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ORIGINAL PAPER

Validation of the performance of a GMO multiplex screening assay based on microarray detection

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Abstract A new screening method for the detection and identification of GMO, based on the use of multiplex PCR followed by microarray, has been developed and is presented. The technology is based on the identification of quite ubiquitous GMO genetic target elements first amplified by PCR, followed by direct hybridisation of the amplicons on a predefined microarray (DualChip[®] GMO, Eppendorf, Germany). The validation was performed within the framework of a European project (Co-Extra, contract no 007158) and in

collaboration with 12 laboratories specialised in GMO detection. The present study reports the strategy and the results of an ISO complying validation of the method carried out through an inter-laboratory study. Sets of blind samples were provided consisting of DNA reference materials covering all the elements detectable by specific probes present on the array. The GMO concentrations varied from 1% down to 0.045%. In addition, a mixture of two GMO events (0.1% RRS diluted in 100% TOPAS19/2) was incorporated in the

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study to test the robustness of the assay in extreme conditions. Data were processed according to ISO 5725 standard. The method was evaluated with predefined performance criteria with respect to the EC CRL method acceptance criteria. The overall method performance met the acceptance criteria; in particular, the results showed that the method is suitable for the detection of the different target elements at 0.1% concentration of GMO with a 95% accuracy rate. This collaborative trial showed that the method can be considered as fit for the purpose of screening with respect to its intra- and inter-laboratory accuracy. The results demonstrated the validity of combining multiplex PCR with array detection as provided by the DualChip[®] GMO (Eppendorf, Germany) for the screening of GMO. The results showed that the technology is robust, practical and suitable as a screening tool.

Keywords GMO · DualChip GMO · Microarray · Screening · Multiplex · Validation · PCR · Inter-laboratory ring trial

Abbreviation

CRL Community Reference Laboratory
EC European Commission
PCR Polymerase Chain Reaction

Introduction

In recent years, the presence of genetically modified organisms (GMOs) in food and animal feed has increased dramatically. Although different countries, such as the USA, consider GMOs as substantially equivalent to non-GMO food and therefore do not require any specific labelling of products containing or deriving from approved GMOs, several other countries enforce the labelling of such products above a threshold level. These adventitious presence thresholds are fixed at 1% in Australia and New Zealand, 3% in South Korea, 4% in Brazil and 5% in Japan, Taiwan and Thailand. To ensure the consumer's choice of freedom, the European Union implemented mandatory rules for labelling food and feed containing GMOs or derived thereof above a threshold of 0.9% with a requirement for the traceability of the GMO in the food and feed chains [1, 2]. In 2006, 26 GMO events were accepted for commercialisation in the EU, of which 24 were plant species. Five of these GMOs are subjected to special restrictions of use within the EU (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm). Therefore, the availability of reliable and validated methods to detect the presence of GMO(s) is a clear necessity.

Until now, validated methods are based on simplex detection, most of them in real-time PCR, focussing on the most common GMOs present in the market or accepted in

different countries (<http://bgmo.jrc.ec.europa.eu/home/docs.htm>). Many methods for GMO testing have been developed, mostly based on PCR or real-time PCR [3–8]. However, the recent increased number of approved GMOs and the outbreaks in Europe of unapproved GMOs raised the need of screening strategies to identify the approved GMOs and to differentiate them from non-approved ones. As a consequence, it becomes essential to develop tests for a reliable and simultaneously specific detection of multiple target elements in a single assay.

Following the valuable results obtained with the GMOChip[®] [9], a new biochip (DualChip[®] GMO, Eppendorf, Germany) was developed for the detection and identification of the EU approved GMOs.

Detection of the amplicons produced by PCR is achieved by their direct hybridisation to capture probes present in spots on a predefined array [10]. The strength of the microarray results from the possibility to obtain multiple detections in one assay, thanks to the presence of multiple capture probes specific for each of the different targets (see the former European GMOchips research program). The present method is based on the detection of individual small GMO-specific DNA sequences, such as promoters, terminators, inserted genes and reference genes for taxa identification rather than on long inserted fragments as in Leimanis et al. 2006 [9].

The detection of these small specific sequences called GM elements is then used as a signature of the GM event. Practically, an algorithm compares the pattern of these elements with a data composition of the EU authorised GM in a “matrix approach” and the correspondence of the experimental data with the genetic composition of the GM allows a unique or restricted identification of the GM event.

Different performance criteria were predefined and first tested in a pre-validation study performed in five different laboratories. Based on the positive results of the pre-validation, the acceptance criteria were confirmed and the inter-laboratory ring trial was organised. The goal of this study was to assess the performance of the DualChip[®] GMO assay as a screening method through a collaborative study. The validation of this detection method was conducted according to ISO 5725 norm and performed in 12 laboratories from different countries with quality assurance systems in place.

Materials and methods

Samples

GMO certified reference materials (CRM) (Maize Bt176, Maize MON810, Maize Bt11 and Roundup Ready[™] Soya)

and the non-GMO plants produced by the Institute for Reference Materials and Measurement (IRMM, Belgium) were purchased from Fluka (Switzerland). The certified concentration value of these CRM is merely based on the mass fraction of GMO to non-GMO dry powder. Control samples of rapeseed TOPAS 19/2 were provided by Bayer BioSciences (Belgium). GA21 Maize seeds were the same as described in Leimanis et al. 2006 [9].

For the validation of the method, test samples were provided to each participating laboratory as blind samples consisting of DNA reference samples specifically adapted to each GM element. The samples were delivered with different concentrations enabling sensitivity testing of the assays. A total of 36 unknown samples, representing six GM levels and five plant DNA levels (see Table 1) were used in the validation study.

All the GMO and non-transgenic plant samples used in the different PCR sets (see Table 1) were prepared from DNA (at concentration of 20 ng/μl) extracted from the certified reference materials by dilution in the pENGL buffer. This buffer contains 20 ng/μl genomic calf thymus DNA (average molecular weight peaking at 5 Kbases) in Tris/HCl 10 mM, pH 7.5, EDTA 1 mM. The use of the pENGL buffer allowed saving large amount of non-transgenic plant DNA. The concentrations in GMO and plant are expressed hereafter in percentage of total DNA present

in the sample and not in percentage per ingredient (see Table 1).

Practically, all the single ingredient DNA samples (see following samples in Table 1: 1, 2, 4–11 and 20–23) were prepared with DNA extracted from certified reference materials at 5% by diluting in pENGL buffer to obtain lower concentrations in GMO (1, 0.5, 0.1 and 0.045%). Samples containing several GMOs (samples: 12–15 and 28–31) were prepared in the same way using pENGL buffer. Maize samples (samples: 16–19) were obtained from pure non-transgenic maize DNA by dilution in the pENGL buffer. Sample 32 containing both rapeseed and soybean at 50% was prepared by mixing identical volumes of both non-transgenic plant DNA (both at 20 ng/μl). Lower concentrations (samples: 33 to 35) were obtained by dilution in pENGL buffer. Sample 36 was prepared by a 50 times dilution of DNA extracted from the 5% CRM of RRS in pure transgenic DNA of TOPAS19/2. Sample 3 is composed of 100 ng of non-plant DNA.

DNA extraction

Genomic DNA was extracted using a CTAB-based method [11]. The genomic DNA was quantified using the “Quant-it™ PicoGreen dsDNA assay kit” (Invitrogen, USA) as described in the manual.

Table 1 Composition of the four PCR (A, B, C, D) in the different PCR sets used for the validation

PCR set	Samples in PCR tubes			
	Reaction tube A (A)	Reaction tube B(B)	Reaction tube C(C)	Reaction tube D (D)
PCR1a	0.1% RRS (1)	0.1% RRS (1)	0.1% RRS (1)	2% soybean (1)
PCR1b	0.1% Bt176 (2)	0.1% Bt176 (2)	0.1% Bt176 (2)	2% maize (2)
PCR2a	Non-plant DNA (3)	Non-plant DNA (3)	Non-plant DNA (3)	Non-plant DNA (3)
PCR3a	1% Bt11 (4)	1% TOPAS19/2 (8)	1% RRS + 1% Bt11 + 1% MON810 (12)	50% maize (16)
PCR3b	0.5% Bt11 (5)	0.5% TOPAS19/2 (9)	0.5% RRS + 0.5% Bt11 + 0.5% MON810 (13)	5% maize (17)
PCR4a	0.1% Bt11 (6)	0.1% TOPAS19/2 (10)	0.1% RRS + 0.1% Bt11 + 0.1% MON810 (14)	1% maize (18)
PCR4b	0.045% Bt11 (7)	0.045% TOPAS19/2 (11)	0.045% RRS + 0.045% Bt11 + 0.045% MON810 (15)	0.5% maize (19)
PCR5a	1% Bt176 (20)	CaMV (500 copies of plasmid) (24)	1% Bt176 + 1% GA21 (28)	50% rapeseed + 50% soybean (32)
PCR5b	0.5% Bt176 (21)	CaMV (100 copies of plasmid) (25)	0.5% Bt176 + 0.5% GA21 (29)	5% rapeseed + 5% soybean (33)
PCR6a	0.1% Bt176 (22)	CaMV (50 copies of plasmid) (26)	0.1% Bt176 + 0.1% GA21 (30)	1% rapeseed + 1% soybean (34)
PCR6b	0.045% Bt176 (23)	CaMV (20 copies of plasmid) (27)	0.045% Bt176 + 0.045% GA21 (31)	0.5% rapeseed + 0.5% soybean (35)
PCR7a	0.1% RRS in 100% TOPAS19/2 (36)	0.1% RRS in 100% TOPAS19/2 (36)	0.1% RRS in 100% TOPAS19/2 (36)	2% soybean in 100% rapeseed (36)

Concentrations of GMO and plant are expressed in percentage of total DNA. A number is given for each sample and placed in brackets after the name of the sample. Amplicons from the four A, B, C, D PCR tubes were mixed for the hybridisation on microarray

Assay

The PCR, hybridisation and detection were performed according to the instructions of the DualChip[®] GMO kit (Eppendorf, Hamburg, Germany).

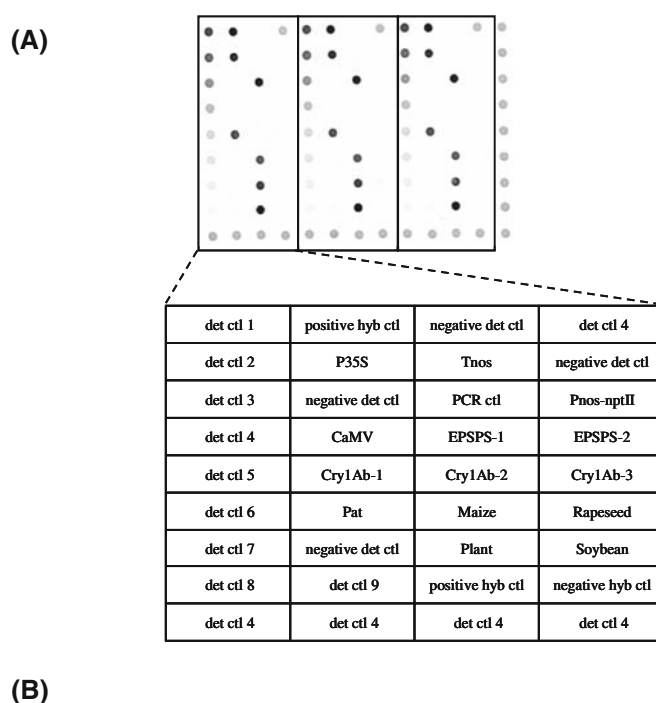
Microarray composition

The microarray consists of a glass slide with capture probes spotted in triplicates on the slide according to a specific pattern that is recognised by the data analysis software. The nucleotide sequences are covalently attached by an amino group at the 5' end onto an aldehyde functionalised slide

[12]. The design of the chip with the location of the different probes present on the array is presented in Fig. 1.

The different capture probes present on the microarray are the following: P35S and Tnos capture probes allow the detection of small DNA fragments present in most GMOs. For example, the P35S capture probe recognises a small conserved part of the CaMV 35S promoter sequence, which is present in at least all EU-accepted GMOs having a 35S promoter. The Pnos-nptII capture probe is specific to a construct-specific sequence present in only 3 (TOPAS19/2, MS1xRF1 and MS1xRF2) out of the 24 GMOs, which are accepted in Europe. Capture probe of EPSPS-2 allows the detection of a specific sequence present in RRS and

Fig. 1 Design of the DualChip GMO. **a** Each capture probe is present in triplicate on the array. Process controls are present on the DualChip GMO for checking the different steps of detection including the PCR (PCR ctl), the hybridisation (*hyb ctl*) and the detection reactions (det ctl). A detection curve is constructed from a series of labelled immobilised probes (*det ctl 1–9*). The *right* column contains only det ctl 4. **b** Specific capture probes present on the DualChip GMO and their corresponding target sequences



NK603. Capture probe of Cry1Ab-3 allows the recognition of a specific sequence present in Bt11, MON531 and MON15985, while the Pat capture probe recognises the sequence of the *pat* gene, which is present in several GMOs (Bt11, T45, DAS59122, T25 and TOPAS19/2). Other capture probes, such as EPSPS-1, Cry1Ab-1, Cry1Ab-2 are specific to a sequence present only in one EU-accepted GMO (GA21, Bt176, MON810, respectively). The plant species, maize, rapeseed and soybean were detected using species-specific genes (*invertase*, *cruciferin* and *lectin* genes, respectively). The plant capture probe allows the detection of the *rbcl* plant universal gene. The capture probe of cauliflower mosaic virus (CaMV) serves as a contamination control for the possible presence of the virus. P35S is a promoter used in many GMO and is present in the CaMV virus. If the P35S screening element is detected together with the presence of CaMV, the probability is high that the P35S signal is coming from the virus infecting the plant and not from a GMO.

A series of controls are provided within the assay in order to detect problems occurring during the method process. The different steps of the assay are checked by three positive controls: the PCR, the hybridisation and the detection controls. When the controls are not correctly detected, the results are discarded.

PCR

The genetic elements were amplified by four separated PCR assays using biotinylated primers. The four different PCR elements amplified the following elements: PCR A: Tnos and P35S; PCR B: Pnos-nptII, CaMV and PCR control; PCR C: Pat, Cry1A(b) and EPSPS; PCR D: conventional maize, soybean, rapeseed and plant. The PCR control is an external DNA spiked into the assay as positive control of the amplification (inhibitors and reagents presence). The four different PCR are also performed with nuclease-free water as negative PCR control for testing of the possible presence of contaminating amplicons or DNA. The PCR assays were carried out in a Mastercycler[®] ep gradient S (Eppendorf, Germany). Into each PCR, 100 ng genomic DNA template was incorporated.

Detection on the array

The microarray hybridisation assays were carried out as follows: 9 µl of PCR product from each of the four PCR reactions were mixed with 4 µl of hybridisation control and 5 µl of SensiHyb solution. A volume of 5 µl of 0.5 N NaOH was added and mixed. After 5 min of incubation at room temperature, the solution was neutralised with 50 µl of Genomic HybriBuffer and loaded into the hybridisation chamber of the slide. The chamber was sealed with a

sealing foil and the slide incubated for 1 h at 60 °C in a Thermomixer comfort (Eppendorf, Germany) under agitation at 1,000 rpm for the hybridisation. After removal of the sealing foil and the hybridisation frame, the slides were labelled using the Silverquant[®] detection kit according to the instruction manual (Eppendorf, Germany). The amplification of the colorimetric signal is based on silver precipitation catalysed by the presence of nanogold particles. The slides were then scanned and quantified with a Silverquant scanner (Eppendorf, Germany) and data obtained were analysed with the Silverquant analysis software as described in Hamels et al. [10]. The analysis software includes the automatic removal of outliers within the triplicates.

Experimental design of the validation

The validation protocol consisted of 12 sets of PCR and hybridisations per laboratory. The 12 PCR were performed in 7 days (PCR 1–7). Negative PCR controls were performed in duplicate for the preliminary tests (PCR 1) and in quadruplicates for each other PCR (PCR 2–7). Each PCR set consisted of 4 PCR tubes (A, B, C, D) containing different primer pairs for the amplification of specific DNA sequences; 9 µl of each of these four PCR was then combined for hybridisation on the array in a final volume of 100 µl. In normal conditions of use of the biochips, a DNA extract is submitted to the four different multiplex PCR (A, B, C and D) and then, after combination of the PCR products of tubes A to D, analysed on the biochips. In this study for validation purposes and in order to reduce the quantity of analyses to be performed, the 36 samples were distributed over the four types of PCR as indicated in Table 1. Each assay (PCR and hybridisation) for validation was performed in quadruplicate. The workload of such a validation study accounted for 3,360 PCR (280 PCR per laboratory: two preliminary PCR sets and its negative PCR control in duplicate plus ten sets distributed over 6 days and the six corresponding negative PCR controls in quadruplicates) and 840 hybridisations (70 hybridisations per laboratory).

The complete DualChip[®] GMO technology was introduced to each laboratory during a “one day demonstration”. The two-first sets of PCR (PCR1a and PCR1b) and hybridisation (with sample controls ctl1 and ctl2, respectively, see Table 1) were performed during the training. The results of these preliminary assays were not part of the evaluation data. The next experiments were then conducted and constitute the results of the evaluation.

A non-plant DNA sample (PCR2a) was included to evaluate the specificity of the assay (false positive results assessment). PCR sets 3, 4, 5 and 6 tested the different GMO concentrations. PCR set 7a tested the ability to detect

a low concentration of a GMO (0.1% RRS) in the presence of 100% concentration of another GMO (TOPAS19/2). Each sample contained 100 ng of purified DNA per PCR tube.

Acceptance criteria

One of the challenges of the validation of a multiplex assay is to determine which statistical parameters should be taken into consideration and the appropriate number of assays to be performed in order to ensure the statistical significance of the study. Since the aim of the validation was to evaluate the significance of the detection of each specific element having a specific capture probe on the array, the data analysis was based on the calculation of the accuracy rate and the confidence threshold was fixed at 95% for each detectable element. The specificity performance (acceptance threshold of false positives) was fixed at 5%. In terms of detection sensitivity, it was decided that the method should be able to detect down to 0.1% of the GMO events and 1% for the taxa. More specific acceptance criteria were: overall validation is accepted if the results of at least 8 laboratories (ISO 5725 recommendation [13]) are reported and considered as valid; the arrays with technical deviations are removed to calculate the total percentage of detection, each target element is validated separately; a signal is considered as positive when its intensity is 2.5 times its standard deviation above the local background intensity and above a threshold value of 1,500. The concentrations over which the method is pre-validated are, for the screening elements, 1, 0.5, 0.1 and 0.045% GM and for the plant species elements, 50, 5, 1 and 0.5%. The false positive results will be determined on a non-plant DNA and

should be lower than 5%. The planning of the validation, i.e. number of laboratories, number of samples, number of replicates, number of GM events per sample and concentrations were then defined accordingly taking these assumptions into account together with the practical feasibility of the validation assay.

Results and discussion

Pre-validation

The performance criteria were first tested in a pre-validation round performed in five laboratories. One laboratory carried out 21 replicates of each sample (1,700 PCR reactions and 425 hybridisations). Each of the four other laboratories analysed three replicates of each sample (240 PCR reactions and 60 hybridisations). A total of 33 replicates were thus performed to assess the repeatability of the method. The results obtained (see Table 2) showed that six screening target elements (P35S, Tnos, Pnos-nptII, Pat, Cry1Ab-1 and Cry1Ab-2) had a limit of detection of 0.045% expressed as mass GMO concentration with an accuracy rate above 95%. One target element (*Cry1Ab-3*) showed a detection limit of 0.1% expressed as GMO concentration with an accuracy rate above 95%, and two screening target elements (*epsps-1* and *epsps-2*) had a detection limit of 0.5% with an accuracy rate above 95%. The four plant species targets were detected in all experiments with accuracy rates above 95% (data not shown). The absolute limit of detection of the CaMV control target was of 50 copies per PCR. To comply with the acceptance criteria, the capture probes of *epsps-1* and *epsps-2* were

Table 2 Pre-validation study of the reproducibility of the method

	Sample composition in PCR tubes							
	GMO (1%)	GMO (0.5%)	GMO (0.1%)	GMO (0.045%)	CaMV (5,000 copies)	CaMV (500 copies)	CaMV (50 copies)	CaMV (10 copies)
Target								
P35S	33/33 (100%)	33/33 (100%)	33/33 (100%)	33/33 (100%)	–	–	–	–
Tnos	33/33 (100%)	33/33 (100%)	33/33 (100%)	33/33 (100%)	–	–	–	–
Pnos-nptII	30/31 (97%)	30/31 (97%)	31/32 (97%)	32/33 (97%)	–	–	–	–
Pat	33/33 (100%)	33/33 (100%)	33/33 (100%)	32/33 (97%)	–	–	–	–
Cry1Ab-1	33/33 (100%)	33/33 (100%)	32/33 (97%)	32/32 (100%)	–	–	–	–
Cry1Ab-2	33/33 (100%)	32/33 (97%)	32/33 (97%)	33/33 (100%)	–	–	–	–
Cry1Ab-3	32/32 (100%)	31/31 (100%)	32/33 (97%)	30/32 (94%)	–	–	–	–
EPSPS-1	32/33 (97%)	33/33 (100%)	26/33 (79%)	21/33 (64%)	–	–	–	–
EPSPS-2	31/32 (97%)	31/32 (97%)	31/33 (94%)	30/32 (94%)	–	–	–	–
CaMV	–	–	–	–	33/33 (100%)	33/33 (100%)	32/32 (100%)	18/33 (55%)

Data are given as the number of positive outcomes per total of replicates. A total of 33 replicates were performed by the five laboratories involved in the pre-validation study. Some data were removed due to technical problems

changed after the pre-validation in order to reach a limit of detection of 0.1% for all screening target elements and the collaborative trial was then organised.

Results of the collaborative trial

The composition of the 12 experimental sets is presented in Table 1. Each set comprised four PCR, which were then detected on one array. The first PCR 1a and b (with samples 1 and 2, respectively) were used to introduce the different laboratories to this new technology (demonstration part). For these preliminary tests, we followed the protocol provided above. Mainly, the DNA samples were processed to the four different multiplex PCR (A, B, C and D) and the resulting amplicons were combined and analysed on the biochip. Out of 12 laboratories, 11 succeeded in performing these preliminary assays and were incorporated into the validation assay.

A non-plant DNA sample (PCR2a, sample 3) was included to test the specificity in terms of false positive results. As shown in Fig. 2, only the PCR control (PCR ctl) was effectively detected after hybridisation. This is in line with the expectation.

In the PCR3a set (Table 1), the PCR tube A contained primers for the amplification of P35S and Tnos DNA fragment from the 1% Bt11 (sample 4). The PCR tube B contained primers for the amplification of the Pnos-nptII fragment from the 1% TOPAS19/2 (sample 8). The PCR primers in the reaction tube C should allow the amplification of the *CryIA(b)*-3 and *Pat* fragments of the 1% Bt11 DNA template, the *epsps*-2 of the 1% RRS DNA template and the *CryIA(b)*-2 of the 1% MON810 (sample 12). The primers in the reaction tube D should allow the amplification of the *ivr* (*invertase*) and *rbcL* reference fragments from the maize genome (sample 16). The results (Fig. 3) show that the simultaneous hybridisation of the four different PCR on a single microarray allowed the detection of all ten expected amplified markers (P35S, Tnos, PCR ctl, Pnos-nptII, *epsps*-2, *CryIA(b)*-2, *CryIA(b)*-3, *Pat*, maize and plant). Similar results were obtained with PCR3b, PCR4a and PCR4b, which were performed with DNA samples having the same GMO but at lower concentrations (see Table 1).

In the PCR5a set (Table 1), the following elements were expected to be amplified in the different PCR tubes: P35S DNA fragment from the 1% Bt176 (tube A, sample 20),

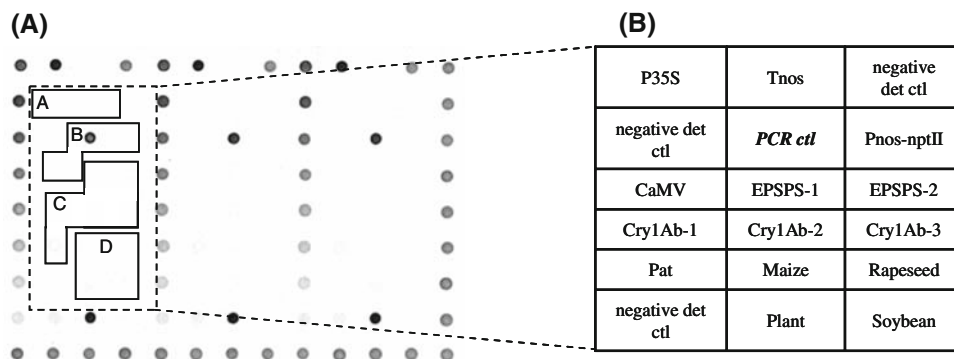
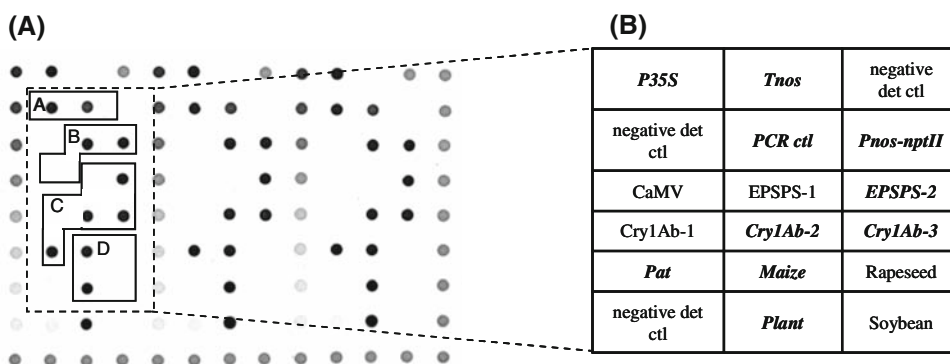


Fig. 2 Array results for PCR2a. **a** A volume of 9 µl of each of the four PCR (A, B, C and D) performed on non-plant DNA sample were hybridised on the DualChip GMO array. PCR B allows the amplification of a DNA fragment (*PCR ctl*), which is detected on

the array. *Box B* shows the positive signal of the PCR control. *Boxes A, C and D* do not show any positive results since the PCR2a sample contains non-plant DNA. **b** The location of the positive element (in **bold and italic**) is indicated. *ctl* control, *det* detection

Fig. 3 Array results for PCR3a. **a** A volume of 9 µl of each of the four PCR (A, B, C and D) were hybridised on a DualChip GMO array. Each PCR allows the amplification of different DNA fragments. *Box A, B, C and D* show the positive signals obtained on the array after hybridisation of PCR products corresponding to the PCR tubes A, B, C and D. **b** The location of the positive elements (in **bold and italic**) is indicated



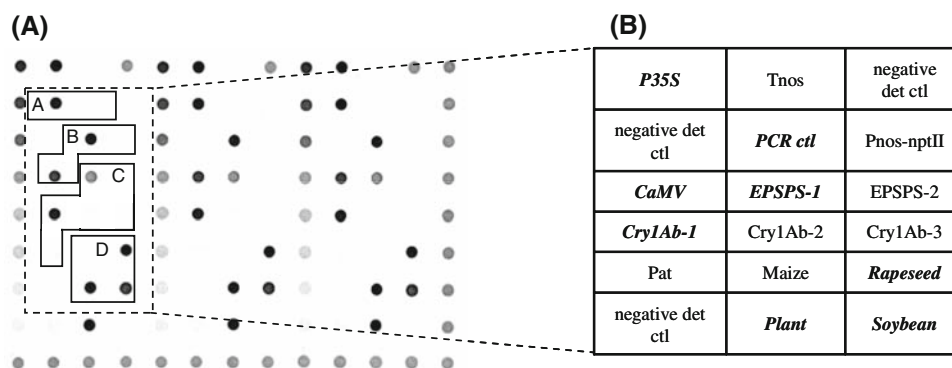


Fig. 4 Array results for PCR5a. **a** A volume of 9 μ l of each of the four PCR (A, B, C and D) were hybridised on a DualChip GMO array. Each of PCR allows the amplification of different DNA fragments. **Box A, B, C and D** show the positive signals obtained on the array

CaMV fragment (500 copies of CaMV plasmid were present in this sample) and the PCR control (tube B, sample 24), *Cry1A(b)-1* of the 1% Bt176 and the *epsps-1* of the 1% GA21 (tube C, sample 28), *cruciferin*, *lectin* and *rbcl* fragments from the sample containing both rapeseed and soybean at a concentration of 50% (tube D, sample 32). The result (Fig. 4) shows that the simultaneous hybridisation of the four different PCR on a single microarray allowed the detection of the eight expected amplified markers (*P35S*, PCR ctl, CaMV, *epsps-1*, *cry1Ab-1*, rapeseed, soybean and plant). PCR5b, PCR6a and PCR6b were performed with DNA samples containing the same GMO at lower concentrations and gave the same results (see Table 1).

The four PCR of the PCR7a set contained the same sample (sample 36), which is a mixture of 0.1% RRS in 100% TOPAS 19/2 (see Table 1). The following elements were expected to be amplified in the different PCR tubes: *P35S* and Tnos DNA fragments from the 0.1% RRS, *P35S* from the 100% TOPAS 19/2 (tube A), *Pnos-nptII* fragment

after hybridisation of PCR products corresponding to the PCR tubes A, B, C and D. **b** The location of the positive elements (in **bold** and *italic*) is indicated

from the 100% TOPAS 19/2 (tube B), *Pat* fragments from the 100% TOPAS 19/2, the *epsps-2* from the 0.1% RRS (tube C), *cruciferin* fragment from the rapeseed DNA template and *lectin* fragment from the soybean DNA template (tube D). The results (Fig. 5) show that the simultaneous hybridisation of the four different PCR on a single microarray allowed the detection of the nine expected amplified markers (*P35S*, Tnos, PCR ctl, *Pnos-nptII*, *epsps-2*, *Pat*, rapeseed, soybean and plant) meaning that both GM events differing by a factor of 1,000 in concentration can be detected simultaneously with the same criteria.

Technical deviations and removal of outliers spots

Some data obtained from experiments showing technical deviations were removed to calculate the total percentage of detection according to the parameters defined in the acceptance criteria (Table 3). Four types of technical deviations were observed. The first kind of deviation,

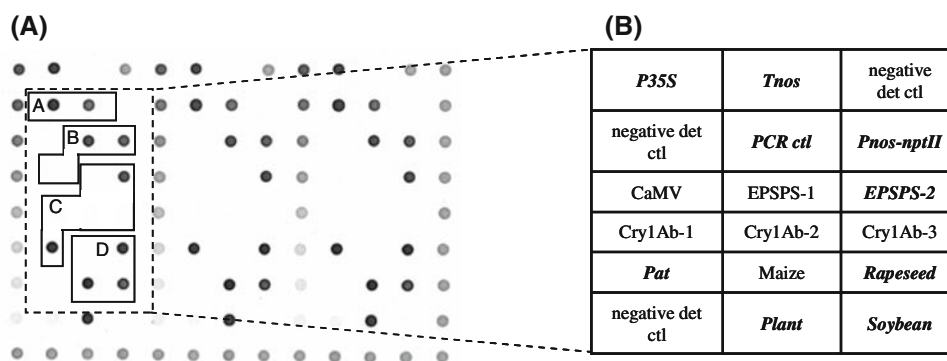


Fig. 5 Array results for PCR7a. **a** A volume of 9 μ l of each of the four PCR reactions (A, B, C and D) were hybridised on a DualChip GMO array. Each of PCR allows the amplification of different DNA fragments. **Box A, B, C and D** show the positive signals obtained on

the array after hybridisation of PCR products corresponding to the PCR tubes A, B, C and D. **b** The location of the positive elements (in **bold** and *italic*) is indicated

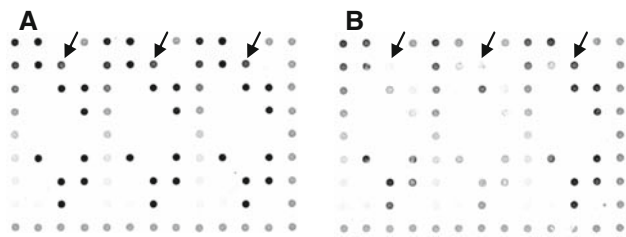


Fig. 6 Example of a technical deviation due to incorrect triplicate analysis. **a** Correct triplicate analysis: the arrows indicate the three replicates of the Tnos marker. **b** Incorrect triplicate analysis: two replicates of the Tnos marker, indicated by the arrows, were affected by the presence of an air bubble in the central upper part of the array, affecting the hybridisation but not the detection curve

called “triplicate analysis deviation”, occurred when two replicates of the same array were affected by a technical artefact. The main cause is the formation of an air bubble during the hybridisation so that part of the array is undetectable or shows a very low density (Fig. 6); of all results, only 3.2% were affected in the analysis of triplicates. A second observed type of technical deviation was negative PCR controls showing a positive signal (0.84% of the total arrays). Finally, 1.2% of the arrays did not show any signal on the positive controls suggesting a problem in one of the detection steps (detection, hybridisation, PCR). Two arrays were also accidentally wiped off. Table 3 summarises the technical deviations and the percentages of affected results.

Outliers [10] that are not covered by these technical errors can be identified based on the ISO 5725. We applied this norm by calculation of the binomial probability distribution of data from all laboratories for each specific PCR. The probability of positive response was estimated across all laboratories as the ratio of positive outcomes over the total number of events. According to the criteria of ISO 5725 (1994), data were identified as outliers if their probability to belong to the same binomial population was lower than 0.01.

Table 3 List and proportion of technical deviations observed during the validation study

Technical deviation	Percentage of affected results (%)
Negative PCR ctl giving a positive response	0.84
Triplicate analysis deviation	3.21
Defective experimental step	
PCR ctl not detected	0.44
Hybridisation ctl not detected	0.15
Detection ctl not detected	0.58
Arrays accidentally wiped off	0.29

Data arrays showing technical deviations have been removed to calculate the total percentage of detection according to the parameters defined in the acceptance criteria

Determination of the accuracy rate

The accuracy rate criterion was set for this study at 95%. Accuracy rates assess the performance of the method on individual genetic target elements. In order to calculate the accuracy rate, the data were first presented as “yes” or “no” result. The binary data obtained for all the replicates in all laboratories were then converted in percentages of detection and the accuracy rate calculated.

The detection accuracy rates for each element and for each PCR were collected from the 11 participating laboratories and are summarised in Table 4. The detection accuracy rates reported in the table are expressed in percentage of total valid assays after removal of the technical deviations and outlying data as described above.

The detection of the GM target elements showed an accuracy rate above the 95% confidence threshold down to 0.1% of GMO concentration for all GM targets (0.1% corresponds to the cut-off established for the microarray). Seven GM target elements out of nine showed an accuracy rate above the 95% confidence threshold even at 0.045% concentration. The *cryIA(b)*-3 element showed an accuracy rate of 94.1%, while the P35S and the *epsps*-1 accuracy rate were respectively 92.5 and 87.5% at the 0.045% concentration. The level reached by these elements is not far away from the 95% threshold and it would be of interest to apply a fuzzy logic analysis [14] in order to evaluate the global performance of the method. The targeted plant reference genes showed an accuracy rate above 95% down to 0.5% plant DNA concentration. For the controls, the CaMV was detected above the accuracy rate of 95% in all samples containing 500–20 copies (lowest number used in the collaborative trial). No false positives were observed in the non-plant extract (PCR2) in the absence of any plant or GM event as proposed in the acceptance criteria. However, a false positive signal at a rate of 5.1% for *epsps*-2 in one GM plant sample and a false positive signal at a rate of 11.9% for maize in another plant sample were observed. False positive results for *epsps*-2 were observed only in one laboratory with two weak signals on four arrays. It was not observed in the 37 other replicates of the other laboratories. This result suggests a possible contamination of the samples during the experiments.

Conclusions

The study is the first validation performed on a multiplex GMO detection assay. The protocol and the validation scheme were based on different assumptions and on statistical thresholds that were set to evaluate the overall method performance. The validation was based on the detection performance of individual elements. The reasons

Table 4 Accuracy rate reported for each element in the different PCR sets

	P35S	Tnos	CaMV	Pnos-nptII	Pat	Cry1Ab-1	Cry1Ab-2	Cry1Ab-3	EPSPS-1	EPSPS-2	Maize	Soybean	Rapeseed	Plant
PCR2a(0% plant)	0.0% (0/24)	0.0% (0/26)	0.0% (0/26)	0.0% (0/26)	0.0% (0/26)	0.0% (0/26)	0.0% (0/26)	0.0% (0/26)	0.0% (0/26)	0.0% (0/26)	0.0% (0/24)	0.0% (0/26)	0.0% (0/26)	0.0% (0/26)
PCR3a(1% GMO, 50% maize)	100.0% (42/42)	100.0% (38/38)	0.0% (0/42)	100.0% (42/42)	100.0% (42/42)	100.0% (0/42)	100.0% (42/42)	100.0% (42/42)	0.0% (0/42)	100.0% (42/42)	97.6% (41/42)	0.0% (0/42)	0.0% (0/42)	100.0% (30/30)
PCR3b(0.5% GMO, 5% maize)	100.0% (43/43)	100.0% (39/39)	0.0% (0/43)	100.0% (43/43)	100.0% (43/43)	0.0% (0/43)	97.7% (42/43)	100.0% (43/43)	0.0% (0/43)	100.0% (43/43)	95.3% (41/43)	0.0% (0/43)	0.0% (0/43)	96.8% (30/31)
PCR4a(0.1% GMO, 1% maize)	100.0% (36/36)	100.0% (36/36)	0.0% (0/36)	100.0% (36/36)	100.0% (36/36)	0.0% (0/36)	100.0% (36/36)	100.0% (36/36)	0.0% (0/36)	100.0% (36/36)	100.0% (32/32)	0.0% (0/36)	0.0% (0/36)	100.0% (28/28)
PCR4b(0.045% GMO, 0.5% maize)	100.0% (34/34)	100.0% (34/34)	0.0% (0/34)	100.0% (34/34)	100.0% (34/34)	0.0% (0/34)	97.1% (33/34)	100.0% (32/34)	0.0% (0/34)	100.0% (34/34)	100.0% (31/31)	0.0% (0/34)	0.0% (0/34)	100.0% (28/28)
PCR5a(1% GMO, plant: 50% rapeseed + 50% soybean)	100.0% (38/38)	0.0% (0/42)	100.0% (42/42)	2.4% (1/42)	0.0% (0/42)	100.0% (42/42)	0.0% (0/42)	0.0% (0/42)	97.6% (41/42)	0.0% (0/42)	11.9% (5/42)	100.0% (42/42)	100.0% (42/42)	100.0% (38/38)
PCR5b(0.5% GMO, plant: 5% rapeseed + 5% soybean)	100.0% (35/35)	0.0% (0/35)	100.0% (39/39)	0.0% (0/35)	0.0% (0/39)	100.0% (39/39)	0.0% (0/39)	0.0% (0/39)	100.0% (39/39)	5.1% (2/39)	0.0% (0/39)	100.0% (39/39)	100.0% (39/39)	100.0% (35/35)
PCR6a(0.1% GMO, plant: 1% rapeseed + 1% soybean)	100.0% (38/38)	0.0% (0/34)	100.0% (38/38)	0.0% (0/34)	0.0% (0/34)	100.0% (38/38)	0.0% (0/38)	0.0% (0/38)	100.0% (38/38)	0.0% (0/38)	0.0% (0/38)	100.0% (38/38)	100.0% (34/34)	100.0% (35/35)
PCR6b(0.045% GMO, plant: 0.5% rapeseed + 0.5% soybean)	92.5% (37/40)	0.0% (0/36)	95.0% (38/40)	0.0% (0/40)	0.0% (0/36)	97.5% (39/40)	0.0% (0/40)	0.0% (0/40)	87.5% (35/40)	0.0% (0/40)	0.0% (0/40)	100.0% (40/40)	100.0% (36/36)	100.0% (37/37)
PCR7a(0.1% RRS in 100% TOPAS19/2)	100.0% (29/29)	97.6% (40/41)	0% (0/41)	97.6% (40/41)	100.0% (41/41)	0% (0/41)	0% (0/41)	0% (0/41)	0% (0/41)	100.0% (41/41)	0% (0/41)	100.0% (41/41)	100.0% (41/41)	100.0% (27/27)

The detection accuracy rates reported in the table are expressed in percentage of total valid assays after removal of technical deviations and outlying data as described above (data given as the number of positive outcomes per total of replicates). For each set of PCR, the expected positive signals, which have an accuracy rate above 95% are indicated in italics. The positive signals having accuracy rate below 95% are indicated in bold. For false positive signals, accuracy rates below 5% are indicated in italics and accuracy rates above 5% are in normal letter. The composition of the PCR sets is presented in Table 1

for such decision are that the elements are common to several GM events and each element whatever the GM event is amplified by the same primers and detected on the same probe. However, the final conclusion of such assay should be the presence or not of the GM event. For this conclusion to be valid, all elements of the same event have to fulfil the criteria as provided in this validation. This is the reason why all elements of the assay had to be validated.

It was decided that each element should be detected with a 95% confidence and the specificity performance (acceptance threshold of false positives) was fixed at 5%. The target detection limit of the method was fixed at 0.1% concentration for each GMO and 1% for the plant reference genes. The other acceptance criteria were a minimum of 8 laboratory reports on 12 considered as valid, using as data the values for each element significantly different from the background and excluding the technical deviations. The validation acceptance criteria and the method performances were set before the conduction of the validation study.

The results clearly showed that the various GM events and reference gene targeted elements can be amplified in separated multiplex PCR and then combined into the same solution for hybridisation. We never encountered interaction between amplicons in the hybridisation step. Additionally, we did not observe significant influence on the LOD of the GMO mixture composition compared to the single assay at least until ratio of 1/1000: in a sample containing 0.1% GMO diluted in another one at 100%, the detections of both GMOs were correctly made with the same 95% accuracy rate.

Probably the most important limiting factor of the method is the risk of carry-over contaminations, which may occur either during or before the assay. We observed some false positive results, mostly concerning the plant species reference genes, when a large amount of DNA of another plant species was present in the sample. The origin of such contamination is difficult to assert. In our experience, such a low level of positive results would come from the presence of very low copy numbers of the contaminant, typically in the range of 1–20 copies in the assay. In such a case, the PCR and the detection are sometimes positive and sometimes negative giving a typical low level of detectable result. Contamination of PCR, especially in routine applications, is always a concern, especially in such a highly sensitive method as this one. This is the reason why it is recommended to limit the number of PCR cycles to 35. The assay can be easily made more sensitive by increasing the PCR cycles to 40, but with a higher risk of false positive since a few contaminant copies will give a positive signal.

The design of the assay was made in such a way as to be able to detect the 0.1% concentration. This corresponds to a range of 40–80 copies in the PCR tube. We have seen that

the 0.045% concentration, which is below the detection limit, was generally detected with good accuracy rate so that the 20–40 copies are reached for most of the elements. The lower sensitivity of the *cry* and *epsps* elements is due to the use of degenerated primers, which limit the real concentrations of specific primers in the PCR solution.

The method allows to detect the presence and also to propose an identification of the event. This identification is based on the fact that the GM event composition differs from one another, so that the combination of positive elements in the assay allows proposing the presence of one or a few events. The identification works best if only one GM event is present in the sample. In complex samples unique identity is not possible, but the method streamlines the identification to a limited number of possible GM events.

This identification method allows the enforcement laboratories to simplify the search for the GM event. Any sample containing more than 0.1% GM will be found positive by the present method and the proposed identity will allow the laboratory to confirm and quantify the GM usually by real-time PCR.

In conclusion, the results of this inter-laboratory trial showed that the method fulfils the requirements in terms of accuracy and detection limits. The method can be considered robust and reliable with respect to its intra- and inter-laboratory accuracy.

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