CO-EXTRA

GM and non-GM supply chains: their CO-EXistence and TRAceability

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Task 4.3: Collaborative method validation

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Final Report

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1 Introduction

A new method based on the use of microarray for the detection of the amplicons produced by PCR was developed allowing the detection and identification of GMOs. This report outlines validation data of the method that was validated in the framework of a European project (Co-Extra project)[1] and with the assistance of the scientific community working on GMOs.

The validation was coordinated by the JRC and organised by Eppendorf Array Technologies. The validation study was performed according to international ISO norms with the participation of twelve laboratories having quality assurance systems in place.

The goal of the study was to assess the performance of the DualChip® GMO assay as a screening method for EU approved GMOs through a collaborative study. The detection method is commercially available as a detection kit (Eppendorf, Hamburg, Germany). The technology is based on the identification of GMO specific DNA sequences first amplified by PCR, followed by hybridisation on a predefined microarray, labelling, detection, data acquisition and data analysis.

The interest of the microarray lies with the possibility to obtain multiple detections within one assay due to the presence of multiple capture probes specific for the different targets. In addition, the microarray is not limited by the number of detection probes. The present method is a qualitative multiple targets detection tool adapted for the screening of multiple GMOs.

The validation was preceded by a pre-validation study performed in five laboratories. The performance criteria evaluated during the pre-validation were mainly the ones reported here under (see chapter 3.2.). Following the evaluation of the pre-validation data, a ring trial was organised and took place from November 2006 to February 2007.

One of the aims of the study was to determine the statistical parameters to be taken into consideration and the appropriate number of assays to be performed in order to obtain the suitable statistical significance of the assay.

The validation was performed to evaluate the significance of the detection of each specific element. In general terms, data analysis was based on the calculation of the accuracy rate and fixed the threshold confidence at 95% for a given element.

The acceptance threshold of false positive results was fixed at 5% while the sensitivity was fixed down to 0.1% for the GMO events and 1% for the plant species. The planning of the validation, number of laboratories, number of samples, number of replicates, number of GM per sample, and concentrations were then defined accordingly taking these assumptions into account together with the practical

feasibility of the validation assay. The validation was further completed by the use of a fuzzy-based approach based on the method of Bellocchi *et al* 2002.

2 General presentation of the DualChip GMO assay

The DualChip® GMO assay is a detection and identification method of multiple DNA sequence targets present in genetically modified (GM) events within a single experiment. The method is based on the amplification of specific GM target sequences and plant specific sequences in multiplex PCR, followed by the hybridisation of the different amplicons directly on the array. The low-density microarray DualChip® GMO is based on the technology developed by Eppendorf Array Technologies (EAT, Namur, Belgium) and is available from the company Eppendorf (Hamburg, Germany).
The presentation of the array and the methods are available at:
http://www.eppendorf.com/int/index.php?l=1&action=document&sitemap=1&docnode=34901&pb=7c1869f9cdab49c5.
The array is fixed on a glass slide with capture probes spotted in triplicates on the slide according to a specific pattern which is recognised by the data analysis software. These nucleotide sequences are covalently attached by an amino group at 5' end onto an aldehyde functionalised slide (Zammatteo *et al.*, 2000).

The DualChip® GMO microarray detects 3 classes of targets: the GMO screening elements, the species specific targets and different control targets. The elements detected on the DualChip GMO are listed hereunder:

Screening target elements

- CaMV 35S promoter (P35S)
- Nopaline synthase terminator (Tnos)
- Phosphinothricin N-acetyltransferase (Pat)
- Cry1Ab delta-endotoxin (Cry1Ab)
- 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)
- The junction between the Nopaline synthase promoter and the neomycin phosphotransferase II gene (Pnos-nptII)

Species specific targets

- Invertase (Maize)
- Cruciferin (Rapeseed)
- Lectin (Soybean)
- rBCL (plant universal)

Control target

- Cauliflower Mosaic Virus (CaMV)

One specific capture probe for each of the DNA target elements is present on the array. However for two elements, Cry1Ab and EPSPS, several capture probes are spotted on the array:

- Cry1Ab1 is specific to the sequence present in Bt176 maize
- Cry1Ab2 is specific to the sequence present in MON810 maize
- Cry1Ab3 is specific to the sequences present in Bt11 sweet maize, MON531 cotton

- and MON15985 cotton
- EPSPS1 is specific to the sequence present in GA21 Maize
- EPSPS2 is specific to the sequences present in NK603 Maize and Roundup Ready ™ soybean) are specific.

The assay starts with the amplification of the following elements in 4 separated PCR assays:

- PCR A: Tnos, P35S
- PCR B: Pnos-nptII, CaMV, PCR control
- PCR C: Pat, Cry1Ab, EPSPS
- PCR D: maize, soybean, rapeseed and plant species

After hybridisation in a thermomixer, the presence of a positive spot is revealed by colorimetric reaction using the Silverquant labeling (Alexandre *et al.*, 2001). The arrays are scanned by a SilverQuant scanner, followed by quantification of the spots present in triplicate on the array and data analysis by the DualChip® evaluation software (see DualChip® GMO Instruction manual).

The outcome of the analysis is the determination of the presence or not of a GMO in the sample and a proposal for its identification based on the detection of the different elements. GM identification is the result of the comparison of the different elements found positive in the assay and their presence in the GMOs approved in the EU. The software makes use of a matrix approach of the different elements which constitutes the GMOs approved according to Regulation (EC) No 1829/2003 at the date of January 2007. The list of GMOs approved is provided in the kit assay and is derived from the list available at http://ec.europa.eu/food/dyna/gm_register/index_en.cfm. Five of these GMO are subjected to special restriction of use within the EU.

3 Organisation of the validation

3.1 List of participants

The method was tested in twelve laboratories with the aim to assess its performance. Each laboratory was requested to carefully follow the protocol provided. The participating laboratories are listed in table 1 in alphabetical order.

Table 1. Laboratories participating in the validation study of the DualChip GMO microarray.

Laboratory	Country
AGES - Österreichische Agentur für Gesundheit und Ernährungssicherheit	Austria
BfR - Bundesinstituts für Risikobewertung	Germany
BIOMI Ltd	Hungary
CSIC - Consejo Superior de Investigaciones Científicas	Spain
CVUA NRW - Chemisches Landes- und Staatliches Veterinäruntersuchungsamt	Germany

DVP-ILVO - Instituut voor Landbouw en Visserijonderzoek	Belgium
IHU – Institut für Hygiene und Umwelt	Germany
INRA - Institut National de la Recherche Agronomique (Versailles)	France
IHCP - Joint Research Centre (JRC) – Community Reference Laboratory for GM Food and Feed, Biotechnology and GMOs Unit	Italy
LGL - Bayer. Landesamt für Gesundheit und Lebensmittelsicherheit	Germany
LIFEPRINT - lifeprint GmbH	Germany
RIKILT – Institute of Food Safety	The Netherlands

3.2 Acceptance criteria

The acceptance criteria considered for the study and data analysis were the following:

1. The analysis was performed if the results of 8 laboratories were available and valid.
2. Arrays with technical problems were removed to calculate the total percentage of detection. The list of such technical problems is as follows:
    - triplicate problem analysis (e.g. presence of a bubble in part of the array)
    - if negative PCR controls were positive or a systematic contamination was suspected
    - defective experimental steps (detection, hybridisation, PCR)
3. Each element was validated separately. The assay was a qualitative detection of each GMO element identified on the array.
4. The detection acceptance criteria was set at the rate of 95% accuracy, ensuring less than 5% false negative and 5 % false positive results.
5. The detection was based on the following cut-offs: a signal was considered as detected when its intensity was above the local background intensity plus 2.5 times its standard deviation and above a fixed value of 1500 on a scale having a maximum of 65,536.
6. The concentrations over which the method was validated were:
    - For the screening elements: 1% GM, 0.5 % GM, 0.1% GM and 0.045% GM. The cut-off established for the microarray was fixed at 0.1% for the GM elements.
    - For the plant-specific elements: 50%, 5%, 1% and 0.5%. The cut-off established for the microarray was fixed at 1% for the plants.
7. One non plant DNA was included in the validation to test the specificity in terms of false positive results.

3.3 Materials

For the validation of the method, test samples were provided to each validation laboratory as blind samples consisting of DNA reference samples, covering the different GM target elements. Samples were provided by the IRMM of the JRC (Certified Reference Materials) except for the epsps-1 target which was a plasmid-inserted sequence. The detection of the GM DNA was performed in presence of the corresponding non GM plant DNA.

The participants received the following materials:

- 36 unknown DNA samples labelled from 1 to 36
- 6 DC GMO kit Box1 (with all PCR reaction reagents) + 3 tubes of UNG (Uracyl N-glycosylase)

- 6 DC GMO kit Box2 (with microarrays and hybridisation reagents)
- 5 Silverquant kits

The participants received the following equipment and consumables:

- 1 Eppendorf Mastercycler Epgradient S PCR cycler (with its control panel)
- PCR microtubes (box of 960 tubes)
- 2 Thermomixers (with Thermoblock)
- 2 Silverquant adapters
- 1 Silverquant scanner
- 1 Silverquant Dongle
- 1 Laptop (software included)

Table 2 shows the GM contents of the unknown samples distributed to the participants. 100 ng of total DNA per PCR were used in the assay.

Table 2. GMO content of validation samples

Sample n°	GMO (w/w)	GM copies Calculated *
ctl 1	RRS 0.1% soybean	88
ctl 2	Bt176 0.1% maize	37
3	non-plant DNA	0
4	Bt11 1% maize	370
5	Bt11 0.5% maize	185
6	Bt11 0.1% maize	37
7	Bt11 0.045% maize	17
8	Topas19/2 1% rapeseed	870
9	Topas19/2 0.5% rapeseed	435
10	Topas19/2 0.1% rapeseed	87
11	Topas19/2 0.045% rapeseed	39
12	RRS 1% soybean + Bt11 1% maize + MON810 1% maize	880 + 370 + 370
13	RRS 0.5% soybean + Bt11 0.5% maize + MON810 0.5% maize	440 + 185 + 185
14	RRS 0.1% soybean + Bt11 0.1% maize + MON810 0.1% maize	88 + 37 + 37
15	RRS 0.045% soybean + Bt11 0.045% maize + MON810 0.045% maize	40 +17 +17
16	Maize diluted in non-plant DNA	0
17	Maize diluted in non-plant DNA	0
18	Maize diluted in non-plant DNA	0
19	Maize diluted in non-plant DNA	0
20	BT176 1% maize	370
21	BT176 0.5% maize	185
22	BT176 0.1% maize	37
23	BT176 0.045% maize	17

24	CaMV (500 copies) in non-plant DNA	0
25	CaMV (100 copies) in non-plant DNA	0
26	CaMV (50 copies) in non-plant DNA	0
27	CaMV (20 copies) in non-plant DNA	0
28	Bt176 1% maize + GA21 1% maize	370 + 370
29	Bt176 0.5% maize + GA21 0.5% maize	185 + 185
30	Bt176 0.1% maize + GA21 0.1% maize	37 + 37
31	Bt176 0.045% maize + GA21 0.045% maize	17 + 17
32	Rapeseed + Soybean 50% diluted in non-plant DNA	0
33	Rapeseed + Soybean 5% diluted in non-plant DNA	0
34	Rapeseed + Soybean 1% diluted in non-plant DNA	0
35	Rapeseed + Soybean 0.5% diluted in non-plant DNA	0
36	RRS 0.1% soybean in Topas 19/2 100% rapeseed	88 + 86956

* The absolute copy numbers in the validation samples are determined by dividing the sample DNA weight (picograms) by the published average 1C value (Arumuganathan and Earle, 1991) for soybean genome (1.13 pg), maize genome (2.73 pg) or rapeseed genome (1.15 pg). Consequently, 100 ng DNA contain 36630 copies of maize, 88496 copies of soybean and 86956 copies of rapeseed when considering the genome in its haploid form.

3.4 Experimental design

The ring-trial was carried out in accordance with the requirements of ISO 5725 (1994).
The DualChip® GMO protocol was followed (DualChip GMO Instruction Manual: http://www.eppendorf.com/int/index.php?l=1&action=document&sitemap=1&docnode=34901&pb=7c1869f9cdab49c5). The protocol includes four independent PCR assays per sample, followed by hybridisation on one microarray. To simplify the experimental design and reduce the time of the assay, the independent PCR assays were reduced to the necessary and the non relevant PCR assays were not performed.
A sample at GM 0.045% was included allowing the evaluation of the sensitivity of the assay below 0.1%.

The method was introduced to each laboratory during a “one day demo”. A first set (PCR1a and 1b) of PCR and hybridisations (samples ctl1 and ctl2) were performed to familiarise the laboratory to the array technology. These preliminary assays are not part of the evaluation data. The validation experiments were performed by the laboratories only in case of positive outcome of the preliminary assays.

Thirty-six blind samples, covering different concentrations of GM and plant DNA were used in the validation study (see table2). These samples were used individually in one or several of the 4 primer mix (A, B, C, D) for PCR amplification (see table 3). Thereafter, the resulting amplicons were mixed for hybridisation on the array. Fourteen working days (7 PCR working days, each of them followed by a hybridisation working day), were planned to complete the experimental work. Overall, 3360 PCR assays (280 PCR reactions per laboratory) and 840 hybridisations (70 hybridisations per laboratory) were needed in this validation.

Each PCR was performed in quadruplicates for all samples and hybridised on microarrays according to the instructions provided in the protocol of the DualChip® GMO kit (Eppendorf, Hamburg, Germany – www.eppendorf-biochip.com).

The four multiplex primer mixes are the ones described in the kit manual. They amplify the following targets.

- PCR A: Tnos, P35S
- PCR B: Pnos-nptII, CaMV, PCR control
- PCR C: Pat, Cry1Ab, EPSPS
- PCR D: maize, soybean, rapeseed and plant species

For time reduction and simplification of the assay, the independent PCR reactions (A B, C, D) were limited to the necessary and the non relevant PCR assays were not performed (see table 3).

Table 3. List of PCR assays and composition in GM and plant species DNA

Steps	PCR assay	Sample tested per PCR	GM % (w/w)	Plant % (w/w)
Step 1	PCR1a	Ctl1(A,B,C and D),	0.1	100
	PCR1b	Ctl2(A,B,C and D)	0.1	100
Step 2	PCR2a	3(A,B,C and D)	0	0
Step 3	PCR3a	4(A)+8(B)+12(C)+16(D),	1	50
	PCR3b	5(A)+9(B)+13(C)+17(D)	0.5	5
Step 4	PCR4a	6(A)+10(B)+14(C)+18(D),	0.1	1
	PCR4b	7(A)+11(B)+15(C)+19(D)	0.045	0.5
Step 5	PCR5a	20(A)+24(B)+28(C)+32(D),	1	50
	PCR5b	21(A)+25(B)+29(C)+33(D)	0.5	5
Step 6	PCR6a	22(A)+26(B)+30(C)+34(D),	0.1	1
	PCR6b	23(A)+27(B)+31(C)+35(D)	0.045	0.5
Step 7	PCR7a	36(A,B,C and D)	0.1 and 99.9	100

The letters A, B, C, D refer to the PCR primer mixes. The numbers (1-36) refer to the sample composition of table 2. Each sample (1-36) is amplified in a separate PCR tube with one of the 4 primer mix and then combined for the hybridisation as indicated in the table.

The PCR2a tested the non-plant DNA sample.
The PCR 3, 4, 5 and 6 tested the different GMO concentrations.
The PCR7a tested the detection of a low concentration of GMO (0.1%) in the presence of high concentration (99.9%) concentration of a second GMO.

3.5 Statistical analysis

The validation of the method was based on the acceptance criteria defined in paragraph 3.2 and the removal of outlying data.

In order to detect possible outliers, a test based on the binomial probability distribution was applied to all data from all laboratories on each of the PCR assay as explained in table 3. For each PCR assay, the probability of positive response was estimated across all laboratories as the ratio of positive results 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.

For this study the accuracy rate criterion was set at 95%. This 95% confidence is also the threshold used in the "Definition of Minimum Performance Requirement for Analytical Methods of GMO Testing" (2005) available at http://gmo-crl.jrc.it/guidancedocs.htm.
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 into percentages of detection and the accuracy rate was determined.

We also propose to use an indicator based on the principle of fuzzy-logic expert system for the purpose of a comprehensive assessment, encompassing the ability of the method to detect the full set of targets. This approach was never applied before in the context of microarray validation. It allows evaluating the global performance of the method using the procedure described in Bellocchi *et al.* (2002) and setting accuracy rate thresholds to 95% (favorable) and 90% (unfavorable).

4 Results

4.1 Pre-analysis of the data

The study was preceded by preliminary tests carried out by participating laboratories to get accustomed to new technology.
Eleven laboratories (out of 12) succeeded in this preliminary test and took part in the validation study.

The calculation of the total percentage of detection (% accuracy) according to the parameters set, was performed after removal of the outliers and of the technical deviations defined in the acceptance criteria (see paragraph 3.2).

The levels of each of the technical deviations were the following:

- 3.2% of the total results were affected in the analysis of triplicates. Two replicates were affected by a technical artefact on the array such as a bubble in part of the array.
- 0.84% showed negative PCR controls as positive, suggesting a possible contamination.
- 1.2% of the tests did not show a signal on the positive controls suggesting a problem in one of the step (detection, hybridization, PCR).

4.2 Accuracy rate

The detection accuracy rates for each element and for each PCR assay was calculated on the data reported by the eleven laboratories and are summarised in table 4. The detection accuracy rates reported in the table are expressed in % of total valid assays after removal of technical deviations and outlying data as described above.

Table 4. Accuracy rate per element in each PCR

PCR event	Group 1: "ProTer"			Group 2: "Screening"							Group 3: "Plant"			
	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%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
PCR3a (1% GMO, 50% plant)	**100.0%**	**100.0%**	0.0%	**100.0%**	**100.0%**	0.0%	**100.0%**	**100.0%**	0.0%	**100.0%**	**97.6%**	0.0%	0.0%	**100.0%**
PCR3b (0.5% GMO, 5% plant)	**100.0%**	**100.0%**	0.0%	**100.0%**	**100.0%**	0.0%	**97.7%**	**100.0%**	0.0%	**100.0%**	**95.3%**	0.0%	0.0%	**96.8%**
PCR4a (0.1% GMO, 1% plant)	**100.0%**	**100.0%**	0.0%	**100.0%**	**100.0%**	0.0%	**100.0%**	**100.0%**	0.0%	**100.0%**	**100.0%**	0.0%	0.0%	**100.0%**
PCR4b (0.045% GMO, 0.5% plant)	**100.0%**	**100.0%**	0.0%	**100.0%**	**100.0%**	0.0%	**97.1%**	**94.1%**	0.0%	**100.0%**	**100.0%**	0.0%	0.0%	**100.0%**
PCR5a (1% GMO, 50% plant)	**100.0%**	0.0%	**100.0%**	2.4%	0.0%	**100.0%**	0.0%	0.0%	**97.6%**	0.0%	11.9%	**100.0%**	**100.0%**	**100.0%**
PCR5b (0.5% GMO, 5% plant)	**100.0%**	0.0%	**100.0%**	0.0%	0.0%	**100.0%**	0.0%	0.0%	**100.0%**	5.1%	0.0%	**100.0%**	**100.0%**	**100.0%**
PCR6a (0.1% GMO, 1% plant)	**100.0%**	0.0%	**100.0%**	0.0%	0.0%	**100.0%**	0.0%	0.0%	**100.0%**	0.0%	0.0%	**100.0%**	**100.0%**	**100.0%**
PCR6b (0.045% GMO, 0.5% plant)	**92.5%**	0.0%	**95.0%**	0.0%	0.0%	**97.5%**	0.0%	0.0%	**87.5%**	0.0%	0.0%	**100.0%**	**100.0%**	**100.0%**
PCR7a (0.1% RRS, 99.9% Topas19/2)	**100.0%**	**97.6%**	0.0%	**97.6%**	**100.0%**	0.0%	0.0%	0.0%	0.0%	**100.0%**	0.0%	**100.0%**	**100.0%**	**100.0%**

ProTer: targets being promoters or terminators
Screening: other GMO specific targets elements
Plant: plant specific targets

The detection of the GM target elements showed an accuracy rate above 95% down to 0.1% GMO concentration for all GM targets (0.1% corresponds to the cut-off sensitivity level established for the microarray).

Seven GM target elements out of nine showed an accuracy rate above 95% also at 0.045% GM concentration. The Cry1Ab-3 element showed an accuracy rate of 94.1 % while for P35S and the EPSPS-1 the accuracy rates were respectively 92.5 % and 87.5%.
The plant elements showed an accuracy rate above the 95% down to 0.5 % plant DNA concentration.

The present validation was conducted in order to assess whether the expected overall performance is in line with the criteria of specificity and sensitivity. Data showed that the method is specific and fulfils the criterion of 95% confidence at the 0.1% GM concentration for the GM target elements and at 1% for the plant targets. Therefore the method fulfils the requirement in term of accuracy and limit of detection.

For the controls, the CaMV was detected above the accuracy rate of 95% in all concentrations ranging from 500 to 20 copies.

The false positive rate was at 0% in the non plant extract (PCR2a) in the absence of any plant or GM event as proposed in the acceptance criteria.
A false positive signal at a rate of 5.1 % for the element 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. EPSPS-2 false signals were observed in one laboratory (lab 12) with 2 weak signals on 4 arrays. It was not observed in the 37 other replicates of the other laboratories. This result suggests a possible contamination of the sample during the experiments carried out by the participating laboratory.

4.3 Evaluation of the global performance of the method

The DualChip® GMO microarray is a multiplex detection assay. The validation was performed to evaluate the detection of each specific element and the performance was assessed by determining the accuracy rate of the method for individual targets.

A further assessment of the global performance of the method, using a novel approach based on the fuzzy-logic principle, was conducted. The purpose of this analysis was to obtain an assessment on the ability of the method to detect a full set of the targets. The fuzzy-logic based indicator of model validity was based on the same principles of the multiple-metrics assessment system originally proposed by Bellocchi *et al*. (2002) and later implemented in software AMPE (Acutis *et al*., 2007).

The approach permits a flexible structure in which accuracy rates of a range of GM elements can be aggregated into a single modular indicator. Aggregation of accuracy rates is based on an expert weighting expression of the balance of importance of the individual elements and their aggregation into modules.

An indicator was elaborated *ad-hoc* for validation of DualChip GMO technology where accuracy rates from different GM targets elements were aggregated into three modules (Table 5). A module is an evaluation measure calculated via a fuzzy-based procedure from accuracy rates of GM target elements. For each module, a dimensionless value between 0 (best model response) and 1 (worst model response) is calculated. The Sugeno method of fuzzy inference was adopted (Sugeno, 1985).

Three membership classes were defined for accuracy rates, according to an expert judgment, namely favourable (F), unfavourable (U) and partial (or fuzzy) membership, using S-shaped curves as transition probabilities in the range F to U (and vice versa). A two-stage design of fuzzy-based rules inferring system is applied: first, accuracy rates of GM events are aggregated into modules and then, using the same procedure, the modules are aggregated in a second-level integrated measure (again, ranging from 0 to 1), called indicator (I_{DCGMO}, Indicator of DualChip Genetically Modified Organisms).

The control rules for estimating module values were based on logic relationships between inputs and outputs, expressed in linguistic terms by *if-then* statements. The expert reasoning runs as follows: *if* all input variables are F, *then* the value of the module is 0 (good method); *if* all indices are U, *then* the value of the module is 1 (bad method), while all the other combinations assume intermediate values.

A combination of favourable and unfavourable accuracy rates in a module (or favourable and unfavourable modules in the indicator) is set up according to a decision rule, that is, the expected weight assigned to a given conjunction (logical statement and) of inputs (accuracy rates or modules).

Each decision rule is derived from the relative importance assigned to each accuracy rate (or module). The genetic elements were grouped into modules according to their similarities. In so doing, the modules represent the features that the evaluation system should consider: (i) the ability of the method to detect promoting and terminating elements (module “ProTer”), (ii) the ability of the method to detect the inserted genes (module “Screening”); (iii) the ability of the method to detect plant markers (module “Plant”).

The same indicator was used to either test positive or false positive accuracy rates (the latter limited to non-plant DNA).

Table 5. List of modules, GM elements and relative weights.

Module	GM element	Description	Weight into the module*
ProTer (Promotor and Terminator)	*P35S*	CaMV 35s promoter	0.40
	Tnos	Nopaline synthase terminator	0.40
	CaMV	Control element	0.20
Screening (Inserted genes)	*Pnos-nptII*	Junction between the Nopaline synthase promoter and the neomycin phosphotransferase II gene	0.14
	PAT	Phosphinothricin acetyltransferase	0.14
	cry1Ab-1	Cry1Ab delta-endotoxin (variant 1)	0.14
	cry1Ab-2	Cry1Ab delta-endotoxin (variant 2)	0.14
	cry1Ab-3	Cry1Ab delta-endotoxin (variant 3)	0.14
	EPSPS-1	5-enolpyruvylshikimate-3-phosphate-synthase (variant 1)	0.14
	EPSPS-2	5-enolpyruvylshikimate-3-phosphate synthase (variant 2)	0.14
Plant (Species reference elements)	*Maize*	Invertase gene	0.30
	Soybean	Lectin gene	0.30
	Rapeseed	Cruciferin gene	0.30
	Plant	universal plant marker	0.10

* Weights were re-arranged when one or more elements were missing in the analysis.

The outlying laboratories excluded from further analysis are reported in Table 6 for each PCR event, together with the probability of rejection. The PCR mixes are described in paragraph 4.

Table 6. Data excluded from the analysis (p<0.01).

PCR sample	Outlying laboratory	probability
PCR4a 0.1%	lab. 1	0.001
PCR4a 0.1%	lab. 10	0.0001
PCR4b 0.045%	lab. 10	~0
PCR5b 0.5%	lab. 10	~0
PCR6a 0.1%	lab. 10	~0

The mean values per PCR for I_{DCGMO} (and its three modules) are given in Table 7.

Table 7. Values of the indicator of global validity of the method (I_{DCGMO}) for modules "ProTer", "Screening", and "Plant".

PCR sample	ProTer	Screening	Plant	I_{DCGMO}
PCR3a 1%	0.0000	0.0000	0.0000	0.0000
PCR3b 0.5%	0.0000	0.0000	0.0000	0.0000
PCR4a 1%	0.0000	0.0000	0.0000	0.0000
PCR4b 0.045%	0.0000	0.0130	0.0000	0.0001
PCR5a 1%	0.0000	0.0000	0.0000	0.0000
PCR5b 0.5%	0.0000	0.0000	0.0000	0.0000
PCR6a 0.1%	0.0000	0.0000	0.0000	0.0000
PCR6b 0.045%	0.3333	0.5000	0.0000	0.2729
PCR7a	0.0000	0.0000	0.0000	0.0000
PCR2a	0.0000	0.0000	0.0000	0.0000

The results from fuzzy-logic based aggregation confirm the fulfilment of the expected acceptance criteria down to 0.1% GM concentration and 1% for the plant targets.

For the 0.045% GM concentration, the results from fuzzy-logic based aggregation produced mixed but mostly favourable results, with only limited inaccuracies (PCR4b and PCR6b). For PCR4b, little inaccuracies came from the screening GM elements, whilst both promoting-terminating and screening GM elements contributed the highest I_{DCGMO} value observed with PCR6b. Even in the latter, however, I_{DCGMO} lower than 0.3 can be regarded as acceptable given the uncertainties associated with low concentrations.

Noteworthy no false positives with the non plant DNA sample (PCR2a) were observed.

5 Conclusions

The overall method performance of the method has been evaluated with respect to the expected method acceptance criteria set before the validation study. All seven criteria were fulfilled, with the main criteria as detection of the different elements at 0.1% concentration of GM and 1% plant with a 95% accuracy rate fully satisfied.

The results obtained during the collaborative trial indicate that the method can be considered as fit for purposes of screening with respect to its intra and inter laboratory accuracy.

The results demonstrate the validity of the DualChip GMO (Eppendorf, Hamburg, Germany) as an array multiplex approach for the screening of GMO. The results showed that the technology is robust, practical and is suitable as a screening tool.

When performed on samples, the detection of the targeted elements leads to the confirmation of the presence or not of a GM. Moreover, using a matrix of the genetic composition of GM events, the identity of the GM can be proposed as the outcome of the data analysis.

The presently validated array can be extended and updated with the growing number of approved and unapproved GMOs for which a detection method is needed.

The performance of the method was assessed on raw material samples; its applicability can be evaluated on and extended to complex samples.

The present work represents, to our knowledge, the first example of inter-laboratory validation of a multiplex assay.

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