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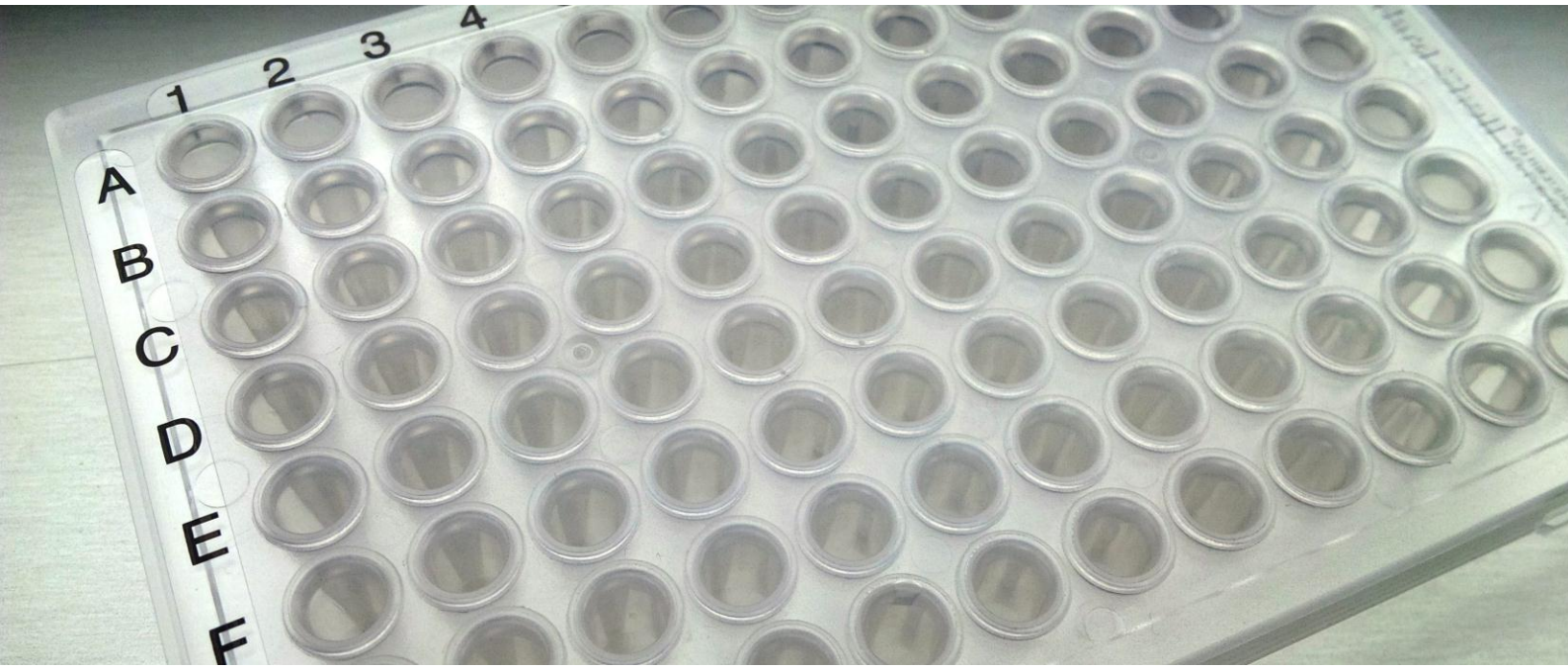

Development and Optimization of the GM Maize Event-Specific Pre-Spotted Plate (MePSP)

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Abstract

This report describes the development of the "GM maize event-specific pre-spotted plates (MePSP)" as a ready-to-use tool for GMO detection and identification.
This MePSP allows the detection of all GM maize events listed in the EU register as of April 2015. The plate includes a total of 20 assays, consisting of 19 event-specific assays and one taxon-specific assay. The assays are spotted in quadruplicate, allowing the simultaneous analysis of one sample 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 maize event-specific PSP (MePSP).

The MePSP allows the detection of all maize GM events listed in the EU register of authorized GMOs[1] as of April 2015 (Table 1). The plate includes a total of 20 assays, consisting of 19 assays for maize GM events and one assay for the maize reference gene. The assays are spotted in quadruplicate, allowing for the analysis of one sample in duplicate plus a negative and a positive control in parallel (Figure 1). The MePSP represents an updated version of the previous release (3) and it contains 1 additional test for the detection of the maize GM event 5307 (SYN-05307-1).

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.

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

Maize GM Events	Unique Identifier	Status
3272	SYN-E3272-5	LLP
5307	SYN-05307-1	LLP
98140	DP-098140-6	LLP
Bt11	SYN-BT011-1	authorized
Bt176	SYN-EV176-9	LLP
DAS40278	DAS-40278-9	LLP
DAS59122	DAS-59122-7	authorized
GA21	MON-00021-9	authorized
LY038	REN-00038-3	not authorized
MIR162	SYN-IR162-4	authorized
MIR604	SYN-IR604-5	authorized
MON810	MON-00810-6	authorized
MON863	MON-00863-5	authorized
MON87460	MON-87460-4	authorized
MON88017	MON-88017-3	authorized
MON89034	MON-89034-3	authorized
NK603	MON-00603-6	authorized
T25	ACS-ZM003-2	authorized
TC1507	DAS-01507-1	authorized

[1] http://ec.europa.eu/food/dyna/gm_register/index_en.cfm

Molecular specificity was reassessed experimentally by testing DNA from each GM maize event and the major crops for each method. Additionally, the method for the detection of the maize GM event 5307 was assessed against all authorized GMOs from soy, 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.

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

		1	2	3	4	5	6	7	8	9	10	11	12
Sample 1	A	HMG	3272	5307	98140	BT11	Bt176	DAS 40278	DAS 59122	GA21	LY038	MIR 162	MIR 604
	B	MON 810	MON 863	MON 87460	MON 88017	MON 89034	NK 603	T25	TC 1507				
Sample 2	C	HMG	3272	5307	98140	BT11	Bt176	DAS 40278	DAS 59122	GA21	LY038	MIR 162	MIR 604
	D	MON 810	MON 863	MON 87460	MON 88017	MON 89034	NK 603	T25	TC 1507				
Sample 3	E	HMG	3272	5307	98140	BT11	Bt176	DAS 40278	DAS 59122	GA21	LY038	MIR 162	MIR 604
	F	MON 810	MON 863	MON 87460	MON 88017	MON 89034	NK 603	T25	TC 1507				
Sample 4	G	HMG	3272	5307	98140	BT11	Bt176	DAS 40278	DAS 59122	GA21	LY038	MIR 162	MIR 604
	H	MON 810	MON 863	MON 87460	MON 88017	MON 89034	NK 603	T25	TC 1507				

Material, methods and experimental design

Sample preparation

DNA was extracted from certified reference materials (CRM) from the Institute for Reference Materials and Measurements (IRMM) and from the American Oil Chemists' Society (AOCS) using the Nucleospin Food kit (Macherey-Nagel GmbH, Düren, Germany), unless the CRM was already available as leaf tissue DNA (T25). The highest nominal level available was used. In case of lack of availability of CRM, control samples provided to the EURL GMFF by applicants were 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 the CTAB-based extraction procedure (soybean) (5).

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

[2] http://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/

Each sample was extracted in duplicate and quantified by fluorescence detection using PicoGreen® ds DNA 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 MePSP were selected among those listed in the EU Database of Reference Methods for GMO Analysis "GMOMETHODS"[3] (7) and are listed in Table 2.

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

Target	Method ID	Target	Method ID
HMG (maize taxon)	qt-tax-zm-002	Event MIR162	qt-eve-zm-022
Event 3272	qt-eve-zm-019	Event MIR604	qt-eve-zm-013
Event 5307	qt-eve-zm-002	Event MON810	qt-eve-zm-020
Event 98140	qt-eve-zm-021	Event MON863	qt-eve-zm-009
Event Bt11	qt-eve-zm-015	Event MON87460	qt-eve-zm-005
Event Bt176	qt-eve-zm-023	Event MON88017	qt-eve-zm-016
Event DAS40278	qt-eve-zm-004	Event MON89034	qt-eve-zm-018
Event DAS59122	qt-eve-zm-012	Event NK603	qt-eve-zm-008
Event GA21	qt-eve-zm-014	Event T25	qt-eve-zm-011
Event LY038	qt-eve-zm-017	Event TC1507	qt-eve-zm-010

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 19 GM maize 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 the SDS 2.4 software.

Additionally, the assay for the GM event 5307 was tested against all available GMOs from soybean (A2704, A5547, CV127, DAS 68416, DP305423, DP356043, FG72, GTS 40-3-2, MON 87701, MON 87705, MON 87708, MON 87769, MON 89788), cotton (281x3006, GHB119, GHB614, LL25, M1445, M15985, M531, M88913, 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 MePSP conditions.

A droplet digital PCR (ddPCR) was first performed to assess the GM copy number of all CRM DNA samples. DNA samples were diluted to 2.5-25 ng of 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 final layout of the maize event-specific PSP (Figure 1) includes the maize taxon-specific assay (hmg) and 19 maize event-specific assays, thus permitting the detection of all maize GMOs currently listed in the EU register of authorized GMOs. The assays are methods validated through collaborative studies by the EURL GMFF and are available in the GMOMETHODS database (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-zm-020 (Event MON810) showed a potential annealing with the soy GM event SYHT0H2 sequence but the experimental assessment did not reveal any signal.
The assays did not display false positive amplification when the simulation was performed against the genome sequence from soybean, *Brassica oleracea*, polish canola, sugar beet, cauliflower mosaic virus strains, cotton progenitor, potato, common rice, common wheat and maize. Four

assays yielded false simulated amplicons due to the recognition of one primer of endogenous sequences but the probes from these assays did not show any potential match.

Experimental specificity

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 assay for the maize event 5307 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 (ENGL) guidance document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (9) (Table 5).

Table 5: LOD expressed in copy number of the 20 assays implemented in the MePSP and mean Cq values.

Method	LOD (cp)	Mean Cq ± SD	Method	LOD (cp)	Mean Cq ± SD
HMG	10	37.07 ± 0.85	MIR 162	10	36.57 ± 0.69
3272	5	36.52 ± 1.11	MIR 604	5	36.14 ± 0.77
5307	5	37.88 ± 0.98	MON 810	10	36.40 ± 0.74
98140	5	37.91 ± 1.16	MON 863	10	36.17 ± 0.74
Bt11	5	37.47 ± 0.49	MON 87460	10	35.99 ± 0.75
Bt176	5	36.66 ± 0.83	MON 88017	5	36.30 ± 1.09
DAS 40278	5	37.27 ± 0.99	MON 89034	10	35.38 ± 2.71
DAS 59122	5	36.13 ± 0.84	NK 603	10	37.17 ± 0.82
GA21	5	37.60 ± 1.20	T25	20	35.38 ± 1.04
LY038	5	35.94 ± 1.41	TC1507	5	36.63 ± 1.15

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 maize samples																		
Target	Ref. GMO Database	Maize	Soy	Canola	Cotton	Rice	Potato	Sugar Beet	Wheat	3272	5307	98140	Bt11	Bt176	DAS 40278	DAS 59122	GA21	LY038	MIR 162	MIR 604	MON 810	MON 863	MON 87460	MON 88017	MON 89034	NK 603	T25	TC 1507
HMG	qt-tax-zm-002	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3272	qt-eve-zm-019	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5307	qt-eve-zm-002	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
98140	qt-eve-zm-021	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bt11	qt-eve-zm-015	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bt176	qt-eve-zm-023	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DAS 40278	qt-eve-zm-004	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
DAS 59122	qt-eve-zm-012	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
GA21	qt-eve-zm-014	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
LY038	qt-eve-zm-017	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
MIR 162	qt-eve-zm-022	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
MIR 604	qt-eve-zm-013	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
MON 810	qt-eve-zm-020	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
MON 863	qt-eve-zm-009	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
MON 87460	qt-eve-zm-005	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
MON 88017	qt-eve-zm-016	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
MON 89034	qt-eve-zm-018	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
NK 603	qt-eve-zm-008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
T25	qt-eve-zm-011	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
TC 1507	qt-eve-zm-010	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Table 4. Specificity assays summary for assays for the detection of E5307. Rows refer to the assays and columns to the samples. In blue, negative results expected and observed.

		GM soy samples													GM cotton samples									GM canola samples								other		
Target	Ref. GMOMETHODS	A2704	A5547	CV127	DAS68416	DP305423	DP356043	FG72	GTS40-3-2	MON87701	MON87705	MON87708	MON87769	MON89788	281x3006	GHB119	GHB614	LL25	MON1445	MON15985	MON531	MON88913	T304	T45	GT73	MS1	MS8	RF1	RF2	RF3	Topas 19/2	EH92	LLRice62	H7-1
5307	qt-eve-zm-002	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Conclusions

The modification brought to the methods in order to adapt them to the plate conditions did not affect the specificity and sensitivity of the GM maize 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 maize events in food and feed samples under the PSP settings.

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Annex 1 – List of plant materials

Event	Provider	ID
5307 (SYN-05307-1)	EURL	EURL-VL-07/11
DAS 59122 (DAS-59122-7)	IRMM	ERM-BF424D
98140 (DP-098140-6)	IRMM	ERM-BF427D
DAS 40278 (DAS-40278-9)	IRMM	ERM-BF433D
MON87460 (MON-87460-4)	AOCS	0709-A
MON88017 (MON-88017-3)	AOCS	0406-D
MON89034 (MON-89034-3)	AOCS	0906-E
LY038 (REN-00038-3)	EURL	CRL-VL-01/06
MIR162 (SYN-IR162-4)	AOCS	1208-A
Bt176 (SYN-EV176-9)	IRMM	ERM-BF411F
NK603 (MON-00603-6)	IRMM	ERM-BF415F
MIR604 (SYN-IR604-5)	IRMM	ERM-BF423D
MON810 (MON-00810-6)	IRMM	BF413GK
MON863 (MON-00863-5)	IRMM	ERM-BF416D
TC1507 (DAS-01507-1)	IRMM	ERM-BF418D
Bt11 (SYN-BT011-1)	IRMM	ERM-BF412F
T25 (ACS-ZM003-2)	AOCS	0306-H4
GA21 (MON-00021-9)	IRMM	ERM-BF414f
3272 (SYN-E3272-5)	IRMM	ERM-BF420C

Material	Provider	ID
Maize kernels	Retailer	
Soy bean kernels	Retailer	
Rice grains	Retailer	
Oilseed rape	EURL	CRL-VL-26/04
Cotton	IRMM	ERM-BF429A
Sugar beet	EURL	CRL-VL-28/04
Potato	EURL	CRL-VL-09/05
Wheat	Retailer	

DNA samples used for testing the specificity of the maize GM event 5307 assay were the ones prepared for the work described in (3) .

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