

New qualitative trait-specific SYBR[®]Green qPCR methods to expand the panel of GMO screening methods used in the CoSYPS

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Received: 10 September 2014 / Revised: 13 March 2015 / Accepted: 18 March 2015 / Published online: 14 April 2015
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Abstract Since 2011, a new Commission Regulation (EU/619/2011) defines that laboratories testing for genetically modified organisms (GMO) need to be able to detect also genetically modified (GM) events pending for authorisation. This, in addition to the fact that the number of GM events authorised in the European Union (EU) that need to be identified multiplies rapidly and that the detection of unauthorised GMO becomes more important, led to the development of a time and cost-effective screening approach. Moreover, the GM elements that are utilised in the transgenic inserts also become increasingly diverse. Consequently, the screening approaches have to be updated to enable full coverage and better discrimination of all these events. To respond to this need, two new qualitative SYBR[®]Green real-time PCR (qPCR) methods were developed and in-house validated: one method is element-specific and targets the Cry3Bb trait, and the other one is a construct-specific method detecting the gat-tpinII junction. Method acceptance parameters such as the sensitivity,

specificity and repeatability were assessed as well as the robustness of the methods. Additionally, the reproducibility was evaluated by transferring the methods to a second laboratory. Both methods allow a specific, sensitive and repeatable detection of the respective targets in food and feed samples and can be easily applied in a routine laboratory. Moreover, they can be used together with previously validated SYBR[®]Green qPCR methods to expand the panel of screening methods. This allows an extended coverage of the GM events authorised in the EU and adds discriminative power to the screening phase.

Keywords GMO · Screening · SYBR[®]Green · qPCR

Introduction

In the 18 years of commercialisation of biotech crops, the hectareage has increased more than a 100-fold making biotech crops the fastest adopted crop technology in recent history [1]. Consequently, many countries have adopted regulations to strictly control the marketing of GM crops and their use in the food and feed chain [2, 3]. In the EU, for example, Regulation EC/1829/2003 [4] ensures traceability of GM products and labelling to ascertain the freedom of choice of the consumer. In this frame, GMO detection plays an important role and the provision of an efficient and reliable detection method by the biotech company is mandatory. To date, these methods rely on the qPCR technology [5], with a specific method for each GM event. As a result, the European Union Reference Laboratory for GM Food and Feed (EURL GMFF; <http://gmo-crl.jrc.ec.europa.eu/>) invests a lot of effort in the validation of these quantitative methods, which can then be implemented by the enforcement laboratories for GMO identification and quantification.

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Electronic supplementary material The online version of this article (doi:10.1007/s00217-015-2454-6) contains supplementary material, which is available to authorized users.

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The number of GM events brought onto the markets is, however, growing steadily. In the EU alone, 50 single and stacked events (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm, update 17/12/2014) are currently authorised under Regulation EC/1829/2003 [4]. In addition, since 2011, the European Commission established a new regulation (EU/619/2011) [6] allowing the limited presence of GM events pending for authorisation in feed. The list of events falling under this legislation presently comprises 29 single and stacked events and makes the number of GM events to be detected by the enforcement laboratories even larger. As a consequence, a one-by-one identification of each GM event becomes clearly unrealistic, and many laboratories have therefore developed a screening approach. Such a screening consists in the detection of taxon- and GM-specific sequences allowing to decide which GM events could potentially be present in a sample based on the combination of the obtained positive elements [7–10]. The main objective of such a screening step is to draw conclusions on the presence/absence of as many GM events as possible with a minimum number of qPCR reactions. This knowledge allows then to reduce the amount of events to be identified in the next step. Further, this kind of approach not only permits evaluating the presence of the events for which all information is available (i.e. the EU-authorized events) but also of the EU-unauthorized ones. This is of uttermost importance as the presence of unauthorized GMO becomes more and more realistic due to the fact that events may be authorized in one country but not in another, that many GM events are being developed for local markets by research institutes, which have no interest in introducing an authorisation dossier in Europe [11] or due to adventitious escapes that can occur [11, 12].

To this aim, several laboratories have developed in-house screening methods, using SYBR[®]Green or TaqMan[®] chemistry, targeting the most common elements in the current GMO such as the *Cauliflower Mosaic Virus* promoter (p35S) [13, 14], the *Agrobacterium tumefaciens* nopaline synthase terminator (tNOS) [13, 15], the phosphotriester-N-acetyltransferases from *Streptomyces viridochromogenes* (*pat*) and from *Streptomyces hygroscopicus* (*bar*) [16, 17] and the 5-enolpyruvylshikimate-3-phosphate synthase from the *Agrobacterium tumefaciens* strain CP4 or from plant origin (*in casu* petunia) (*epsps*) [16, 18]. In some cases, multiplex qPCR assays have been established to decrease the number of reactions to be performed (e.g. [19–21]). However, it should be noted that multiplexing using qPCR has its limitations [22], i.e. the number of dyes that can currently be detected, the occurrence of false positives when the targets to be multiplexed become too numerous and/or may interact and the loss of sensitivity. Furthermore, high-throughput technologies, as, for instance, NASBA implemented microarray analysis (NAIMA) [23, 24],

microdroplet PCR implemented capillary gel electrophoresis (MPIC) [25], multiplex ligation detection methods [26] (and references therein), a combined microchip-PCR and microarray system (MACRO) [27], digital PCR [28, 29] and next-generation sequencing (NGS) [30–32], have been developed and their possible use in GMO detection was demonstrated. These methods are, however, still too costly and/or cumbersome for routine use and often require expensive equipment and/or specialised data analysis tools and staff. Consequently, presently, qPCR remains the method of choice to be used by the GMO detection laboratories. To take into account the growing diversity of the authorised and unauthorised GMO (i.e. new taxa are being transformed and different elements in the transgenic constructs are used), to allow full coverage of all events that are requested to be identified by the competent authorities, to enable sufficient discrimination in order to reduce the number of identifications to be performed and to keep the screening approach up to date, new screening methods targeting other sequences are needed.

Here, the authors present the development and in-house validation of two new qualitative SYBR[®]Green qPCR methods. The first qPCR method is a GM element-specific one targeting the *Bacillus turingiensis* (Bt) δ -endotoxin encoding gene *cry3Bb* (Cry3Bb). The second method is a construct-specific method aiming at the detection of the junction sequence between the glyphosate N-acetyltransferase of *Bacillus licheniformis* and the terminator of the *Solanum tuberosum* proteinase inhibitor (*gat-tpinII*). The SYBR[®]Green chemistry has been chosen for these methods to enable adding them to the already available screening SYBR[®]Green methods to form a panel of methods, which can be run in a single 96-well plate [7] and consequently build a more efficient and complete screening phase. In addition, SYBR[®]Green chemistry only uses two primers rendering it cheaper than TaqMan[®] for which also a labelled probe is needed. Furthermore, performing a melt curve analysis after the amplification renders the SYBR[®]Green methods specific while keeping a certain flexibility to also detect the presence of closely related targets. The different method acceptance parameters [33], necessary to enable declaring a method fit for purpose, were evaluated for each method.

These two new methods can be integrated in the decision support system that is used in the laboratory for GMO detection (i.e. the Combinatory SYBR[®]Green qPCR Screening (CoSYPS) system) [7] to form a whole with the previously validated and published qualitative SYBR[®]Green methods targeting reference genes [34], generic elements [13, 35, 36] and trait sequences [16]. On the one hand, the *gat-tpinII* method will allow covering and discriminating GM events that do not possess any of the targets that are currently included in the CoSYPS, i.e.

EC/1829/2003 authorised soy event 356043, EU/619/2011 authorised maize event 98140 and oilseed rape event 73496. On the other hand, the method targeting Cry3Bb, as present in the EC/1829/2003 authorised maize events MON 863 and MON 88017, permits to further discriminate p35S/tNOS possessing events, especially within the maize events, which is the largest group of authorised events.

Materials and methods

Design and selection of primer pairs

Bioinformatics development of primers and in silico specificity analysis was performed as outlined in [13].

For each method, different primers were designed using the wEMBOSS “Primer 3” program [37] with the “product size range” specification set at “60–120 bp” and the “primer size” set at an optimum of 22 bases. Relevant DNA sequences, containing the targeted element, were collected from public databases (NCBI and EMBL), patents and scientific literature as well as from in-house DNA sequencing. Primer pair complementarities, potential hairpin formation and self-annealing were evaluated in silico (“Oligo Calc” software <http://www.basic.northwestern.edu/biotools/oligo-calc.html>). For the Cry3Bb and gat-tpinII targets, respectively, 5 and 4 primer combinations were obtained.

Further, an in silico specificity test of the primer pairs was performed. This test consisted of a bioinformatics analysis using “primersearch” (<https://wemboss.uio.no/wEMBOSS/>, <http://emboss.bioinformatics.nl/cgi-bin/emboss/primer-search>) [38, 39], which mimics the PCR amplification of the tested primers on a list of relevant DNA sequences retrieved from public databases, patents and scientific literature and from in-house DNA sequence analyses. Only primer pairs that gave the expected in silico amplification were retained to test the specificity experimentally.

Plant material, DNA extraction and characterisation

All plant materials used in this study were purchased as Certified Reference Material (CRM) from the American Oil Chemists’ Society (AOCS; Urbana, USA; <http://www.aocs.org/LabServices/content.cfm?ItemNumber=19248>) and the Institute for Reference Materials and Measurements (IRMM; Geel, Belgium; <https://ec.europa.eu/jrc/en/reference-materials/catalogue/order>). The certificates, stating amongst others the origin, zygosity and certified GM %, can be found on the websites of the respective CRM producers. The CRM were either sold as powders or under the form of genomic DNA (gDNA).

Genomic DNA from maize, oilseed rape, sugar beet, rice and potato materials was extracted using a CTAB-based

procedure [40]. gDNA from cotton and soybean materials was extracted using a CTAB-based procedure in combination with the Genomic Tip20/G (QIAGEN; protocol adapted from the EU-RL GMFF validated method at http://gmo-crl.jrc.ec.europa.eu/summaries/281-3006%20Cotton_DNAExtr.pdf).

All materials were thoroughly characterised by using the CoSYPS system [7] developed at the WIV-ISP GMO-lab under ISO 17025 accreditation [41], and an inhibition test [42] was performed using the TaqMan[®] event-specific qPCR methods (<http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). All plant materials used are listed in Table 1A.

DNA concentration and purity

The DNA concentration was determined by means of fluorimetry using a VersaFluor[™] Fluorometer (Biorad) and the Quant-iT[™] PicoGreen[®] dsDNA Assay kit (Molecular Probes). The purity (A260/A280 and A260/A230 ratios) of the DNA was checked by spectrophotometry using the Nanodrop[®] 2000 device (Thermo Scientific).

qPCR reaction

The qPCR reaction mix, the qPCR cycling conditions and the program for melting curve analysis were carried out as described previously [13]. The reactions were performed using the SYBR[®]Green I master mix (Diagenode DMSG-2X-A300) and a final primer concentration of 250 nM. All runs were performed on an ABI 7300 qPCR system (Applied Biosystems) except for the gat-tpinII specificity assessment, which was done on an iQ[™]5 cycler (BioRad).

The selected primer sequences and the amplicon size for the two methods are presented in Table 2.

Method acceptance parameters

Method acceptance parameters were evaluated as described in the recently published validation guidelines [33] unless described differently. Calculation of the number of haploid genome equivalents (HGE) was done using the 1C values from [43].

Specificity, sensitivity and repeatability [35] were tested in-house. The specificity was tested using 25 ng of the CRM gDNA in the qPCR reaction. In addition to the LOD₆, also the LOD_{95%} was determined. Hereto, the same CRM as used for the LOD₆ determination (i.e. 100 % soybean MON 88017 for Cry3Bb and 10 % soybean 356043 for gat-tpinII) was utilised at three GM levels (below LOD₆, at LOD₆ and above LOD₆). Each level was tested in 60 replicates, and the LOD_{95%} is set at the HGE where all 60 replicates give a specific amplification. Dilutions

Table 1 Results from the specificity assessment for the Cry3Bb and gat-tpinII SYBR®Green qPCR screening methods

Species	CRM	Certified GM % (%)	Origin	Cry3Bb expected signal	C _q	T _m	gat-tpinII expected signal	C _q	T _m
<i>A. Use of gDNA (25 ng) from Certified Reference Materials to assess the specificity of each SYBR®Green qPCR method</i>									
Maize	WT	0	AOCS	–	–	–	–	–	–
	MON 810	5	IRMM	–	–	–	–	–	–
	MON 89034	100	AOCS	–	– ^b	– ^b	–	–	–
	MON 863	9.86	IRMM	+	+	+	–	–	–
	MON 88017	100	AOCS	+	+	+	–	–	–
	Bt176	5	IRMM	–	–	–	–	–	–
	3272	100	AOCS	–	–	–	–	–	–
	DAS59122	9.87	IRMM	–	–	–	–	–	–
	1507	9.86	IRMM	–	–	–	–	–	–
	T25	100	AOCS	–	–	–	–	–	–
	Bt11	5	IRMM	–	–	–	–	–	–
	GA21	4.29	IRMM	–	–	–	–	–	–
	NK603	4.91	IRMM	–	–	–	–	–	–
	MIR604	100	AOCS	–	–	–	–	–	–
	MIR162 ^a	100	AOCS	–	–	–	–	–	–
	98140 ^a	10	IRMM	–	–	–	+	+	+
	MON 87460 ^a	100	AOCS	–	–	–	–	–	–
DAS40278-9 ^a	1	IRMM	–	–	–	–	–	–	
Soybean	WT	0	IRMM	–	–	–	–	–	–
	GTS40-3-2	10	IRMM	–	–	–	–	–	–
	A2704-12	100	AOCS	–	–	–	–	–	–
	A5547-127	100	AOCS	–	–	–	–	–	–
	MON 89788	100	AOCS	–	–	–	–	–	–
	305423 ^a	10	IRMM	–	–	–	–	–	–
	356043	10	IRMM	–	–	–	+	+	+
	MON 87701	100	AOCS	–	–	–	–	–	–
	FG72 ^a	100	AOCS	–	–	–	–	–	–
	MON 87705 ^a	100	AOCS	–	–	–	–	–	–
	MON 87708 ^a	100	AOCS	–	–	–	–	–	–
	BPS-CV127-9 ^a	100	AOCS	–	–	–	–	–	–
Oilseed rape	WT	0	AOCS	–	–	–	–	–	–
	GT73	100	AOCS	–	–	–	–	–	–
	MS8	100	AOCS	–	–	–	–	–	–
	Rf3	100	AOCS	–	–	–	–	–	–
	T45	100	AOCS	–	–	–	–	–	–
	Ms1 ^a	100	AOCS	–	–	–	–	–	–
	Rf1 ^a	100	AOCS	–	–	–	–	–	–
	Rf2 ^a	100	AOCS	–	–	–	–	–	–
	Topas 19/2 ^a	100	AOCS	–	–	–	–	–	–
	73496 ^a	10	IRMM	–	–	–	+	+	+
	Cotton	WT	0	AOCS	–	–	–	–	–
MON 1445		100	AOCS	–	–	–	–	–	–
MON 15985		100	AOCS	–	–	–	–	–	–
MON 531		100	AOCS	–	–	–	–	–	–
LL25		100	AOCS	–	–	–	–	–	–
GHB614		100	AOCS	–	–	–	–	–	–
281-24-236 × 3006-210-23		10	IRMM	–	–	–	–	–	–

Table 1 continued

Species	CRM	Certified GM % (%)	Origin	Cry3Bb expected signal	Cq	T_m	gat-tpinII expected signal	Cq	T_m
Sugar beet	MON 88913 ^a	100	AOCS	–	–	–	–	–	–
	GHB119 ^a	10	IRMM	–	–	–	–	–	–
	T304-40 ^a	1	IRMM	–	–	–	–	–	–
	WT	0	IRMM	–	–	–	–	–	–
	H7-1	100	IRMM	–	–	–	–	–	–
Rice	WT	0	AOCS	–	–	–	–	–	–
	LL62 ^a	100	AOCS	–	–	–	–	–	–
Sybricon	BCCM number		Cq		T_m (°C)				
<i>B. Use of Sybricon plasmids (200 cp) to determine the nominal T_m value for each SYBR[®]Green qPCR method</i>									
Sybricon 026 (Cry3Bb)	LMBP 6933		31.2		81.7				
Sybricon 030 (gat-tpinII)	LMBP 8353		32.4		74.6				

+ Exponential amplification observed, T_m within the T_m interval (nominal $T_m \pm 1$ °C)

– No exponential amplification observed, T_m not within the T_m interval (nominal $T_m \pm 1$ °C)

^a GM event falling under Regulation EU/619/2011

^b Weak positive signal: presence of traces of other GM events

Table 2 Primer sets and amplicon sizes for each screening qPCR method

Target	Primer name	Primer sequence (5' → 3')	Amplicon size (bp)	Reference
Cry3Bb	Cry3Bb1_Fwd_3	CTACCAGTCCTTCTCGAACACC	105	This study
	Cry3Bb1_Rev_1	GCGTACTCCTCGATCTTCTTGT		
gat-tpinII	gatpin-F2	GGACCTCACATCCTGATGTATAA	86	This study
	gatpin-R1	AAGTTGGCCAATCCAGAAGA		

were made in nuclease-free water (instead of background DNA as stated in the guidelines). The reproducibility of the methods was assessed by transferring them to a second laboratory (JRC-IHCP).

The robustness of the methods was tested on the CRM from the sensitivity determination using a fractional factorial approach [44]. The different factors that were evaluated were as follows: qPCR instrument (ABI7300 versus StepOnePlus), qPCR master mix (Diagenode DMSG-2X-A300 versus Promega GoTaq[®] qPCR), primer concentration (30 % less), annealing temperature (± 1 °C) and reaction volume (± 1 μ l with a constant DNA amount). Eight different combinations were run (Table 3). Twenty HGE were used in six replicates per combination.

Cloning, sequencing and plasmid registration

The amplicon obtained for each of the SYBR[®]Green methods was cloned into the pUC18 vector according to [13] to obtain the plasmids called “Sybricon”. Sequencing of the amplicon and plasmid registration was performed as outlined in [35]. The Sybricon names of the plasmids can be found in Table 1B.

CoSYPS screening analysis

Two samples, which were formerly used in proficiency and comparative tests (GeM SU35 containing event MON 88017; JRC CT1/13 containing events 98140 and MON 863), were utilised to check additional method performance parameters such as applicability and practicability. In addition, to mimic food/feed matrices encountered in GMO routine analysis, a real-life sample called “Tostitos” and composed of maize tortilla chips was tested using a panel of screening methods including the one for Cry3Bb developed and validated in this study.

Results and discussion

Due to the increasing amount of GM events to be detected and the growing diversity of the elements used in the transgenic inserts, the panel of screening methods applied in GMO detection needs to be updated regularly to allow continuous coverage of this list of events and to provide sufficient discrimination to reduce the number of identifications to be performed in a second step. Two new qualitative

Table 3 Scheme for robustness testing and results for the Cry3Bb and gat-tpinII screening qPCR methods

Tested factors	Standard condition	Tested combinations							
		1	2	3	4	5	6	7	8
PCR instrument	ABI7300	ABI7300	ABI7300	ABI7300	ABI7300	StepOnePlus	StepOnePlus	StepOnePlus	StepOnePlus
Master Mix	Diagenode	Diagenode	Diagenode	Promega	Promega	Diagenode	Diagenode	Promega	Promega
Primer concentration (nM)	250	250	175	250	175	250	175	250	175
Volume (μ l) mix + DNA	20 + 5	19 + 5	19 + 5	21 + 5	21 + 5	21 + 5	21 + 5	19 + 5	19 + 5
Annealing temperature ($^{\circ}$ C)	60	61	59	61	59	59	61	59	61
<i>Cry3Bb</i>									
C_q^a	30.7	33.0 \pm 0.4	32.2 \pm 0.4	30.4 \pm 0.4	31.5 \pm 0.3	34.6 \pm 0.5	34.6 \pm 0.7	31.2 \pm 0.6	31.7 \pm 0.8
T_m^b	81.6	81.0–81.6	80.7–81.7	85.6–86	85.6–85.9	81.1–81.2	80.9–81.1	84.1–84.7	84.2–84.6
<i>gat-tpinII</i>									
C_q^a	32.4	32.5 \pm 0.3	32.7 \pm 0.3	30.0 \pm 0.5	30.8 \pm 0.3	33.6 \pm 0.3	34.5 \pm 0.4	31.8 \pm 0.1	30.7 \pm 0.4
T_m^b	74.7	74.2–74.5	74.2–74.5	78.8–79.4	78.7–79.1	74.2–74.4	73.9–74.1	77.7–78.0	77.6–77.9

^a Average C_q and standard deviation (in italics) over six replicates

^b Minimal and maximal T_m taking all six replicates into account (in bold)

trait-specific SYBR[®]Green qPCR methods were developed and in-house validated. One method targets the *Bacillus thuringiensis* (Bt) δ -endotoxin encoding gene *cry3Bb* (Cry3Bb), present in the maize events MON 863 and MON 88017. The other one, a construct-specific method, aims at the detection of the junction sequence between the glyphosate N-acetyltransferase of *Bacillus licheniformis* and the terminator of the *Solanum tuberosum* proteinase inhibitor (*gat-tpinII*) as found in maize event 98140, soybean event 356043 and oilseed rape event 73496.

Development of the new screening qPCR methods

For both methods presented here, the designed primer combinations were first tested in silico against target and non-target containing GM events and their wild-type (WT) counterparts using bioinformatics tools (data not shown). For the method targeting the Cry3Bb sequence, this analysis resulted in the elimination of one primer pair, which gave a positive reaction for the non-target containing GM event MIR604. For the *gat-tpinII* method, results were as expected (i.e. a positive reaction was obtained for all target containing materials and not for the non-target containing ones) for all four primer sets.

The remaining four pairs for both methods were subsequently experimentally tested against a limited number of GM and WT materials, i.e. the GM events that contain the respective targets and their conventional counterparts. For each method, the best performing pair (i.e. the primer pair giving a specific positive signal considering the C_q and T_m values as described in [13]) was retained and its

specificity was further tested on all available GM and WT CRM (Table 1A). This primer pair was also used to evaluate the acceptance parameters of each method. The Cry3Bb method amplifies a fragment of 105 bp, while the *gat-tpinII* method amplifies a sequence of 86 bp (Table 2).

Sybricon plasmids carrying the targeted sequence amplified from a positive CRM (MON 88017 for Cry3Bb, 356043 for *gat-tpinII*) were constructed and sequenced. This showed a perfect homology of the insert (100 % identity at nucleic acid level) with the sequences as reported in the patents EP1698699 (GenBank CS409981.1) for Cry3Bb and WO2008002872 (GenBank FB727464.1) for *gat-tpinII*. These plasmids were also used to determine the nominal T_m value for each target: 81.7 $^{\circ}$ C for Cry3Bb and 74.6 $^{\circ}$ C for *gat-tpinII* (Table 1B). The latter values were subsequently used as a criterion (with an allowed variation of ± 1 $^{\circ}$ C) [7, 33] in the decision whether the amplification in a sample is positive and specific [13].

In-house validation of the new screening qPCR methods

To ensure a newly developed qPCR method is fit for purpose, a number of method acceptance parameters need to be tested against previously set criteria. Besides the specificity of a method, the sensitivity is of uttermost importance to allow detection of low amounts of GM events in relation to, for example, Regulation EU/619/2011 [6] and the fact that unauthorised GMO may be present at trace level. In addition, parameters such as the repeatability and the robustness of the two new methods were evaluated.

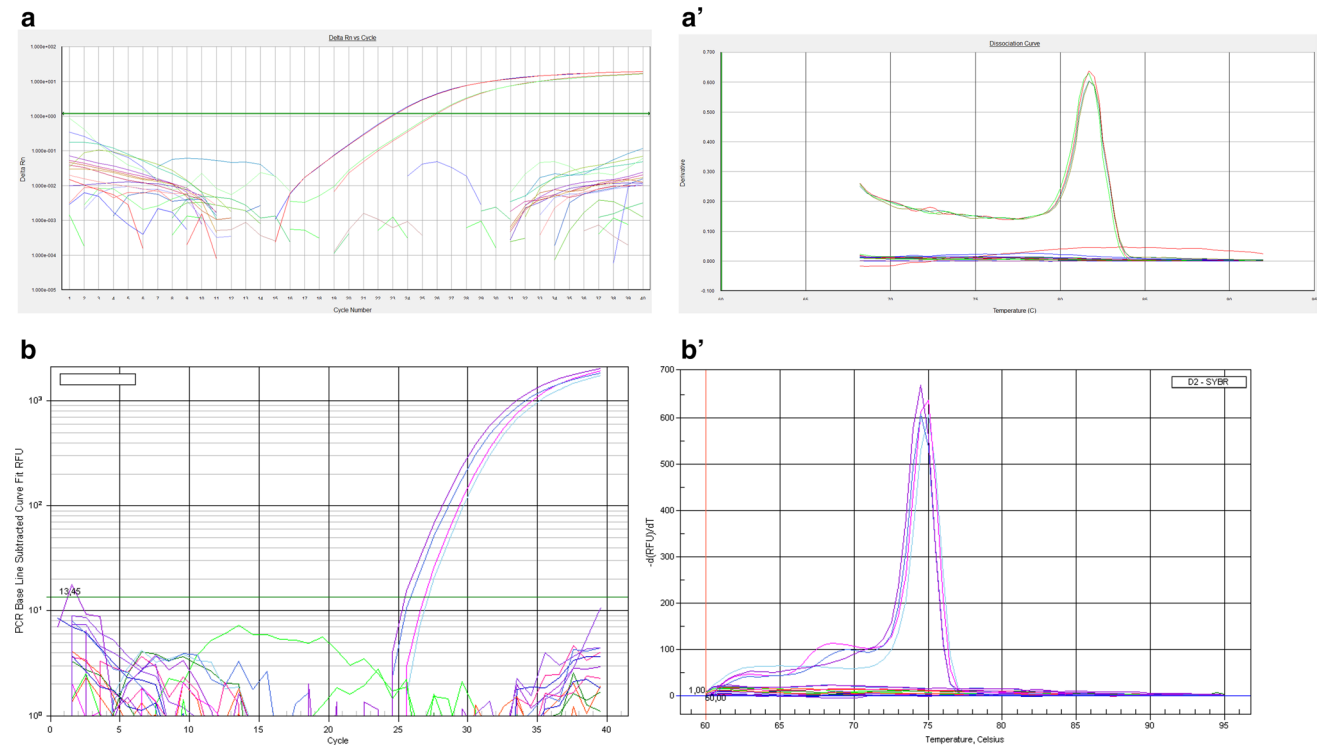


Fig. 1 Amplification curves (**a**, **b**) and melting curves (**a'**–**b'**) for the Cry3Bb (**a**–**a'**) and gat-tpinII (**b**–**b'**) targeting SYBR[®]Green qPCR methods

Specificity

For both screening methods, all the GM events containing the respective targeted sequence showed a clear amplification with a T_m value within the nominal $T_m \pm 1^\circ\text{C}$ interval with a single melting curve peak (Fig. 1). Additionally, nor the tested WT materials neither the non-target containing GM events gave a specific amplification (Table 1). For the Cry3Bb method, however, one GM event (MON 89034) that is not expected to contain the targeted Bt endotoxin gene resulted in a late but specific amplification (T_m 81.4 °C, C_q at 30 compared to C_q values of 18 and 21 for the 100 % MON 88017 and 10 % MON 863 materials, respectively). It was nevertheless demonstrated, using the EU-RL validated event-specific method (http://gmo-crl.jrc.ec.europa.eu/summaries/MON88017_validated_Method_correctedversion1.pdf), that this is due to a low-level presence of the MON 88017 event (C_q value of 34) in the used CRM. Indeed, the available CRM is intended for calibration purposes and/or as positive controls but not for specificity studies and are therefore only certified for the presence of a specific GM event and not for the absence of any other events (ERM Application note 4—http://irmm.jrc.ec.europa.eu/reference_materials_catalogue/user_support/erm_application_notes/application_note_4/Documents/application_note_4_english.pdf). Low levels of contaminating events may thus occur. In summary, neither false

positives nor false negatives were observed for any of the present methods.

The specificity of the methods was further confirmed by agarose gel electrophoresis. Indeed, a band of the correct size was only seen for the target containing CRM for both methods (except for the Cry3Bb target where a less intense band was also seen for the non-target containing CRM MON 89034 due to contamination).

Sensitivity

The sensitivity of the two newly developed qPCR methods was assessed via the determination of the limit of detection (LOD) of the methods. Two types of LOD, namely the LOD_6 and the $\text{LOD}_{95\%}$, were determined.

The LOD_6 was established for each target by testing a dilution series (from 5 ng till the theoretical zero HGE) of a positive CRM (i.e. MON 88017 for Cry3Bb and 356043 for gat-tpinII). Each dilution point was tested in six replicates, and four independent experiments were run under repeatability conditions [45]. The LOD_6 of each run was set at the lowest HGE level at which all six replicates were still positive and the LOD_6 of the method was established as the highest LOD_6 over the four runs [33]. Using a Grubbs test, the outlying C_q values were identified (1 outlier for Cry3Bb, no outliers for gat-tpinII) and excluded from all calculations. In addition, the first run for the Cry3Bb target

Table 4 Summary of results for the assessment of the different parameters during method validation

Target	Parameter	Developing laboratory (WIV-ISP)					Second laboratory (IHCP)				
		Run 1	Run 2	Run 3	Run 4	Method	Run 1	Run 2	Run 3	Run 4	Method
Cry3Bb	LOD ₆ (C _q)	31.1	34.5	34.4	34.9	34.6	35.3	35.0	33.4	35.8	33.4
	LOD ₆ (HGE)	6	2	2	2	2	2	2	6	2	6
	T _m (°C)	–	–	–	–	81.7 ^a /81.3 ^b	–	–	–	–	–
	ε (%)	95	105	97	101	101	108	103	100	101	103
	R ²	0.999	0.997	1.000	0.996	0.999	0.999	1.000	0.999	1.000	1.000
gat-tpinII	LOD ₆ (C _q)	35.0	34.9	34.7	33.0	34.2	36.0	35.3	36.9	36.0	36.1
	LOD ₆ (HGE)	2	1	4	4	4	1	3	1	1	3
	T _m (°C)	–	–	–	–	74.6 ^a /74.2 ^b	–	–	–	–	–
	ε (%)	93	101	103	97	97	109	96	105	102	100
	R ²	0.991	0.995	0.999	0.999	0.999	0.997	0.998	0.997	0.982	0.999

Run 1 of the developing laboratory for the Cry3Bb marker was excluded [due to lower C_q values (in bold)]

^a Nominal T_m determined on the respective Sybricon plasmid

^b Average T_m determined on the measurements from the sensitivity experiment over the 4 runs until the LOD₆

was excluded due to consistently lower C_q values compared to the other three runs. The results are summarised in Table 4. For each target, the amplification and melting curves at the LOD₆ for each of the four runs are given in Online Resource 1. For both screening methods, the LOD₆ was in accordance with the set criterion of 20 HGE ([33]; 2 HGE for Cry3Bb and 4 HGE for gat-tpinII). In all cases, the 0.1 HGE level showed not more than 1 positive indicating sufficient accuracy of the dilutions and thus correct estimation of the initial DNA concentration [46].

The LOD_{95 %} is defined as the LOD at which the analyte is detected with 95 % confidence, meaning that <5 % false negatives are allowed [33, 47]. The CRM as described above was tested in three levels around the LOD₆ (2–6–12 HGE for Cry3Bb and 2–4–10 HGE for gat-tpinII), each in 60 replicates. In both cases, 60 positive results were obtained for the two highest levels. The 2 HGE level, on the contrary, gave only 59 positives for Cry3Bb and 52 for gat-tpinII out of 60 replicates. The obtained data allowed thus to set the effective LOD of the methods at 6 and 4 HGE, respectively, for Cry3Bb and gat-tpinII (i.e. the lowest level at which all 60 replicates are positive).

Amplification efficiency and linearity

Even if these parameters are in principle not requested for qualitative qPCR methods, they can easily be calculated from the data obtained for the determination of the LOD₆ if wished. The validation guidelines [33] propose to use the upper end of the dilution series (e.g. 5,000–100 HGE) for this purpose.

Applying this to the data obtained for the Cry3Bb method leads to an overall PCR efficiency of 101 % and an

R² coefficient of 0.999 (Table 4). As no acceptance criterion is set for these parameters for qualitative qPCR methods, one could apply those for quantitative methods. In this case, the values calculated for the Cry3Bb qPCR method (on the range 1925–96 HGE) comply with the predefined criteria for amplification efficiency (90–110 %) and linearity (≥0.98) [48].

For the gat-tpinII method, however, using only the upper end of the dilution series would not be correct. As the in-house method validation was performed before the publication of the validation guidelines, the LOD₆ experiment was started at 5 ng template DNA. For the 10 % soybean event 356043, used as positive material, this corresponds only to 431 HGE. Consequently, only two dilution points are available in the upper end of the series. Using these data would lead to an incorrect calculation of the amplification efficiency and linearity. Therefore, these parameters were calculated using the points until the LOD₆ of each run (431–4 HGE except for runs 1 and 2 were the lowest HGE was, respectively, 2 and 1 HGE) as described previously [35]. The results, as presented in Table 4, show that the efficiency (ε) of the method is 97 % and the R² coefficient 0.999, which still fulfil the set criteria.

Repeatability

As the LOD₆ experiment was done under repeatability conditions [45], this allowed to calculate a relative repeatability standard deviation using the 24 obtained results (excluding the outliers) for each dilution point. For both the Cry3Bb and gat-tpinII methods, the RSD_r was below 25 % along the dynamic range. The methods are thus repeatable and conform the preset criterion for quantitative qPCR methods [48].

Robustness

Five factors were tested using a fractional factorial design to reduce the number of tests to be carried out. For each combination separately, the average C_q value with its standard deviation was calculated over the six tested replicates. In each case, all six replicates gave a C_q in the same range and below 40. Also for the T_m , all six replicates were considered (even if they do not fall in the set T_m interval) and minimum and maximum values are given. This allows to see that the variation between T_m values is always ≤ 1 °C. Further, taking into account, all six replicates enables seeing possible differences due to the changes in the tested factors. The obtained results (Table 3) show that changes in the primer concentration, the reaction volume and the annealing temperature have no big impact on the obtained C_q and T_m values. Also the use of another qPCR instrument did not really influence the data when the same master mix was used (Table 3). However, when switching from one master mix to another on a same instrument, the data were affected. The observation that the C_q values in the Promega mix compared to the Diagenode mix are lower can be due to the fact that the master mixes do not use exactly the same dye: Promega uses the BRYTGreen® dye, while Diagenode uses SYBR®Green I. Indeed, it has been reported that the BRYTGreen® dye shows greater fluorescence enhancement upon binding to double-stranded DNA than SYBR®Green I (Promega webpage: <http://be.promega.com/products/qpcr-and-rt-qpcr/gotaq-real-time-qpcr-and-rt-qpcr-systems/?origUrl=http%3a%2f%2fdk.promega.com%2fproducts%2fqpcr%2fqpcr-and-rt-qpcr%2fgotaq-real-time-qpcr-and-rt-qpcr-systems%2f>). The upwards shift in the T_m can be due to the ionic composition of the master mix or to the fact that the emission spectrum of the BRYTGreen® dye may be different from the SYBR®Green one.

Method transferability

The experiment as designed for the determination of the LOD_6 was repeated in a second laboratory (JRC-IHCP) under reproducibility conditions [45]. The relative reproducibility standard deviation was calculated using the 48 results per dilution point (excluding the outliers) obtained by both laboratories and was below 25 % for both methods along the dynamic range. Additionally, the LOD_6 was in the same range as the one obtained in the developing laboratory (6 HGE for Cry3Bb; 3 HGE for gat-tpinII; Table 4). Furthermore, the amplification efficiency (103 % for Cry3Bb, 100 % for gat-tpinII; Table 4) and R^2 (1.000 for Cry3Bb, 0.999 for gat-tpinII; Table 4), calculated on the same dilution range as for the developing laboratory, complied with the set criteria. These results allow to conclude

that the developed screening methods targeting the Cry3Bb and gat-tpinII sequences are straightforward to implement in another laboratory.

Applicability and practicability

To show the applicability of both screening methods, they were tested on different matrices. Firstly, both the gDNA extracted from the CRM and the Sybricon plasmid were compared. Both showed a specific amplification and a band of the right size (105 bp for Cry3Bb and 86 bp for gat-tpinII) upon agarose gel electrophoresis. In addition, proficiency test (PT) samples identified as containing GM events that comprise Cry3Bb and/or gat-tpinII were used to further demonstrate the applicability of the methods. Together with the newly developed methods, previously validated qPCR methods for p35S and tNOS [13] as well as for the respective species-specific marker [34] were used for the screening as a control. The results are summarised in Table 5.

The GeMMA SU35 sample (100 % Maize Flour Test Material), which was identified to contain the maize GM event MON 88017 using the event-specific qPCR method (http://gmo-crl.jrc.ec.europa.eu/summaries/MON88017_validated_Method_correctedversion1.pdf), was shown to contain the control markers p35S and tNOS as well as the Cry3Bb element. This is in accordance with the available information on the introduced transgenic cassettes in this GM event (http://ec.europa.eu/food/food/biotechnology/gmo_reference_lab_en.htm). A C_q difference was observed between the control markers and the one under investigation. This can, however, be explained by the fact that the sample also contains other events, namely GTS40-3-2 (containing p35S and tNOS) and MON 810 (possessing p35S). For all detected elements, a specific T_m , as compared to the respective Sybricons, was obtained. Furthermore, analysis of a real-life sample (“Tostitos”; Table 5) using the WIV-ISP GMolab panel of screening methods including those described here showed the detection of the Cry3Bb target amongst others (e.g. p35S, tNOS, *epsps*, maize endo-gene ADH). These positive markers could be explained by the presence of the maize event MON 88017 as detected by using the event-specific method (http://gmo-crl.jrc.ec.europa.eu/summaries/MON88017_validated_Method_correctedversion1.pdf).

Similarly, a JRC comparative study CT01/13 sample (biscuit powder) was analysed. All analysed markers (ADH, p35S, tNOS, Cry3Bb, gat-tpinII) were found positive. They could be explained by the presence of the maize GM events 98140 (gat-tpinII) and MON 863 (p35S, tNOS, Cry3Bb) as confirmed using the respective event-specific methods (http://gmo-crl.jrc.ec.europa.eu/summaries/DP-098140-6_validated_Method.pdf, <http://gmo-crl.jrc.ec.europa.eu/summaries/MON863-WEB-Protocol-Validation.pdf>).

Table 5 Applicability of new qPCR screening methods on PT and real-life samples

	Species	p35S		tNOS		Cry3Bb		gat-tpinII		Additional markers	Event(s) identified
		C _q	T _m	C _q	T _m	C _q	T _m	C _q	T _m		
<i>PT sample</i>											
GeMMa SU35	ADH+	+	+	+	+	+	+	nt	nt	nt	MON 88017
CT01/13	ADH+	+	+	+	+	+	+	+	+	nt	98140 MON 863
<i>Real-life sample</i>											
Tostitos	ADH+ LEC+	+	+	+	+	+	+	nt	nt	CP4-EPSPS +	MON 88017

nt not tested

The two new screening methods presented here are developed using the same conditions as the methods previously validated by the WIV-ISP GMOLab [13, 16, 34–36]. The standard qPCR reagents and instrument/infrastructure can be used and no additional training of the staff is thus needed. This way of working allows to analyse samples with a full panel of screening methods in just one run as was also shown by the PT sample analyses. The ease of handling of the SYBR[®]Green screening methods was further confirmed by the successful transfer of the methods to another laboratory.

Taking together all the results obtained for the Cry3Bb and gat-tpinII targeting methods show their validity for use in routine GMO detection of food and feed samples as well as their transferability. Consequently, the methods were added to the CoSYPS decision support system (DSS) as used by the WIV-ISP GMOLab [7]. Previously, the CoSYPS included nine in-house validated GM-specific qPCR methods targeting generic sequences (p35S, tNOS, pNOS, pFMV, t35S pCAMBIA) [13, 35, 36] as well as trait-specific elements (*pat*, *bar*, *cryIAb*, *epsps*) [16]. In addition, it contained six qPCR methods that allow detecting crop species representing the taxa commonly used in EU-authorized GMO, i.e. maize (ADH), soybean (LEC), oilseed rape (CRU), rice (PLD), sugar beet (GLU3) and cotton (SAD1) [34]. These markers are further combined with a plant kingdom marker (RBCL) based on the chloroplastic *rbcL* gene sequence [34]. Furthermore, to allow determining whether a positive result for the p35S marker is due to the presence of a p35S-containing GMO or of the donor organism *Cauliflower Mosaic Virus* (CaMV), a method (CRT) specific for the CaMV reverse transcriptase gene was included. All methods were designed to function under the same conditions permitting to apply them all together for the analysis of a sample in a single run. Using this full set of seventeen methods, 85.4 % of the GM events authorized under 1829/2003 and 619/2011 are covered.

The addition of the two new methods to this panel of screening methods allows to cover newly commercialised events and in this way supports the discriminative power of

the DSS. Indeed, when adding the gat-tpinII method to the CoSYPS, the coverage of the system increases to 89.6 % (additional coverage of authorised events soy 356043, maize 98140 and oilseed rape 73496). The Cry3Bb method, on the other hand, adds more discriminative power to the DSS. In many GMO detection laboratories, p35S and tNOS are the two most commonly applied screening markers. A positive outcome for p35S will result in a list of 23 events to be identified. When adding tNOS, this number can be decreased to 14 when both markers are positive. A positive outcome for the additionally applied Cry3Bb allows reducing the list further to two events. This marker allows thus a substantial diminution of the amount of work that needs to be carried out after the screening phase (i.e. GM event identification), especially for the maize events which constitute the largest group (17 single events) of GMO to be identified. Furthermore, it is useful in the detection of EU-unauthorised GM events.

Conclusion

The screening approach, applied by many laboratories, is very useful as a first step in GMO detection. However, to cope with the increasing number of commercialised GM events and the growing diversity of elements [1] and species, these screening systems need not only to be as complete as possible to give sufficient discriminative power to the system but also to be complemented regularly with new markers. In the WIV-ISP GMOLab, currently seventeen in-house validated screening markers are available for routine detection of GMO in food and feed samples [42]. Eleven of these methods have been fully validated in an EU inter-laboratory test [49] and will be added to the EU Compendium (<http://gmo-crl.jrc.ec.europa.eu/gmomethods/>). To supplement this list, two new trait-specific SYBR[®]Green screening methods have been developed and in-house validated. Method acceptance parameters as described in the validation guidelines [33] were assessed.

Both qualitative SYBR[®]Green methods, as designed here, can be regarded as specific for their respective target and

allow sensitive detection of low copy numbers (below 20 HGE) of the targets. Also parameters such as the amplification efficiency, linearity, repeatability and reproducibility are in compliance with the set acceptance criteria, and the methods can be applied on proficiency/comparative test samples as well as on real-life samples. The methods show to be robust to changes in running conditions and qPCR instruments but tend to show some variation when another master mix is used. This issue can be resolved by the use of a dynamic system in which the sample is compared to a positive control.

In conclusion, the two newly developed methods were found to be fit for purpose and suitable for routine analysis under ISO 17025 accreditation [41]. The methods were added to the CoSYPS decision support system [7] applied in the WIV-ISP GMOLab. This consists of the introduction of the LOD_6 (as C_q value) to decide on the presence/absence of an element and the T_m interval (nominal $T_m \pm 1$ °C) to evaluate the specificity of an amplification. Together with the previously published methods targeting species-specific markers [34], generic sequences [13, 35, 36] and trait elements [16], these new methods complete the set of screening methods already present in the CoSYPS DSS [7]. Additionally, the Cry3Bb method was chosen to be fully validated in a collaborative trial in the frame of the GMOval project (UK Food Standards Agency contract FS244027). As all CoSYPS methods have been developed under the same conditions, they enable analysis of a sample in a single 96-well plate. Consequently, this system forms an efficient and cost-/time-saving tool for the detection of authorised and unauthorised GMO in food and feed samples. This type of screening tools can be applied in other GMO detection laboratories but can also be adapted to other scientific fields such as the detection of food-borne pathogens [50, 51]. Additionally, these methods can serve as a basis to develop other GMO detection tools using new technologies such as xMAP [52–54]. Furthermore, the detection of a certain screening marker in a sample can be exploited for the application of technologies such as NGS [55] and DNA/chromosome walking [36, 55–57], that are especially useful for the characterisation of events for which little or no information (i.e. classes 2, 3 and 4) [9] is available (e.g. characterisation of genomic insertions and flanking regions, identification of transgenic sequences).

Acknowledgments This work was financially supported by the British Food Standard Agency (FSA, contract G03032) and the German Federal Office of Consumer Protection and Food Safety (BVL) through the Project GMOseek, under the European ERA-NET consortium SAFEFOODERA; and by the Federal Public Service Health, Food Chain Safety and Environment in the frame of the UGMMONITOR project (convention RF 11/6242). Sequencing was performed at the Platform Biotechnology and molecular Biology of the Scientific Institute of Public Health (WIV-ISP). The authors like to thank L. Lefèvre and N. Papazova for, respectively, technical support and guidance during the gat-tpinII validation.

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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