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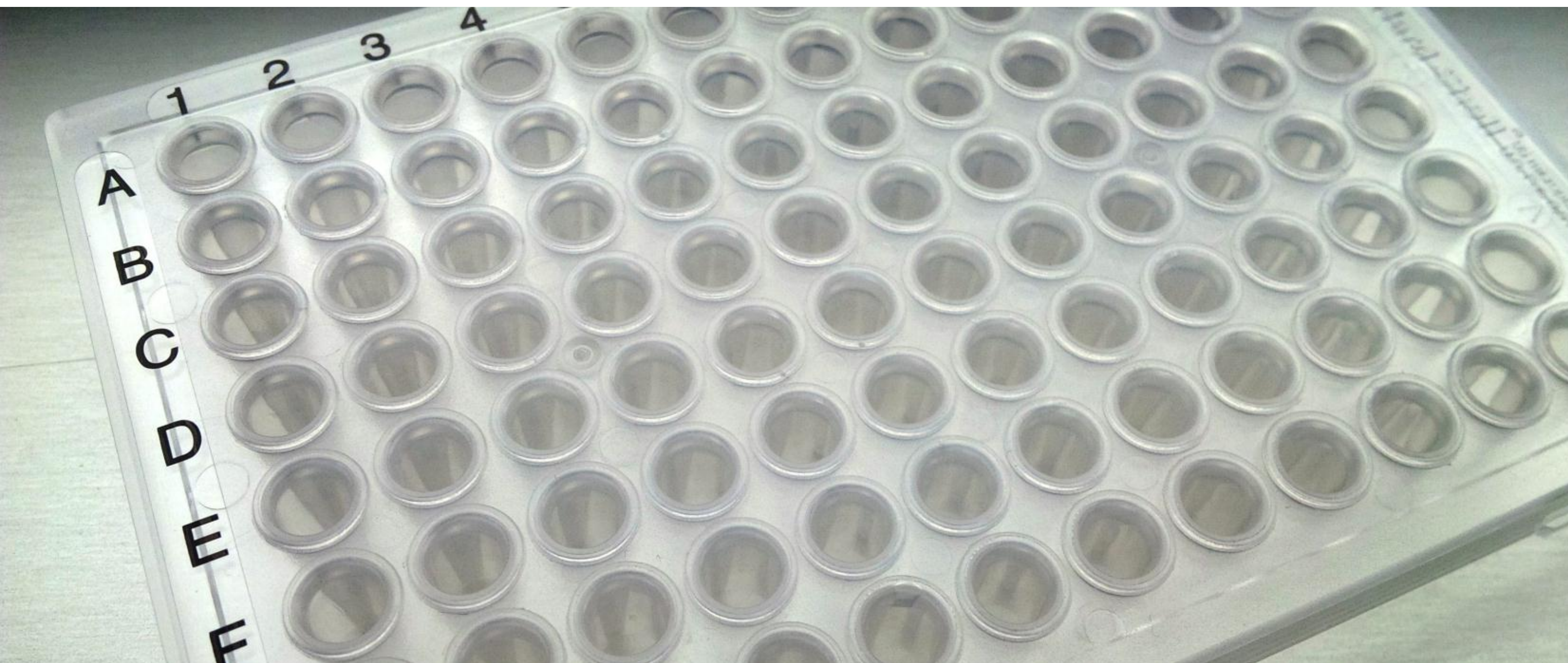

Development and Optimization of the GM Soybean Event-Specific Pre-Spotted Plate (SePSP)

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Abstract

This report describes the development of the "GM soybean event-specific pre-spotted plate (SePSP)" as a ready-to-use tool for GMO detection and identification.
This SePSP allows the detection of all soy GM events listed in the EU register as of April 2015. The plate includes a total of 14 assays, consisting of 13 event-specific assays and one taxon-specific assay. The assays are spotted in 6 replicates, allowing the simultaneous analysis of two samples in duplicate plus a negative and a positive control.
The performance of the assays in terms of specificity and sensitivity is in line with requirements for GMO testing; they therefore can be used for the detection of single and stacked soy GM events in food and feed samples.

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Background

Pre-spotted plates (PSPs) consist in ready-to-use real-time PCR plates whose wells have been spotted with specific primers and probes targeting sequences of interest. They present the advantage of being time- and cost-efficient and offer a straightforward tool to face high-throughput testing needs. The first application of pre-spotted plates was developed by the JRC in 2009 (1, 2) and the latest PSP version was released in 2013 (3) in response to the introduction of new GMOs on the market.

To improve the economical aspect of the PSP tools and to make them better suited for routine application, the Molecular Biology and Genomics (MBG) Unit of the JRC has now developed a GM soybean event-specific PSP (SePSP).

The SePSP allows the detection of all soy GM events listed in the EU register of authorised GMOs[1] as of April 2015 (Table 1). The plate includes a total of 14 assays, consisting of 13 assays for soy GM events and one assay for the soy reference gene. The assays are spotted in six replicates, allowing for the analysis of two samples in duplicate plus a negative and a positive control in parallel (Figure 1). The SePSP represents an updated version of the previous release (3) and it contains assays for the detection of 4 additional soy GM events: DAS-68416-4, MON87705, MON87708 and MON87769.

To reduce the associated costs, the reaction volume was scaled down from 50 µl to 25 µl. A bridging study is here described for the verification of performance of all the validated methods included in the previous version with the new reaction conditions.

Molecular specificity was reassessed experimentally by testing DNA from each GM soy event and the major crops for each method. Additionally, the methods for the detection of the soy GM events DAS-68416-4, MON87705, MON87708 and MON87769 were assessed against all authorized GMOs from maize, canola, cotton, rice, potato, sugar beet. Moreover, specificity was assessed *in silico* using the "JRC GMO-Matrix"[2] . Finally, the sensitivity of the modified assays was reassessed by evaluating their limit of detection (LOD) under the new reaction conditions.

Table 1. List of authorized GM events (not including stacked GM events) as of April 2015 (EU register) detected by the SePSP. LLP: low level presence in feed (4).

Soybean GM Events	Unique Identifier	Status
A2704-12	ACS-GM005-3	authorized
A5547-127	ACS-GM006-4	authorized
CV127	BPS-CV127-9	authorized
DAS68416	DAS-68416-14	LLP
DP305423	DP-305423-1	authorized
DP356043	DP-356043-5	authorized
FG72	MST-FG072-2	LLP
GTS40-3-2	MON-04032-6	authorized
MON87701	MON-87701-2	authorized
MON87705	MON-87705-6	authorized
MON87708	MON-87708-9	authorized
MON87769	MON-87769-7	authorized
MON89788	MON-89788-1	authorized

[1] http://ec.europa.eu/food/dyna/gm_register/index_en.cfm
[2] http://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/

Figure 1. Layout of the GM soybean event-specific pre-spotted plate.

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
	1	2	3	4	5	6	7	8	9	10	11	12
A	Lec	GTS 40-3-2	Lec	GTS 40-3-2	Lec	GTS 40-3-2	Lec	GTS 40-3-2	Lec	GTS 40-3-2	Lec	GTS 40-3-2
B	A2704	MON 87701	A2704	MON 87701	A2704	MON 87701	A2704	MON 87701	A2704	MON 87701	A2704	MON 87701
C	A5547	MON 87705	A5547	MON 87705	A5547	MON 87705	A5547	MON 87705	A5547	MON 87705	A5547	MON 87705
D	CV127	MON 87708	CV127	MON 87708	CV127	MON 87708	CV127	MON 87708	CV127	MON 87708	CV127	MON 87708
E	DAS 68416	MON 87769	DAS 68416	MON 87769	DAS 68416	MON 87769	DAS 68416	MON 87769	DAS 68416	MON 87769	DAS 68416	MON 87769
F	DP 305423		DP 305423		DP 305423		DP 305423		DP 305423		DP 305423	
G	DP 356043		DP 356043		DP 356043		DP 356043		DP 356043		DP 356043	
H	FG72		FG72		FG72		FG72		FG72		FG72	

Material, methods and experimental design

Sample preparation

DNA was extracted using a CTAB-based extraction procedure (5) from certified reference materials (CRM) from the Institute for Reference Materials and Measurements (IRMM) and from the American Oil Chemists' Society (AOCS). The highest nominal level available was used. The complete list of materials is listed in Annex 1.

Wild-type control samples (maize, canola, wheat, sugar beet, potato and cotton) were extracted using the Foodproof GMO Sample Preparation kit (Biotecon Diagnostics GmbH, Postdam, Germany) according to the manufacturer's instructions or a CTAB-based extraction procedure (soybean) (5).

Seeds and grains were first ground to fine powder using Grindomix GM 200 (Retsch GmbH, Haan, Germany).

Each sample was extracted in duplicate and then quantified by fluorescence detection using PicoGreen® dsDNA quantitation kit (Invitrogen, Molecular Probes, Eugene, OR, USA). All extracts were examined on agarose gel to verify the DNA integrity and tested for the absence of PCR inhibitors (6).

Methods

Assays for use in the SePSP were selected among those listed in the database of EU reference methods for GMO analysis "GMOMETHODS"[3] (7) and are listed in Table 2.

Table 2. Assays included in the SePSP. "Method ID" refers to the GMOMETHODS database identification code.

Target	Method ID	Target	Method ID
Soybean Taxon-sp.	qt-tax-gm-002	Event FG72	qt-eve-gm-001
Event A2704-12	qt-eve-gm-004	Event GTS40-3-2	qt-eve-gm-005
Event A5547-127	qt-eve-gm-007	Event MON87701	qt-eve-gm-010
Event CV127	qt-eve-gm-011	Event MON87705	qt-eve-gm-003
Event DAS68416	qt-eve-gm-013	Event MON87708	qt-eve-gm-012
Event DP305423	qt-eve-gm-008	Event MON87769	qt-eve-gm-002
Event DP356043	qt-eve-gm-009	Event MON89788	qt-eve-gm-006

Molecular specificity of the assays

In silico analyses

In silico specificity of the event-specific assays was assessed using the JRC GMO-Matrix[4], which simulates PCR amplifications against all GMOs and genome sequences available in the restricted Central Core Sequence Information System (CCSIS) database[5] (8).

Experimental verification

Each assay was tested in duplicate against the DNA extracts from the 13 GM soybean CRMs and the wild-type samples. RTi-PCR methods were tested in a volume of 25 µL containing 100 copies of GM target at the extracted nominal level or 100ng of wild type gDNA, 1×TaqMan® Universal PCR Master Mix no UNG (Applied Biosystems), and primers and probes at a concentration of 900nM and 250nM, respectively. The thermal profile used was: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. Data acquisition was set on the 60°C step. Runs were performed using the 7900 Real-Time PCR System (Life Technologies) and data analysed using the SDS 2.4 software.

Additionally, the assays for the newly authorized events DAS-68416-4, MON87705, MON87708 and MON87769 were tested against all available GMOs from maize (3272, 5307, 98140, Bt11, Bt176, DAS40278, DAS59122, GA21, LY038, MIR162, MIR604, MON810, MON863, MON87460, MON88017, MON89034, NK603, T25, TC1507), cotton (281x3006, GHB119, GHB614, LL25, MON1445, MON15985, MON531, MON88913, T304), canola (T45, GT73, MS1, MS8, RF1, RF2, RF3, TOPAS 19/2), potato (EH92), rice (LLRice62) and sugar beet (H7-1).

[3] http://gmo-crl.jrc.ec.europa.eu/gmomethods/
[4] http://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/
[5] https://ec.europa.eu/jrc/en/scientific-tool/central-core-dna-sequence-information-system-ccsis

Determination of the limit of detection (LOD)

The limit of detection (LOD) was evaluated to assess the sensitivity of each assay under the SePSP conditions.

A droplet digital PCR (ddPCR) was first performed to assess the GM copy number of all CRM DNA samples. 2.5-25 ng DNA was quantified in 20 µl reaction mix containing 2X QX200™ ddPCR SuperMix for Probes (no dUTP) (Biorad 186-3023), 900 nM of each primer and 250 nM of the fluorescently-labelled probe. The thermocycling consisted of 10 minutes at 95°C for enzyme activation, 40 cycles of denaturation at 94°C for 30 seconds and annealing/elongation at 60°C for 1 minute, an enzyme deactivation step at 98°C for 10 minutes. Droplet PCRs were run on the Biorad QX200™ Droplet Digital PCR System and data analysed using the associated QuantaSoft Software.

Using the ddPCR-defined concentrations, two DNA solutions were prepared: one containing 6 copies/µl of all GM targets and one containing 6 copies/µl of the taxon target. From these dilution levels, the 4, 2, 1 and 0.2 copies/µl levels were obtained.

Each dilution level was analysed on 10 PCR plates that were pre-dispensed with a mixture of primers and probes using a robotic liquid handler (Starlet, Hamilton Robotics) and that contained a total amount of 30, 20, 10, 5 or 1 copie(s) of each target.

RTi-PCR reactions were run as described above (*Experimental verification*). The LOD was determined as the last dilution level at which no negative results were observed in the 10 replicates.

Results

The layout of the SePSP (Figure 1) includes the soybean taxon-specific assay (Lec) and 13 GM soy event-specific assays, thus permitting the detection of all GM soybean currently listed in the EU register of authorised GMOs. The assays are methods validated through collaborative studies by the EU Reference Laboratory for GM Food and Feed (EURL GMFF) and are available in the EU Database of Reference Methods for GMO Analysis (7).

In order to be used on PSPs, modifications of the PCR reaction conditions were needed in terms of oligonucleotide concentration, volume of reaction, and reaction mixture composition. Therefore, additional experiments were performed to confirm that the modifications did not affect the methods performance in terms of specificity and sensitivity (LOD).

Molecular specificity of the assays

In silico specificity

In silico specificity tests confirmed that no cross-reactivity was to be expected between the chosen assays and other GM events or plant genomes. The assay qt-eve-gm-005 (Event GTS40-3-2) showed a potential annealing with the soy GM event SYHT0H2 sequence but further investigations showed that the annealing concerned only the sequence of one primer of the assay.

The assays did not display false positive amplification when the simulation was performed against the genome sequences from soybean, *Brassica oleracea*, polish canola, sugar beet, cauliflower mosaic virus strains, cotton progenitor, potato, common rice, common wheat, maize and flax.

Experimental specificity

The molecular specificity was confirmed under the PSP reaction and cycling conditions for each method. Amplification was observed for all expected positive assays and no cross-reaction occurred (Table 3), confirming that the modifications introduced to the original PCR reaction conditions did not affect the performance of the methods in terms of specificity. Similarly, the assays for the soybean events DAS68416-4, MON87705, MON87708 and MON87769 did not show any false amplification signal when tested against GM events from the other crops (Table 4).

Determination of the limit of detection (LOD)

The sensitivity, in terms of limit of detection (LOD), was in line with the minimum performance requirements for analytical methods as defined by the EURL GMFF and the European Network of GMO Laboratories guidance document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (9) (Table 5).

Table 5. LOD expressed in copy number for the 14 assays implemented in the SePSP.

Method	LOD (cp)	Mean Cq ± SD
Lec	5	37.10 ± 0.67
A2704	5	35.90 ± 0.49
A5547	10	35.51 ± 0.65
CV127	5	37.73 ± 1.10
DAS68416	5	37.91 ± 0.46
DP305423	5	37.93 ± 0.45
DP356043	5	36.98 ± 1.06
FG72	5	36.23 ± 0.82
GTS40-3-2	5	36.57 ± 0.59
MON87701	5	37.07 ± 1.33
MON87705	5	37.27 ± 0.60
MON87708	5	37.50 ± 0.75
MON87769	5	37.79 ± 1.00
MON89788	20	38.49 ± 0.46

Table 3: Summary of the specificity assessment. Rows refer to the assays and columns to the samples. In green, positive results expected and observed; in blue, negative results expected and observed.

		Wt samples								GM soybean samples												
Target	Ref. GMOMETHODS	Maize	Soy	Canola	Cotton	Rice	Potato	SugarBeet	Wheat	A2704	A5547	CV127	DAS68416	DP305423	DP356043	FG72	GTS40-3-2	MON87701	MON87705	MON87708	MON87769	MON89788
Lec	qt-tax-gm-002	-	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
A2704	qt-eve-gm-004	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A5547	qt-eve-gm-007	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
CV127	qt-eve-gm-011	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
DAS68416	qt-eve-gm-013	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
DP305423	qt-eve-gm-008	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
DP356043	qt-eve-gm-009	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
FG72	qt-eve-gm-001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
GTS40-3-2	qt-eve-gm-005	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
MON87701	qt-eve-gm-010	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
MON87705	qt-eve-gm-003	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
MON87708	qt-eve-gm-012	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
MON87769	qt-eve-gm-002	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
MON89788	qt-eve-gm-006	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Table 4. Specificity assays summary for assays for the detection of DAS68416, MON87705, MON87708, MON87769. Rows refer to the assays and columns to the samples. In blue, negative results expected and observed.

		GM maize samples																			GM cotton samples									GM canola samples								other		
Target	Ref. GMOMETHODS	3272	5307	98140	Bt11	Bt176	DAS40278	DAS59122	GA21	LY038	MIR162	MIR604	MON810	MON863	MON87460	MON88017	MON89034	NK603	T25	TC1507	281x3006	GHB119	GHB614	LL25	MON1445	MON15985	MON531	MON88913	T304	T45	GT73	MS1	MS8	RF1	RF2	RF3	Topas 19/2	EH92	LLRice62	H7-1
DAS68416	qt-eve-gm-013	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MON87705	qt-eve-gm-003	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MON87708	qt-eve-gm-012	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MON87769	qt-eve-gm-002	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Conclusions

The modification brought to the methods in order to adapt them to the PSP conditions did not affect the specificity and sensitivity of the GM soybean event-specific assays. Indeed, *in silico* and experimental molecular specificity were confirmed for all methods and the modified assays displayed an adequate LOD.

In conclusion, the performance of all modified methods is in line with the minimum performance requirements as established by the EURL GMFF/ENGL (9) . Methods can therefore be used for the detection of single and stacked GM soybean events in food and feed samples under the PSP settings.

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Annex 1 – List of plant materials

Event	Provider	ID
A2704	AOCS	0707-B6
A5547	AOCS	0707-C4
CV127	AOCS	0911-C
DAS68416	IRMM	ERM-BF432c
DP305423	IRMM	ERM-0311-A
DP356043	IRMM	ERM-BF426c
FG72	AOCS	0610-A2
GTS40-3-2	IRMM	ERM-BF410dk
MON87701	AOCS	0809-A
MON87705	AOCS	0210-A
MON87708	AOCS	0311-A
MON87769	AOCS	0809-B
MON89788	AOCS	0906-B

Material	Provider	ID
Maize kernels	Retailer	
Soybean kernels	Retailer	
Rice grains	Retailer	
Oilseed rape	EURL	VL26/04
Cotton	IRMM	ERM-BF429A
Sugar beet	EURL	VL28/04
Potato	EURL	VL09/05
Wheat	Retailer	

DNA samples used for testing the specificity of soy GM events DAS-68416-14, MON87705, MON87708 and MON87769 were the same as the ones prepared for the work described in (3).

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