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Development of a ready-to-use, multi-target screening pre-spotted plate (sPSP) for GMO detection.

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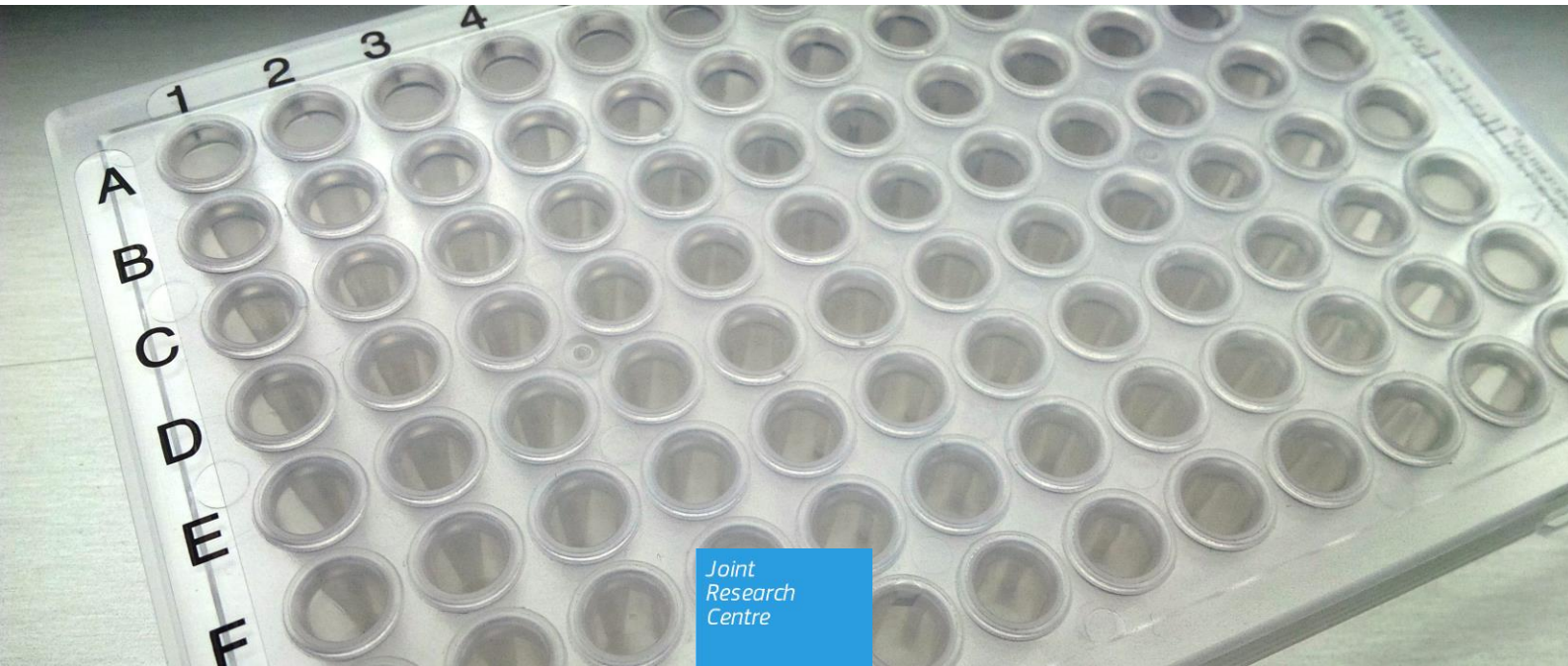

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Abstract

In this report, we describe the development of the screening pre-spotted plate (sPSP) as a ready-to-use and multi-target tool for GMO screening. This new sPSP allows the detection of all but one of the authorized GM events as listed in the EU register in one single experiment, thus providing a fast screening system that reduces both workload and starting material. To achieve this goal, a combination of 7 taxon-specific methods, 6 element-specific methods and 3 event-specific methods was carefully selected from the available validated methods and assessed for specificity and sensitivity under the sPSP conditions. With a layout of only 16 methods, repeated 6 times throughout the plate, this sPSP can accommodate the analysis of two samples in duplicate as well as a positive and negative control. The sPSP offers several advantages when compared to the previously used event-specific pre-spotted plates (ePSP). First, only 16 methods are needed for a complete screening, as opposed to 48 for the ePSP. In the laboratory, this translates into the possibility to include positive and negative control in the same plate and into a reduced need of starting DNA material. Additionally, the reaction volume is reduced by half, which translates into a significant reduction in reagent cost. The sPSP constitutes a novel and fast screening approach that could potentially be adopted by the GMO reference laboratories in the EU. Upon reception and quality control of the first batch this January 2014, the sPSP will be distributed to selected National Reference Laboratories for evaluation of their performance and practicability on a routine testing basis. Adoption of this screening tool by the EU reference laboratories would constitute another step toward the harmonization of GMO detection approaches in the EU.

1. Introduction

In the European legal context, one of the missions of the European Commission Joint Research Centre (JRC) is to work in close collaboration with the European Network of GMO Laboratories (ENGL) in the harmonization of GMO testing approaches, including GMO detection techniques [1]. Working towards this aim, the first application of pre-spotted plates was developed by the JRC, with a pilot application published in Querci *et al.* [2].

Pre-spotted plates (PSP) consist in ready-to-use real-time (RTi) PCR plates whose wells have been spotted with specific primers and probes targeting a sequence of interest. They present the advantage of being time- and cost-efficient while offering a straightforward tool to face high-throughput testing needs. The pilot application included 48 TaqMan® RTi-PCR assays covering 7 plants species and 39 GM events, most of which derived from the methods submitted by applicants seeking commercial approval of GMO and validated by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF). This new ready-to-use and multi-target tool for GMO detection proved to be performing well: a collaborative trial involving 389 samples, with a broad coverage of food and feed matrices, and a total of 31 ENGL laboratories demonstrated the feasibility of using PSPs for GMO detection (2008, data not published). Furthermore, Kluga *et al.* [3] demonstrated that the detection of low amount of GMOs was possible, therefore confirming the applicability of the PSPs to the processed food matrices typically involved in laboratory testing.

A survey conducted within the ENGL members raised several drawbacks in the use of the PSP for GMO detection. Major drawbacks include for example the lack of flexibility in testing scheme and the necessity to obtain high quality DNA in considerable amount. Also, the increasing number of GMO introduced in the market calls for periodical updates of such a support, and, as a consequence, introduces a challenge of space limitation. To cope with these problems and to provide the GMO community with tools that better meet their expectations, the MBG unit undertook the development of a novel GMO screening 96-well PSP. This new tool, the screening pre-spotted plate (sPSP) offers the possibility to screen for the presence of all possible GMOs listed in the EU register (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm) at the exception of one (Table 1), combining results from only 6 element-specific, 7 taxon-specific and 3 event-specific methods (Table 2) and thus decreasing both the workload and the amount of DNA material needed. Each sPSP allows the analysis of two samples in duplicate, as well as of one negative and one positive control and thus may represents an advantage over the event-specific pre-spotted plate (ePSP, [4]), on which controls cannot be analysed due to lack of space.

All methods spotted on the sPSP are methods that were validated by the EURL GMFF, at the exception of one (cry1Ab – see Table 2). Those methods were re-evaluated in-house according to the needs of the PSP development, *i.e.* uniformity of the different amplification protocols throughout the plate and reduced reaction volume. Specificity and limit of detection (LOD) were hence re-assessed experimentally by testing each GM event for each method while implementing the different changes: final reaction volume of 25 µL instead of 50 µL, use of TaqMan® Universal Master Mix for all methods, use of FAM-labelled probes with TAMRA quencher, and use of a standard cycling program. Additionally, the molecular specificity of the assays was assessed *in silico* using the "JRC GMO-Matrix" tool developed by the Molecular Biology and Genomics Unit, which simulates the PCR amplification against all GMO sequences available in restricted Central Core Sequence Information System (CCSIS) database.

Table 1. List of authorized GM events (not including stacked events) as of November 2013 (EU register) detected by the sPSP. Only GHB614 is not detected by the sPSP. LLP1: low level presence of 0.1% tolerated in feed; LLP2: low level presence of 0.9% tolerated in food and feed

	Event	Taxon	Status
Maize	GMO event B176 (SYN-EV176-9)	Maize	LLP 1
	GMO Event 1507 Maize (DAS-01507-1)	Maize	authorized
	GMO Event 3272 Maize (SYN-E3272-5)	Maize	LLP 1
	GMO Event GA21 Maize (MON-00021-9)	Maize	authorized
	GMO Event 59122 Maize (DAS-59122-7)	Maize	authorized
	GMO Event Bt11 Maize (SYN-BT011-1)	Maize	authorized
	GMO Event 98140 Maize (DP-098140-6)	Maize	LLP 1
	GMO Event MIR604 Maize (SYN-IR604-5)	Maize	authorized
	GMO Event MIR162 Maize (SYN-IR162-4)	Maize	authorized
	GMO Event MON89034 Maize (MON-89034-3)	Maize	authorized
	GMO Event MON87460 Maize (MON-87460-4)	Maize	LLP 1
	GMO Event MON810 Maize (MON-00810-6)	Maize	authorized
	GMO Event NK603 Maize (MON-00603-6)	Maize	authorized
	GMO Event MON88017 Maize (MON-88017-3)	Maize	authorized
	GMO Event T25 Maize (ACS-ZM003-2)	Maize	authorized
	GMO Event MON863 Maize (MON-00863-5)	Maize	authorized
	GMO Event DAS-40278-9 Maize (DAS-40278-9)	Maize	LLP 1
Soybean	GMO Event MON87705 Soybean (MON-87705-6)	Soybean	authorized
	GMO Event MON87701 Soybean (MON-87701-2)	Soybean	authorized
	GMO Event FG72 Soybean (MST-FG072-3)	Soybean	LLP 1
	GMO Event MON89788 Soybean (MON-89788-1)	Soybean	authorized
	GMO Event DAS-68416-4 Soybean (DAS-68416-4)	Soybean	authorized
	GMO Event A5547-127 Soybean (ACS-GM006-4)	Soybean	authorized
	GMO Event 356043 Soybean (DP-356043-5)	Soybean	authorized
	GMO Event A2704-12 Soybean (ACS-GM005-3)	Soybean	authorized
	GMO Event 40-3-2 Roundup Ready Soybean (MON-04032-6)	Soybean	authorized
	GMO Event CV127 Soybean (BPS-CV127-9)	Soybean	LLP 1
	GMO Event 305423 Soybean (DP-305423-1)	Soybean	LLP 1
Rapeseed	GMO Event GT73 Rapeseed (MON-00073-7)	Rapeseed	authorized
	GMO Event Rf1 Rapeseed (ACS-BN001-4)	Rapeseed	LLP 2
	GMO Event Topas 19/2 Rapeseed (ACS-BN007-1)	Rapeseed	LLP 2
	GMO Event Ms1 Rapeseed (ACS-BN004-7)	Rapeseed	LLP 2
	GMO Event Rf2 Rapeseed (ACS-BN002-5)	Rapeseed	LLP 2
	GMO Event T45 Rapeseed (ACS-BN008-2)	Rapeseed	authorized
	GMO Event MS8 Rapeseed (ACS-BN005-8)	Rapeseed	authorized
	GMO Event RF3 Rapeseed (ACS-BN003-6)	Rapeseed	authorized
Cotton	GMO Event MON88913 Cotton (MON-88913-8)	Cotton	LLP 1
	GMO Event MON15985 Cotton (MON-15985-7)	Cotton	LLP 1
	GMO Event GHB119 Cotton (BCS-GH005-8)	Cotton	LLP 1
	GMO Event MON1445 Cotton (MON-01445-2)	Cotton	authorized
	GMO Event LLCotton25 Cotton (ACS-GH001-3)	Cotton	authorized
	GMO Event MON531 Cotton (MON-00531-6)	Cotton	authorized
	GMO Event 281-24-236 Cotton (DAS-24236-5)	Cotton	authorized
	GMO Event 3006-210-23 (DAS-21023-5)	Cotton	authorized
	GMO Event T304-40 Cotton (BCS-GH004-7)	Cotton	LLP 1
	GMO Event GHB614 Cotton (BCS-GH002-5)	Cotton	authorized
Potato	GMO Event EH92-527-1 Potato (BPS-25271-9)	Potato	authorized
Rice	GMO Event LLRICE62 Rice (ACS-OS002-5)	Rice	LLP 1
Sugar beet	GMO Event H7-1 Sugar Beet (KM-000H71-4)	Sugarbeet	authorized

Following RTi-PCR, the analysis of the pattern of positive and negative results returned by the 16 reactions of sPSP follows a decision support system (DSS) that is based on a matrix approach (Annex 1), such that the occurrence of a negative test excludes the presence of the pertaining event. All events remaining after the negative selection are possibly contained in the analysed sample and are to be investigated using a limited number of event-specific methods. The matrix approach was implemented in a web-based tool, the PSP JRC GMO-Matrix [9], that allows users to quickly interpret results.

Adoption of the sPSP by the GMO community as a routine GMO screening tool would imply an increased harmonization of GMO analysis approaches at the EU level.

2. Material, methods and experimental design

2.1 Sample preparation

Certified reference materials (CRM) from the Institute for Reference Materials and Measurements (IRMM) and from the American Oil Chemists' Society (AOCS) were used in this study. Unless not available, the CRM at 1% nominal level was used. In the case another nominal level was used, samples were diluted to 1% GM content using non-GM material. The complete list of CRMs is given in Annex 2.

DNA from maize (N=15) and soybean (N=9) CRMs was extracted according to EURL GMFF standard operating procedures using the Nucleospin Food kit and CTAB extraction protocol, respectively, unless CRMs were provided as leaf tissue DNA (N=1 for maize; N=3 for soybean). DNA from cotton CRMs (N=7) was extracted using the Foodproof GMO sample preparation kit from Biotecon Diagnostics according to the manufacturer's instructions, at the exception of 2 samples provided as leaf tissue DNA. Rapeseed CRMs (N=8) were mostly provided as leaf tissue DNA and hence did not require extraction, at the exception of one for which the EURL GMFF CTAB extraction protocol was used. Rice (N=1) and sugar beet (N=1) CRMs were extracted using a modified CTAB protocol to increase yield. Each sample was extracted in duplicate.

The DNA was quantified by fluorescence detection using PicoGreen® ds DNA quantitation kit (Molecular Probes) and was examined on agarose gel to verify its integrity. Inhibition runs were performed for all samples to ensure that no PCR inhibitor was present in the extracts. The above activities were performed following EURL GMFF standard operating procedures [5].

All DNA samples were then diluted to a concentration of 20 ng/µL for use in the molecular specificity tests as described in paragraph 2.3, and stored at -20°C until needed.

2.2 Method selection

sPSP were developed with the aim of achieving a rapid detection of all possible GMOs authorized in the EU. As of November 2013, this concerned seven plant species and a total of 79 GM events. The selection of the methods had to fulfil this requirement while maintaining the number of assays to be performed as low as possible to reduce both workload and starting material.

The seven selected plant-specific assays correspond to the reference gene assays validated by the EURL GMFF and already implemented in the previous and current ePSP versions (Table 2).

The element-specific assays were selected primarily amongst the existing validated methods listed in the EU Database of Reference Methods for GMO Analysis (http://gmo-crl.jrc.ec.europa.eu/gmomethods/) so that the combination of a minimum number of assays would detect a maximum number of authorized GM events. A total of 6 element-specific methods were chosen (Table 2), allowing the detection of all but 4 of the listed authorized GMO. Three event-specific methods were added (Table 2), bringing to 16 the

total number of reactions needed to analyse one sample (hence 2 columns on a plate). Only one GM event was therefore not included, cotton GHB614, for practical reason. If needed, this event, which is however rarely screened for according to a survey conducted within the ENGL, will require the use of an independent event-specific method to be detected.

Table 2. Methods selected for the development of the screening pre-spotted plate

	Target	Reference*
Taxon-specific	hmg (maize)	QT-TAX-ZM-002
	lec (soy)	QT-TAX-GM-002
	cruA (rapeseed)	QT-TAX-BN-012**
	sah7 (cotton)	QT-TAX-GH-016
	ugp (potato)	QT-TAX-ST-010
	pld (rice)	QT-TAX-OS-017
	gs (sugar beet)	QT-TAX-BV-013
Element-specific	p35S	QT-ELE-00-004
	tNos	QL-ELE-00-013
	CTP2-Cp4EPSPS	QL-CON-00-008
	pat	QT-ELE-00-002
	bar	QL-ELE-00-014
	cryIAb**	http://www.bvl.bund.de/
Event-specific	DAS40278 (maize)	QT-EVE-ZM-004
	CV127 (soybean)	QT-EVE-GM-011
	DP-305423	QT-EVE-GM-008

* Method available at http://gmo-crl.jrc.ec.europa.eu/gmomethods/

** Sampling and analysis guidelines for the detection of genetically modified flax; http://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/Fachmeldungen/GVO_Flax.pdf?__blob=publicationFile

2.3 Molecular specificity of the chosen methods

2.3.1 General

Given that the development of the pre-spotted plates required adaptations of the selected validated methods, the molecular specificity of these assays had to be re-assessed by testing all CRM samples against all methods in real-time PCR experiments. The adaptations that were required to achieve method uniformity throughout the plate were as follow: reaction volume decreased from 50 to 25 µL, modification of the probe labelling from VIC to FAM for the CruA method, use of the TaqMan® Universal Master Mix for all method, and use of a standard cycling protocol for all methods. While using real-time techniques for detecting the presence of GMO, it should be noted that the use of the PSP is exclusively of a qualitative nature.

2.3.2 In silico specificity analyses

The molecular specificity of the 16 assays was assessed in silico using a bioinformatics tool implemented in the EURL GMFF. The ePCR tool [6] was used to perform simulation of PCR amplifications using primers and probes from existing methods against all the GMO sequences available in the EURL GMFF restricted database, the Central Core Sequence Information System (CCSIS). The analysis results in a matrix where it is possible to visualize whether given methods show perfect matches, mismatches, or no match at all against any GM sequence.

2.3.3 Experimental verification, plate set-up and real-time PCR reactions

The experimental conditions (reaction volume and reagent concentrations) foreseen to use in the pre-spotted plates were simulated in the specificity analyses. For each extract (two per CRM sample), a 25 µL amplification mix containing 100 ng of 1% CRM DNA, 2x TaqMan® Universal PCR Master Mix (Life Technologies), 600 nM of each primer and 200 nM of the FAM/TAMRA-labelled probe for the tested method (Table 3) was prepared and loaded onto a 96-well plate. This was performed for the 16 methods. The thermocycling consisted of a 2 minutes UNG step at 50°C and a 10 minutes denaturation step at 95°C, followed by 45 cycles of denaturation step at 95°C and an annealing/elongation step of 1 minute at 60°C. RTi-PCR runs were performed using the 7900HT Fast Real-Time PCR System and the 7500 Real Time PCR System (Life Technologies) and data analysed using the SDS 2.4 and 7500 software v2.0.6, respectively.

Table 3. Methods implemented in the sPSP (*VIC labelling replaced by FAM labelling).

	Target	Primers/Probe	Sequence	Reference
Taxon-specific	hmg (maize)	ZM1-F	5'-TTGGACTAGAAATCTCGTGCTGA-3'	QT-TAX-ZM-002
		ZM1-R	5'-GCTACATAGGGAGCCTTGTCCT-3'	
		ZM1-P	5'-FAM-CAATCCACACAAACGCACGCGTA-TAMRA-3'	
	lec (soy)	lec F	5'-CCAGCTTCGCCGCTTCCTTC-3'	QT-TAX-GM-002
		lec R	5'-GAAGGCAAGCCCATCTGCAAGCC-3'	
		lec P	5'-FAM-CTTCACCTTCTATGCCCCTGACAC-TAMRA-3'	
	cruA (rapeseed)	CruA-F	5'-GGCCAGGGTTTCCGTGAT-3'	QT-TAX-BN-012*
		CruAR	5'-CCGTCGTTGTAGAACCATTGG-3'	
		TM003-P	5'-FAM-AGTCCTTATGTGCTCCACTTTCTGGTGCA-TAMRA-3'	
	sah7 (cotton)	SAH7-uni-f1	5'-AGTTTGTAGGTTTTGATGTTACATTGAG-3'	QT-TAX-GH-016
		SAH7-uni-r1	5'-GCATCTTTGAACCGCCTACTG-3'	
		SAH7-uni-s1	5'-FAM-AAACATAAAATAATGGGAACAACCATGACATGT-TAMRA-3'	
	ugp (potato)	UGP-af7	5'-GGACATGTGAAGAGACGGAGC-3'	QT-TAX-ST-010
		UGP-ar8	5'-CCTACCTCTACCCCTCCGC-3'	
		UGP-sf1	5'-FAM-CTACCACCATTACCTCGCACCTCCTCA-TAMRA-3'	
	pld (rice)	KVM159	5'- TGGTGAGCGTTTTGCAGTCT-3'	QT-TAX-OS-017
		KVM160	5'- CTG ATC CAC TAG CAG GAG GTC C-3'	
		TM013	FAM-5'-TGTTGTGCTGCCAATGTGGCCTG -TAMRA-3'	
	gs (sugar beet)	GluA3-F	5'-GACCTCCATATTACTGAAAGGAAG-3'	QT-TAX-BV-013
		GluA3-R	5'-GAGTAATTGCTCCATCCTGTTCA-3'	
		GluD1	5'-FAM-CTACGAAGTTTAAAGTATGTGCCGCTC-TAMRA-3'	
Element-specific	p35S	35S-FTM	5'-GCCTCTGCCGACAGTGGT-3'	QT-ELE-00-004
		35S-RTM (2)	5'-AAGACGTGGTTGGAACGTCTTC-3'	
		35S-TMP	5'-FAM-CAAAGATGGACCCCCACCCACG-TAMRA-3'	
	tNos	180-F	5'-CATGTAATGCATGACGTTATTTATG-3'	QL-ELE-00-013
		180-R	5'-TTGTTTTCTATCGCGTATTAAATGT-3'	
		TM-180YY	5'-FAM-ATGGGTTTTTATGATTAGAGTCCCGCAA-TAMRA-3'	
	CTP2-Cp4EPSPS	GT73-TmF	5'-GGGATGACGTTAATTGGCTCTG-3'	QL-CON-00-008
		GT73-TmR	5'-GGCTGCTTGCACCGTGAAG-3'	
		GT73-TmP	5'-FAM-CACGCCGTGGAAACAGAAGACATGACC-TAMRA-3'	
	pat	KVM-5	5'-TTGAGGGTGTTGTGGCTGGTA-3'	QT-ELE-00-002
		KVM-6	5'-TGTCCAATCGTAAGCGTTCCT-3'	
		TaqMan Pat1	5'-FAM-CTTCCAGGGCCCAGCGTAAGCA-TAMRA-3'	
	bar	RapB-F1	5'-ACAAGCACGGTCAACTTCC-3'	QL-ELE-00-014
		RapB-R1	5'-GAGGTCGTCCGTCCACTC-3'	
		RapB-S1	5'-FAM-TACCGAGCCGCAGGAACC-TAMRA-3'	
	cryIAb	Bt-F1(mod)	5'-GAGGAAATGCGTATTCAATTCAAC-3'	www.bvl.bund.de
		Bt-R	5'-TTCTGGACTGCGAACAATGG-3'	
		Bt-P2	5'-FAM-ACATGAACAGCGCCTTGACCACAGC-TAMRA -3'	
Event-specific	DAS40278	DAS-40278-9_5'-f1	5'-CACGAACCATTGAGTTACAATC-3'	QT-EVE-ZM-004
		DAS-40278-9_5'-r3	5'-TGGTTCATTGTATTCTGGCTTTG-3'	
		DAS-40278-9_5'-S2	5'-FAM-CGTAGCTAACCTTCATTGTATTCCG-TAMRA-3'	
	CV127	SE-127-f4	5'-AACAGAAGTTTCCGTTGAGCTTTAAGAC-3'	QT-EVE-GM-011
		SE-127-r2	5'-CATTCGTAGCTCGGATCGTGTAC-3'	
		SE-127-p3	5'-FAM-TTTGGGGAAGCTGTCCCATGCCC-TAMRA-3'	
	DP-305423	DP305-f1	5'-CGTGTTCTCTTTTTGGCTAGC-3'	QT-EVE-GM-008
		DP305-r5	5'-GTGACCAATGAATACATAACACAAACTA-3'	
		DP305-p	5'-FAM-TGACACAAATGATTTTCATACAAAAGTCGAGA-TAMRA-3'	

2.4 Determination of the limit of detection (LOD_{10})

The sensitivity of each taxon-, element- and event-specific method was re-assessed using the sPSP reaction conditions as described in 2.3.3. DNA samples containing the pertaining genetic elements were prepared at the dilutions of 20, 10, 5, 2.5, and 1.25, 0.625, and 0.312 copies of transgene per reaction by mixing DNA from GM events with background lambda DNA for a total of 100 ng DNA per reaction. Each GM dilution level was then analysed in 10 replicates by RTi-PCR and the LOD was determined as the last dilution level at which no negative result is observed.

3. Results

3.1 Molecular specificity of the chosen methods

The molecular specificity of each method was confirmed under the new reaction and cycling conditions. Amplification was observed for all expected positive assays (Figure 1). In the few cases in which amplification was observed in assays for which a negative result was expected, further investigation was conducted in order to verify if this was due to unspecific reaction or to (sample) contamination and, if so, to identify the source of the contamination. A summary of all the observed cases of false positives is contained in Table 4. These few cases were resolved by repeating the assays using a different DNA sample and, if still positive, by submitting the problematic samples to a screening with the sPSP in order to identify all contaminants. Wild-type samples used to dilute the CRMs were also checked for contamination using the ePSP. Additionally, because IRMM CRMs are known to contain contaminants in certain cases [7], the unexpected results were cross-referenced with the IRMM records. From those investigations, it was concluded that the false positive results arose from CRM or wild-type sample contaminations and not from a lack of specificity in the chosen method.

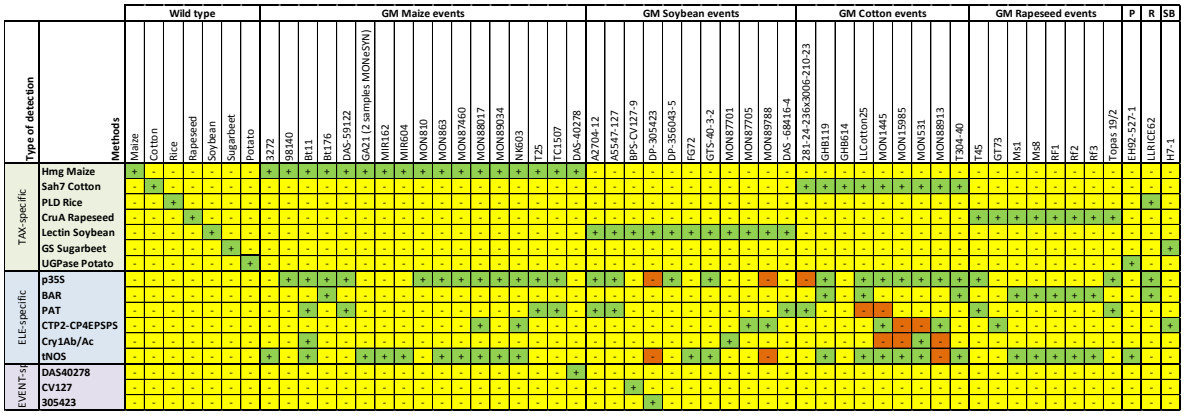

Figure 1. Summary of the specificity assays. In green, positive results expected and observed; in yellow, negative results expected and observed; in orange, solved unexpected positive results.

3.2 Determination of the limit of detection (LOD)

In all cases, the LOD of the assays (Table 5) was in line with the MPR for analytical methods as defined by the EURL GMFF and the ENGL guidance document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing", stating that for individual modules the LOD should be < 25 copies [8]. In the case of the potato, an LOD inferior to the highest dilution level was found due to the fact that the reference gene is present in multiple copies in the genome. As different methods showed different LOD, a sensitivity of 10 copies was established for the system as a whole.

Table 4. Resolved cases of false positive during specificity assays

Sample	False positive result	Explanation
1. Soybean DP-305423	p35S, tNOS	ePSP showed contamination with soy 40-3-2
2. Soybean M89788	p35S, tNOS	Use of event-specific method showed contamination of reference material with soy 40-3-2 (contains both events)
3. Cotton 281-24-236 x 3006-210-23	p35S	ePSP showed contamination with soy 40-3-2 (which contains p35s), also, previous report of p35s contamination of IRMM sample is found
4. LLcotton25	PAT	Wild-type sample used for dilution was found
5. Cotton M1445	PAT, Cry1Ab/Ac	Wild-type sample used for dilution was found to contain cotton 281-3006 (contains PAT); Use of event-specific method showed contamination of reference material with M531 (contains Cry1Ab/Ac)
6. Cotton M15985	CTP2-CP4EPSPS, Cry1Ab/Ac	ePSP showed contamination with cotton M1445 and M531 (contains CTP2-CP4EPSPS and Cry1Ab, respectively)
7. Cotton MON88913	Cry1Ab/Ac, tNOS	Use of event-specific method showed contamination of reference material with M531 (contains both events)
8. Cotton M531	CTP2-CP4EPSPS	ePSP showed contamination with cotton M1445 (contains CTP2-CP4EPSPS)

Table 5. LOD evaluation under PSP amplification conditions

Method	Sample used*	LOD (copy number)
hmg	MON810	2.5
lec	MON-04032-6	2.5
cruA	Rf2	2.5
sah7	GHB119	1.25
ugp	wild type potato	< 0.312
pld	wild type rice	10
gs	wild type sugar beet	1.25
p35s	Bt11	5
tNOS	Bt11	5
cry1Ab	Bt11	10
bar	Rf2	10
ctp2-cp4epsps	NK603	2.5
pat	Bt11	2.5
DAS-40278	DAS-40278	5
CV127	CV127	5
DP-305423	DP-305423	5

* Refer to Annex 2 for CRM details

Note that after PSP production, the LOD_{10} was assessed again on the sPSP themselves. Sample preparation using droplet digital PCR and results can be found in Annex 3. The LOD of all assays were in line with the MPR.

3.3 Plate layout

Figure 2 displays the plate layout that was adopted and ordered for production (Eurogentec, Liège, BE). All 16 methods are contained in two columns and the layout consists of six repeats of the 16 assays. The plate thus allows the analysis of two samples in duplicate plus one negative and one positive control.

	1	2	3	4	5	6	7	8	9	10	11	12
A	HMG	p35S	HMG	p35S	HMG	p35S	HMG	p35S	HMG	p35S	HMG	p35S
B	Lec	bar	Lec	bar	Lec	bar	Lec	bar	Lec	bar	Lec	bar
C	CruA	pat	CruA	pat	CruA	pat	CruA	pat	CruA	pat	CruA	pat
D	Sah7	CTP2-EPSPS	Sah7	CTP2-EPSPS	Sah7	CTP2-EPSPS	Sah7	CTP2-EPSPS	Sah7	CTP2-EPSPS	Sah7	CTP2-EPSPS
E	UGP	CrylAb	UGP	CrylAb	UGP	CrylAb	UGP	CrylAb	UGP	CrylAb	UGP	CrylAb
F	Pld	tNOS	Pld	tNOS	Pld	tNOS	Pld	tNOS	Pld	tNOS	Pld	tNOS
G	Gs	CV127	Gs	CV127	Gs	CV127	Gs	CV127	Gs	CV127	Gs	CV127
H	DAS40278	305423	DAS40278	305423	DAS40278	305423	DAS40278	305423	DAS40278	305423	DAS40278	305423

Figure 2. Layout of the sPSP. In blue, the taxon-specific methods; in purple, the element-specific methods; in yellow and green, the event-specific methods for maize and soybean, respectively.

3.4 Decision Support System (PSP JRC GMO-Matrix)

The EURL GMMF developed a user-friendly DSS associated to the screening PSP based on a matrix approach. The DSS consists of a specific interface for the JRC GMO-Matrix web application (http://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/). In short, a pre-selected array of assays, reflecting the methods used for the screening PSP, was uploaded on a dedicated webpage and then used for the interpretation of screening PSP results. By filling in the pattern of positive/negative results obtained, the algorithm provides the list of GM events potentially present in the analysed sample (see Event Finder Functionality in (9)).

4. Conclusions

This work describes the development, using a combination of laboratory and bioinformatics approaches, of the screening PSP. This multi-target system allows the detection of all but one EU authorized GMO in a single experiment through the combination of 16 assays. Assays selected among the EU reference methods were successfully adapted for use on PSP with no change in method specificity and sensitivity (LOD). To facilitate results' interpretation, the screening PSP can be combined with a web-based DSS developed in our laboratory (PSP JRC GMO-Matrix) that provides an exhaustive list of GM events to be taken into consideration for the identification phase based on the screening results obtained.

Such strategy can provide clear advantages for laboratories in terms of analysis time, costs and increased laboratory capacity. Indeed, considering that laboratories often screen for a more limited array of GM targets, the adoption of screening PSP would

improve the likelihood to detect all GMO present while, at the same time, further harmonise the implementation of GMO testing.

Upon reception of the sPSP in January 2014, an inter-laboratory pilot project will be launched to assess the tool's performance in routine GMO testing scenarios.

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List of abbreviations and definitions

CRM: Certified Reference Material

DSS: Decision Support System

ENGL: European Network of GMO Laboratories

ePSP: Event-specific Pre-Spotted Plate

GMO: Genetically Modified Organism

LOD: Limit of Detection

MPR: Minimum Performance Requirements

PSP: Pre-Spotted Plate

RTi-PCR: Real-Time Polymerase Chain Reaction

sPSP: Screening Pre-Spotted Plate

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Annex 1

Matrix of authorized GM events as of November 2013 (EU register). Not including stacked events.

	Taxon	ELE-p35S	ELE-tNOS	CON-CTP2-CP4EPSPS	ELE-PAT	ELE-BAR	ELE-cry1Ab/Ac	Status
GMO Event DAS-40278-9 Maize (DAS-40278-9)	Maize	-	-	-	-	-	-	LLP 1
GMO Event Bt176	Maize	+	-	-	-	+	-	LLP 1
GMO Event 1507 Maize (DAS-01507-1)	Maize	+	-	-	+	-	-	authorized
GMO Event 3272 Maize (SYN-E3272-5)	Maize	-	+	-	-	-	-	LLP feed
GMO Event GA21 Maize (MON-00021-9)	Maize	-	+	-	-	-	-	authorized
GMO Event 59122 Maize (DAS-59122-7)	Maize	+	-	-	+	-	-	authorized
GMO Event Bt11 Maize (SYN-BT011-1)	Maize	+	+	-	+	-	+	authorized
GMO Event 98140 Maize (DP-098140-6)	Maize	+	-	-	-	-	-	LLP 1
GMO Event MIR604 Maize (SYN-IR604-5)	Maize	-	+	-	-	-	-	authorized
GMO Event MIR162 Maize (SYN-IR162-4)	Maize	-	+	-	-	-	-	authorized
GMO Event MON89034 Maize (MON-89034-3)	Maize	+	+	-	-	-	-	authorized
GMO Event MON87460 Maize (MON-87460-4)	Maize	+	+	-	-	-	-	LLP 1
GMO Event MON810 Maize (MON-00810-6)	Maize	+	-	-	-	-	-	authorized
GMO Event NK603 Maize (MON-00603-6)	Maize	+	+	+	-	-	-	authorized
GMO Event MON88017 Maize (MON-88017-3)	Maize	+	+	+	-	-	-	authorized
GMO Event T25 Maize (ACS-ZM003-2)	Maize	+	-	-	+	-	-	authorized
GMO Event MON863 Maize (MON-00863-5)	Maize	+	+	-	-	-	-	authorized
GMO Event CV127 Soybean (BPS-CV127-9)	Soybean	-	-	-	-	-	-	LLP 1
GMO Event 305423 Soybean (DP-305423-1)	Soybean	-	-	-	-	-	-	LLP 1
GMO Event MON87705 Soybean (MON-87705-6)	Soybean	-	-	+	-	-	-	authorized
GMO Event MON87701 Soybean (MON-87701-2)	Soybean	-	-	-	-	-	+	authorized
GMO Event FG72 Soybean (MST-FG072-3)	Soybean	-	+	-	-	-	-	LLP feed
GMO Event MON89788 Soybean (MON-89788-1)	Soybean	-	-	+	-	-	-	authorized
GMO Event DAS-68416-4 Soy (DAS-68416-4)	Soybean	-	-	-	+	-	-	authorized
GMO Event A5547-127 Soybean (ACS-GM006-4)	Soybean	+	-	-	+	-	-	authorized
GMO Event 356043 Soybean (DP-356043-5)	Soybean	+	-	-	-	-	-	authorized
GMO Event A2704-12 Soybean (ACS-GM005-3)	Soybean	+	-	-	+	-	-	authorized
GMO Event 40-3-2 Roundup Ready Soybean (MON-04032-6)	Soybean	+	+	-	-	-	-	authorized

GMO Event LLRICE62 Rice (ACS-OS002-5)	Rice	+	-	-	-	+	-	LLP 1
GMO Event GT73 Rapeseed (MON-00073-7)	Rapeseed	-	-	+	-	-	-	authorized
GMO Event Rf1 Rapeseed (ACS-BN001-4)	Rapeseed	-	+	-	-	+	-	LLP 2
GMO Event Topas 19/2 Rapeseed (ACS-BN007-1)	Rapeseed	+	-	-	+	-	-	LLP 2
GMO Event Ms1 Rapeseed (ACS-BN004-7)	Rapeseed	-	+	-	-	+	-	LLP 2
GMO Event Rf2 Rapeseed (ACS-BN002-5)	Rapeseed	-	+	-	-	+	-	LLP 2
GMO Event T45 Rapeseed (ACS-BN008-2)	Rapeseed	+	-	-	+	-	-	authorized
GMO Event MS8 Rapeseed (ACS-BN005-8)	Rapeseed	-	+	-	-	+	-	authorized
GMO Event RF3 Rapeseed (ACS-BN003-6)	Rapeseed	-	+	-	-	+	-	authorized
GMO Event EH92-527-1 Potato (BPS-25271-9)	Potato	-	+	-	-	-	-	authorized
GMO Event H7-1 Sugar Beet (KM-000H71-4)	Sugarbeet	-	-	+	-	-	-	authorized
GMO Event MON88913 Cotton (MON-88913-8)	Cotton	+	-	+	-	-	-	LLP 1
GMO Event MON15985 Cotton (MON-15985-7)	Cotton	+	+	-	-	-	-	LLP 1
GMO Event GHB119 Cotton (BCS-GH005-8)	Cotton	+	+	-	-	+	-	LLP 1
GMO Event MON1445 Cotton (MON-01445-2)	Cotton	+	+	+	-	-	-	authorized
GMO Event LLCotton25 Cotton (ACS-GH001-3)	Cotton	+	+	-	-	+	-	authorized
GMO Event MON531 Cotton (MON-00531-6)	Cotton	+	+	-	-	-	+	authorized
GMO Event 281-24-236 Cotton (DAS-24236-5)	Cotton	-	-	-	+	-	-	authorized
GMO Event 3006-210-23 (DAS-21023-5)	Cotton	-	-	-	+	-	-	authorized
GMO Event T304-40 Cotton (BCS-GH004-7)	Cotton	+	+	-	-	+	-	LLP 1
GMO Event GHB614 Cotton (BCS-GH002-5)*	Cotton	-	-	-	-	-	-	authorized

* Only event not detected by the ePSP.

Annex 2

List of CRMs used for the development of the screening pre-spotted plate.

GM Event	Distributor	Code (Nominal Level)
GMO Event 1507 Maize (DAS-01507-1)	IRMM	ERM-BF418c (1%)
GMO Event 3272 Maize (SYN-E3272-5)	IRMM	ERM-BF420b (1%)
GMO Event GA21 Maize (MON-00021-9)	IRMM	ERM-BF414d (1%)
GMO Event 59122 Maize (DAS-59122-7)	IRMM	ERM-BF424c (1%)
GMO Event Bt176 Maize (SYN-BT011-1)	IRMM	ERM-BF411d (1%)
GMO Event Bt11 Maize (SYN-BT011-1)	IRMM	ERM-BF412d (1%)
GMO Event 98140 Maize (DP-098140-6)	IRMM	ERM-BF427c (2%)
GMO Event MIR604 Maize (SYN-IR604-5)	IRMM	ERM-BF423c (1%)
GMO Event MIR162 Maize (SYN-IR162-4)	AOCS	1208-A (100%)
GMO Event MON89034 Maize (MON-89034-3)	AOCS	0906-E (100%)
GMO Event MON87460 Maize (MON-87460-4)	AOCS	0709-A (100%)
GMO Event MON810 Maize (MON-00810-6)	IRMM	ERM-BF413d (1%)
GMO Event NK603 Maize (MON-00603-6)	IRMM	ERM-BF415d (1%)
GMO Event MON88017 Maize (MON-88017-3)	AOCS	0406-D (100%)
GMO Event T25 Maize (ACS-ZM003-2)	AOCS	0306-H4 (100%)
GMO Event MON863 Maize (MON-00863-5)	IRMM	ERM-BF416c (1%)
GMO Event DAS-40278-9 Maize (DAS-40278-9)	IRMM	ERM-BF433c (1%)
GMO Event MON87705 Soybean (MON-87705-6)	IRMM	0210-A (100%)
GMO Event MON87701 Soybean (MON-87701-2)	AOCS	0809-A (100%)
GMO Event FG72 Soybean (MST-FG072-3)	AOCS	0610-A2 (100%)
GMO Event MON89788 Soybean (MON-89788-1)	AOCS	0906-B (100%)
GMO Event DAS-68416-4 Soy (DAS-68416-4)	IRMM	ERM-BF432c (1%)
GMO Event A5547-127 Soybean (ACS-GM006-4)	AOCS	0707-C3 (100%)
GMO Event 356043 Soybean (DP-356043-5)	IRMM	ERM-BF425c (1%)
GMO Event A2704-12 Soybean (ACS-GM005-3)	AOCS	0707-B4 (100%)
GMO Event 40-3-2 Roundup Ready Soybean	IRMM	ERM-BF410d (1%)
GMO Event CV127 Soybean (BPS-CV127-9)	AOCS	0911C (100%)
GMO Event 305423 Soybean (DP-305423-1)	IRMM	ERM-BF426c (10%)
GMO Event GT73 Rapeseed (MON-00073-7)	AOCS	0304-B (100%)
GMO Event Rf1 Rapeseed (ACS-BN001-4)	AOCS	0711-B (100%)
GMO Event Topas 19/2 Rapeseed (ACS-BN007-1)	AOCS	0711-D (100%)
GMO Event Ms1 Rapeseed (ACS-BN004-7)	AOCS	0711-A (100%)
GMO Event Rf2 Rapeseed (ACS-BN002-5)	AOCS	0711-C (100%)
GMO Event T45 Rapeseed (ACS-BN008-2)	AOCS	0208-A4 (100%)

GMO Event MS8 Rapeseed (ACS-BN005-8)	AOCS	0306-F3 (100%)
GMO Event RF3 Rapeseed (ACS-BN003-6)	AOCS	0306-G3 (100%)
GMO Event MON88913 Cotton (MON-88913-8)	AOCS	0906-D (100%)
GMO Event MON15985 Cotton (MON-15985-7)	AOCS	0804-D (100%)
GMO Event GHB119 Cotton (BCS-GH005-8)	IRMM	ERM-BF428b (1%)
GMO Event MON1445 Cotton (MON-01445-2)	AOCS	0804-B (100%)
GMO Event LLCotton25 Cotton (ACS-GH001-3)	AOCS	0306-E2 (100%)
GMO Event MON531 Cotton (MON-00531-6)	AOCS	0804-C (100%)
GMO Event 281-24-236 x Cotton (DAS-24236-5) 3006-210-23 (DAS-21023-5)	IRMM	ERM-BF422c (1%)
GMO Event T304-40 Cotton (BCS-GH004-7)	IRMM	ERM-BF429b (1%)
GMO Event GHB614 Cotton (BCS-GH002-5)	AOCS	1108-A3 (100%)
GMO Event EH92-527-1 Potato (BPS-25271-9)	IRMM	ERM-BF421b (100%)
GMO Event LLRICE62 Rice (ACS-OS002-5)	AOCS	0306-I4 (100%)
GMO Event H7-1 Sugar Beet (KM-000H71-4)	IRMM	ERM-BF419b (100%)

Annex 3

LOD assessment on sPSP (Eurogentec Lot 13077/P01).

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). In case of lack of availability of CRM, control samples provided to the EURL GMFF by applicants or samples from retails were used. The complete list of materials is reported in Table 1.

Table 1: List of material used for preparation of the positive control sample series for taxon-specific assays (Txn) and screening-specific assays (Scr)

Taxon Target	sample	Screening Target	sample
Hmg	wt maize retail	**p35s/bar**	LLrice62 AOCS 0306-I5
Lec	wt soy retail	**tNOS**	MIR162 AOCS 1208-A
CruA	DP73496 (blank) ERM-BF434a	**CTP2-EPSPS**	MON87705 AOCS 0210-A
Sah7	T304 (blank) ERM-BF429a	**PAT**	281x3006 ERM-BF422D
UGP	wt potato CRL VL09/05	**Cry1Ab/Ac**	MON87701 AOCS 0809-A
PLD	wt rice retail	**CV127**	CV127 AOCS 0911-C
GS	wt s.beet CRL VL28/04	**DP-305423**	DP-305423 ERM-BF426D
		DAS-40278	DAS40278 ERM-BF433d

Unless the CRM was available as DNA solution, DNA was extracted using the Nucleospin Food kit (Macherey-Nagel GmbH, Düren, Germany), Foodproof GMO sample preparation kit (Biotecon Diagnostics GmbH, Postdam, Germany) according to the manufacturer's instructions or a CTAB-based extraction protocol. Seeds and grains were first ground to fine powder using Grindomix GM 200 (Retsch GmbH, Haan, Germany).
Each sample was quantified by fluorescence detection using PicoGreen® ds DNA quantitation kit (Invitrogen, Molecular Probes, Eugene, OR, USA) and tested for absence of PCR inhibitors.

A droplet digital PCR (ddPCR) was first performed to assess the GM copy number of all DNA samples.

1-50 ng DNA (except potato 0.03 ng) were quantified in 20 µl reaction mix containing 2X QX200™ ddPCR SuperMix for Probes (no dUTP) (Biorad 186-3023), 600 nM of each primer and 200 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 concentration, two dilution levels of 6 copies/µl of each GM/taxon targets were prepared. From these levels the corresponding 4, 2, 1 and 0.2 copies/µl levels were obtained. All samples were diluted in 10ng/µl of Lambda DNA (Invitrogen).
5 µl of each dilution level was analysed on 10 PSP (Eurogentec SA) containing a total amount of 30, 20, 10, 5 and 1 copie(s) as described in the Standard Operating Procedure: Screening Pre-Spotted Plates (Sc-PSP) JRC.I3.S67/EURL.

The LOD was determined as the last dilution level at which no negative result was observed in the 10 replicates (Table 2).

Table 2: LOD observed for each assay and corresponding Cq observed.

Method	LOD (cp)	Mean Cq ± SD	Method	LOD (cp)	Mean Cq ± SD
HMG	5	37.04 ± 0.84	p35s	5	37.21 ± 0.77
Lec	5	37.36 ± 0.87	tNOS	10	36.82 ± 0.43
CruA	20	37.28 ± 0.44	CTP2-EPSPS	5	37.09 ± 0.62
Sah7	5	38.40 ± 0.70	PAT	5	35.51 ± 0.61
UGP	5	37.01 ± 0.84	BAR	5	37.60 ± 0.86
PLD	5	38.33 ± 0.84	Cry1Ab/Ac	5	36.46 ± 1.04
GS	5	37.97 ± 0.64	CV127	5	36.69 ± 0.77
DAS-40278	10	36.45 ± 0.49	DP-305423	5	37.54 ± 0.53

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