composition of such mixes could be found and then detailed discussions/conclusions about the influence of it on the results cannot be draw. Therefore, a simple test of a regular q-PCR with Fluidigm mastermix will already identify some of the assays that are not compatible without adaptation. Adaptations might be made to the primer probe concentrations or sequences or to the mastermix itself.

In order to test the power of low-level detection of the system, the detection of a number of low level targets was performed, firstly, after the preparation of the test samples and after the preamplifications using conventional q-PCR. These data were, then, compared to the data obtained in the Fluidigm run. Targets were successfully detected in both systems, including the ones at low concentrations, showing that results are in perfect concordance (see "Fluidigm run"). The low SEM values (Table 4) give an indication of the precision of the preamplification reactions as well as the accuracy of the amplification reactions performed in the Fluidigm as already observed by Del Gaudio et al. [31] and Devonshire et al. [23].

A matrix approach can be used to detect UGMs that contain identical elements present in authorized GMOs [3, 4, 8, 38]. By screening for the presence or absence of candidate GMO elements common to multiple GMOs, the potential presence of UGMs can be investigated. The detection of a combination of GMO elements that cannot be explained by authorized GMOs present in the same sample may be indicative for the presence of UGMs. The Fluidigm system has high potential for rapid incorporation of this matrix approach because of its high-throughput nature.

Performance comparisons between conventional q-PCR and the Fluidigm system for single nucleotide polymorphism genotyping have been reported [40] with high correlations between these platforms. The present study did not aim to correlate data between platforms but the results obtained showed that the Fluidigm system is as good as the conventional q-PCR in detecting low level targets. As such, the Fluidigm system may prove a useful tool in the screening methods for detection of authorised and unauthorised GMO in food and feed. Especially laboratories with a large number of samples are likely to benefit from reduced amounts of analysis time and chemicals per sample. Based on data from the CRM samples in this study, 23 out of the 28 tested assays (82 %) seem directly transferable from conventional to nanolitre format. Adding real-life samples to check for matrix effects would greatly contribute to a more validated suitability of the Fluidigm system as a high-throughput method for GMO multi-detection.

Acknowledgments FCAB, LHSG and AZD were supported by fellowships from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Ministry of Education, Brazil, according to cooperation program CAPES-Wageningen (project 005/09).

Conflict of interest The authors have declared no conflict of interest

References

1. Clive J (2012) Global status of commercialized biotech/GM crops: 2012. ISAAA, Ithaca
2. Holst-Jensen A (2009) Testing for genetically modified organisms (GMOs): past, present and future perspectives. Biotechnol Adv 27(6):1071–1082. doi:10.1016/j.biotechadv.2009.05.025
3. Waiblinger HU, Grohmann L, Mankertz J, Engelbert D, Pietsch K (2010) A practical approach to screen for authorised and

unauthorised genetically modified plants. Anal Bioanal Chem 396(6):2065–2072. doi:10.1007/s00216-009-3173-2
4. Scholtens I, Laurensse E, Molenaar B, Zaaijer S, Gaballo H, Boleij P, Bak A, Kok E (2013) Practical experiences with an extended screening strategy for genetically modified organisms (GMOs) in real-life samples. J Agric Food Chem. doi:10.1021/jf4018146
5. Querci M, Van den Bulcke M, Zel J, Van den Eede G, Broll H (2010) New approaches in GMO detection. Anal Bioanal Chem 396(6): 1991–2002. doi:10.1007/s00216-009-3237-3
6. Raymond P, Gendron L, Khalf M, Paul S, Dibley K, Bhat S, Xie V, Partis L, Moreau M, Dollard C, Cote M, Laberge S, Emslie K (2010) Detection and identification of multiple genetically modified events using DNA insert fingerprinting. Anal Bioanal Chem 396(6):2091–2102. doi:10.1007/s00216-009-3295-6
7. Permingeat HR, Reggiardo MI, Vallejos RH (2002) Detection and quantification of transgenes in grains by multiplex and real-time PCR. J Agric Food Chem 50(16):4431–4436. doi:10.1021/jf020081d
8. Germini A, Zanetti A, Salati C, Rossi S, Forre C, Schmid S, Marchelli R (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(11):3275–3280. doi:10.1021/jf035052x
9. 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(9):3333–3337. doi:10.1021/jf049192y
10. 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 Methods 2(4):325–336. doi:10.1007/s12161-009-9093-0
11. Mano J, Shigemitsu N, Futo S, Akiyama H, Teshima R, Hino A, Furui S, Kitta K (2009) Real-time pcr array as a universal platform for the detection of genetically modified crops and its application in identifying unapproved genetically modified crops in Japan. J Agric Food Chem 57(1):26–37. doi:10.1021/jf802551h
12. Bordoni R, Germini A, Mezzelani A, Marchelli R, De Bellis G (2005) A microarray platform for parallel detection of five transgenic events in foods: a combined polymerase chain reaction-ligation detection reaction-universal array method. J Agric Food Chem 53(4): 912–918. doi:10.1021/jf0486949
13. Germini A, Rossi S, Zanetti A, Corradini R, Fogher C, Marchelli R (2005) Development of a peptide nucleic acid array platform for the detection of genetically modified organisms in food. J Agric Food Chem 53(10):3958–3962. doi:10.1021/jf050016e
14. Leimanis S, Hernandez M, Fernandez S, Boyer F, Burns M, Bruderer S, Glouden T, Harris N, Kaeppeli O, Philipp P, Pla M, Puigdomenech P, Vaitilingom M, Bertheau Y, Remacle J (2006) A microarray-based detection system for genetically modified (GM) food ingredients. Plant Mol Biol 61(1–2):123–139. doi:10.1007/s11103-005-6173-4
15. Leimanis S, Hamels S, Naze F, Mbella GM, Sneyers M, Hochegger R, Broll H, Roth L, Dallmann K, Micsinai A, La Paz JL, Pla M, Brunen-Nieweler C, Papazova N, Taverniers I, Hess N, Kirschneit B, Bertheau Y, Audeon C, Laval V, Busch U, Pecoraro S, Neumann K, Rosel S, van Dijk J, Kok E, Bellocchi G, Foti N, Mazzara M, Moens W, Remacle J, Van Den Eede G (2008) Validation of the performance of a GMO multiplex screening assay based on microarray detection. Eur Food Res Technol 227(6):1621–1632. doi:10.1007/s00217-008-0886-y|10.1007/s00217-008-0886-y
16. Hamels S, Glouden T, Gillard K, Mazzara M, Debode F, Foti N, Sneyers M, Nuez TE, Pla M, Berben G, Moens W, Bertheau Y, Audeon C, Van den Eede G, Remacle J (2009) A PCR-microarray method for the screening of genetically modified organisms. Eur Food Res Technol 228(4):531–541. doi:10.1007/s00217-008-0960-5|10.1007/s00217-008-0960-5
17. Prins TW, van Dijk JP, Beenen HG, Van Hoef AMA, Voorhuijzen MM, Schoen CD, Aarts HJM, Kok EJ (2008) Optimised padlock probe ligation and microarray detection of multiple (non-authorised) GMOs in a single reaction. BMC Genomics. doi:10.1186/1471-2164-9-584
18. Ujhelyi G, Dijk JP, Prins TW, Voorhuijzen MM, Hoef AM, Beenen HG, Morisset D, Gruden K, Kok EJ (2012) Comparison and transfer testing of multiplex ligation detection methods for GM plants. BMC Biotechnol 12:4. doi:10.1186/1472-6750-12-4
19. Morisset D, Dobnik D, Hamels S, Zel J, Gruden K (2008) NAIMA: target amplification strategy allowing quantitative on-chip detection of GMOs. Nucleic Acids Res 36 (18). doi:10.1093/nar/gkn524|10.1093/nar/gkn524
20. Dobnik D, Morisset D, Gruden K (2010) NAIMA as a solution for future GMO diagnostics challenges. Anal Bioanal Chem 396(6): 2229–2233. doi:10.1007/s00216-009-3197-7
21. Nadal A, Coll A, La Paz JL, Esteve T, Pla M (2006) A new PCR-CGE (size and color) method for simultaneous detection of genetically modified maize events. Electrophoresis 27(19):3879–3888. doi:10.1002/elps.200600124|10.1002/elps.200600124
22. 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(3):765–772
23. Devonshire A, Elaswarapu R, Foy C (2011) Applicability of RNA standards for evaluating RT-qPCR assays and platforms. BMC Genomics 12. doi:10.1186/1471-2164-12-118|10.1186/1471-2164-12-118
24. Weaver S, Dube S, Mir A, Qin J, Sun G, Ramakrishnan R, Jones R, Livak K (2010) Taking qPCR to a higher level: analysis of CNV reveals the power of high throughput qPCR to enhance quantitative resolution. Methods 50(4):271–276. doi:10.1016/j.ymeth.2010.01.003
25. Spurgeon SL, Jones RC, Ramakrishnan R (2008) High throughput gene expression measurement with real time PCR in a microfluidic dynamic array. Plos One 3 (2). doi:10.1371/journal.pone.0001662|10.1371/journal.pone.0001662
26. de Boer M, Berg S, Timmermans M, den Dunnen J, van Straalen N, Ellers J, Roelofs D (2011) High throughput nano-liter RT-qPCR to classify soil contamination using a soil arthropod. BMC Mol Biol 2. doi:10.1186/1471-2199-12-11|10.1186/1471-2199-12-11
27. ENGL (2011) European Network of GMO Laboratories. Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials
28. Ruttink T, Demeyer R, Van Gulck E, Van Droogenbroeck B, Querci M, Taverniers I, De Loose M (2010) Molecular toolbox for the identification of unknown genetically modified organisms. Anal Bioanal Chem 396(6):2073–2089. doi:10.1007/s00216-009-3287-6
29. Chaouachi M, Chupeau G, Berapd 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(24):11596–11606. doi:10.1021/jf801482r|10.1021/jf801482r
30. Liu J, Hansen C, Quake SR (2003) Solving the "world-to-chip" interface problem with a microfluidic matrix. Anal Chem 75(18): 4718–4723. doi:10.1021/ac0346407|10.1021/ac0346407
31. Del Gaudio S, Cirillo A, Di Bernardo G, Galderisi U, Cipollaro M (2010) A preamplification approach to GMO detection in processed foods. Anal Bioanal Chem 396(6):2135–2142. doi:10.1007/s00216-009-3199-5
32. Davydova N, Streltsov VA, Roufail S, Lovrecz GO, Stacker SA, Adams TE, Achen MG (2012) Preparation of human vascular endothelial growth factor-D for structural and preclinical therapeutic studies. Protein Expr Purif 82(1):232–239. doi:10.1016/j.pep.2012.01.001
33. Huelga SC, Vu AQ, Arnold JD, Liang TY, Liu PP, Yan BY, Donohue JP, Shiue L, Hoon S, Brenner S, Ares M, Yeo GW (2012) Integrative genome-wide analysis reveals cooperative regulation of alternative

splicing by hnRNP proteins. Cell Rep 1(2):167–178. doi:10.1016/j.celrep.2012.02.001
34. Sanchez-Freire V, Ebert AD, Kalisky T, Quake SR, Wu JC (2012) Microfluidic single-cell real-time PCR for comparative analysis of gene expression patterns. Nat Protoc 7(5):829–838. doi:10.1038/nprot.2012.021
35. Whale AS, Huggett JF, Cowen S, Speirs V, Shaw J, Ellison S, Foy CA, Scott DJ (2012) Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. Nucleic Acids Res 40(11):e82. doi:10.1093/nar/gks203
36. Burns M, Burrell A, Foy C (2010) The applicability of digital PCR for the assessment of detection limits in GMO analysis. Eur Food Res Technol 231(3):353–362. doi:10.1007/s00217-010-1277-8
37. Corbisier P, Bhat S, Partis L, Xie V, Emslie K (2010) Absolute quantification of genetically modified MON810 maize (*Zea mays* L.) by digital polymerase chain reaction. Anal Bioanal Chem 396(6):2143–2150. doi:10.1007/s00216-009-3200-3
38. Dinon A, Prins T, van Dijk J, Arisi A, Scholtens I, Kok E (2011) Development and validation of real-time PCR screening methods for detection of cry1A.105 and cry2Ab2 genes in genetically modified organisms. Anal Bioanal Chem 400(5):1433–1442. doi:10.1007/s00216-011-4875-9
39. Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8(19):4321–4325
40. Wang J, Lin M, Crenshaw A, Hutchinson A, Hicks B, Yeager M, Berndt S, Huang WY, Hayes RB, Chanock SJ, Jones RC, Ramakrishnan R (2009) High-throughput single nucleotide polymorphism genotyping using nanofluidic dynamic arrays. BMC Genomics 10. doi:10.1186/1471-2164-10-561
41. Kuribara H, Shindo Y, Matsuoka T, Takubo K, Futo S, Aoki N, Hirao T, Akiyama H, Goda Y, Toyoda M, Hino A (2002) Novel reference molecules for quantitation of genetically modified maize and soybean. J AOAC Int 85(5):1077–1089
42. Shindo Y, Kuribara H, Matsuoka T, Futo S, Sawada C, Shono J, Akiyama H, Goda Y, Toyoda M, Hino A, Asano T, Hiramoto M, Iwaya A, Jeong SI, Kajiyama N, Kato H, Katsumoto H, Kim YM, Kwak HS, Ogawa M, Onozuka Y, Takubo K, Yamakawa H, Yamazaki F, Yoshida A, Yoshimura T (2002) Validation of real-time PCR analyses for line-specific quantitation of genetically modified maize and soybean using new reference molecules. J AOAC Int 85(5):1119–1126
43. 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(7):2100–2109. doi:10.1021/jf011157t|10.1021/jf011157t
44. Status of dossiers-EU-RL GMFF validation process. http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx
45. Ronning SB, Vaitilingom M, Berdal KG, Holst-Jensen A (2003) Event specific real-time quantitative PCR for genetically modified Bt11 maize (*Zea mays*). Eur Food Res Technol 216(4):347–354. doi:10.1007/s00217-002-0653-4