Journal of Food Composition and Analysis 125 (2024) 105722

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Set-up and in-house validation of two multiplex event-specific PCR methods to simplify screening for GMOs without common screening elements

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ARTICLE INFO

Keywords:
GMO detection
Routine screening
Multiplex quantitative PCR
Tetraplex qPCR
In-house validation
Soybean
Maize
Cotton

ABSTRACT

Since commercialization, the ever growing number of Genetically Modified (GM) crops make routine screening by European enforcement laboratories more expensive, labour-intensive and challenging every year. GM events, with elements for which no detection methods are available, further extend this screening because they require for event-specific methods to be included in the screening. Here, we describe the set-up and in-house validation of two tetraplex qPCR methods for simultaneous detection of six GM events that are not covered by common element-specific screening methods. The two qualitative multiplex PCR methods comprise previously developed and interlaboratory validated singleplex detection assays for GHB614 cotton, DP305423 soybean, CV127 soybean, DAS40278 maize, VCO1981 maize and 98140 maize. A plant-specific actin PCR was included in both multiplex PCR methods as a positive control. The validation data show that the assessed performance parameters are in accordance with the minimum performance requirements suggested by European Network of GMO Laboratories. The two multiplex PCR methods are reliable, ready to use and will lead to a faster and more efficient diagnostic routine GMO screening.

1. Introduction

At present, a substantial number of Genetically Modified Organisms (GMOs) is authorised in the EU. This includes 17 soybean (26 including stacks; conventional crossings between two GM organisms that each contain one or more GM events), 20 maize (124 including stacks), six canola (13 including stacks), nine cotton (15 including stacks) and one sugar beet (EUginius, 2023; European Commission (2023)). In addition, there are products subject to European Commission (EC) Decisions on withdrawal from the market that need to be screened for, since they are only allowed for up to 0.1% in feed. GMOs that currently fulfil the requirements of Commission Regulation (EU) No. 619/2011 (European Commission, 2011) are also allowed up to 0.1% in animal feed. Products containing GMOs must be labelled as such with the exception that adventitious presence of up to 0.9% of a GM ingredient is allowed without labelling (EC, 2003). Testing for the presence of all of these GM events in each sample is laborious and costly, hence several strategies for GMO screening have been developed and published (European Committee for Standardization [CEN], 2014; Köppel et al., 2015; Morisset et al., 2014). It is essential to design a broad enough screening method that enables detection of all authorised and unauthorised GMOs in the EU, but is as cost-effective as possible simultaneously. Furthermore, as positive screening results can also give information on the presence of unknown GMOs, it might be beneficial to apply a broader screening to certain samples.

Currently, many element- and construct-specific methods are available to screen for the presence of GMOs (Debode et al., 2013; Liang et al., 2014; Prins et al., 2017; Waiblinger et al., 2010). In order to cover all (un)authorised and unknown GMOs that contain one or more detectable elements, an extended screening approach has been applied for feed samples that are predominantly labelled as GMO (Scholtens et al., 2017). However, continuously more GMOs appear on the market that are challenging to screen for by using element- or construct-specific methods. The reason is that they do not contain any of the available common screening elements, or they contain elements that cannot be used to distinguish GMO from non-GMO because they are derived from the same species, or from other common crops like maize. Examples are I-rAct1, the rice actin intron (Scholtens et al., 2013) that is also present in unmodified rice, or P-Ubiquitin, the maize *ubi-1* promoter that is also present in unmodified maize (Debode et al., 2013). For these GMOs, screening with their separate event-specific methods is an option. A drawback is that the number of tests rapidly increases and are often

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https://doi.org/10.1016/j.jfca.2023.105722
Received 7 June 2023; Received in revised form 22 September 2023; Accepted 25 September 2023
Available online 29 September 2023

negative. This is time consuming and expensive. Therefore, multiplex PCR screening can be helpful to reduce the number of reactions. Already several multiplex PCR methods have been described (Eum et al., 2019; Grohmann et al., 2017). Grohmann et al. describe a hexaplex qPCR that allows detection of six GM events with only two fluorescent labels; the 6-Carboxyfluorescein (FAM) label, as used in the original singleplex methods, and the 6-Hexachlorofluorescein (HEX) label. These GM event-specific tests are expected to be mostly negative but in case the PCR results are positive, singleplex methods need to be performed in order to identify the specific GM event(s).

In this paper we present two tetraplex qPCR assays that were successfully set-up and in-house validated to simultaneously detect six cotton, soybean and maize GMOs without common screening elements. Each assay consists of a plant-specific actin PCR (Laube et al., 2010) and three event-specific methods with different fluorescent labels. The multiplex PCR 1 (mpPCR 1) method consists of GHB614 cotton, DP305423 soybean, CV127 soybean and actin and the mpPCR 2 method consists of DAS40278 maize, VCO1981 maize, 98140 maize and actin. Previously developed event-specific methods were used. These methods, together with certified reference materials (CRMs), are supplied by the manufacturer of the GMO and have been interlaboratory validated by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) in collaboration with the European Network of GMO Laboratories (ENGL) (EURL GMFF, 2023a). All methods have been developed with a FAM label, but in this study the label colours were designed differentially to be able to distinguish between GM events present. The actin PCR has been included in both mpPCR methods as a positive control to confirm that the DNA is of sufficient quality for qPCR. The GM events included in the two mpPCR methods were selected to complement the extended routine element screening described before (Scholtens et al., 2017).

2. Materials and methods

2.1. Reference materials

CRMs for cotton (MON15985 (AOCS 0804-D), GHB614 leaf DNA (AOCS 1108-A6), conventional cotton leaf DNA (AOCS 0306-A3)), soybean (MON89788 (AOCS 0906-B), A5547–127 (AOCS 0707-C6), MON87701 (AOCS 0809-A), CV127 (AOCS 0911-C), conventional soybean (AOCS 0906-A)), maize (5307 (AOCS 0411-D)) and canola (88302 (AOCS 1011-A), RT73 (AOCS 0304-B2)) were purchased from the American Oil Chemist's Society (AOCS; Illinois, United States). Cotton (GHB119 (ERM-BF428c), T304–40 (ERM-BF429c), 281–24–236 × 3006–210–23 (ERM-BF422b)), soybean (GTS 40–3–2 (ERM-BF410gk), DP305423 (ERM-BF426d)), maize (TC1507 (ERM-BF418d), NK603 (ERM-BF415f), VCO1981 (ERM-BF438b), DAS40278 (ERM-BF433d), 98140 (ERM-BF427d), conventional maize (ERM-BF414a)), potato (EH92–527–1 (ERM-BF421b)) and sugar beet (H7–1 (ERM-BF419b)) were obtained from the Joint Research Centre (JRC; Geel, Belgium). The Bt63 rice control material is a plasmid obtained from the ENGL. The pea and flax materials were purchased in a local supermarket.

2.2. DNA extractions

For all but one of the CRMs (GHB614 cotton), 100 ± 10 mg of material was used to obtain genomic DNA by performing the cetyltrimethylammonium bromide (CTAB) DNA isolation method as described previously (Murray and Thompson, 1980). After dissolving the DNA in 100 µl of PCR-grade water (UltraPure distilled water; Invitrogen), the DNA concentration and purity was determined by the ratio of the absorbance at 260/280 and 260/230 nm wavelengths using a NanoDrop (ND-1000; NanoDrop Technologies, Delaware, United States). The DNA extracts were diluted to 10 ng/µl in PCR-grade water and stored at 4 °C until further use.

2.3. Oligonucleotide primers and probes and multiplex qPCR procedure

Two qualitative mpPCR methods were set-up. The first mpPCR method targets GHB614 cotton, DP305423 soybean, CV127 soybean and the endogenous gene actin. The second mpPCR method targets DAS40278 maize, VCO1981 maize, 98140 maize and actin. The oligonucleotide primers and TaqMan probes used in this work have been previously reported and were synthesized by Integrated DNA Technologies (Leuven, Belgium), except for the DP305423 probe, which was produced by LGC Biosearch Technologies (Middlesex, United Kingdom). Each probe is labelled with a different fluorescent reporter dye at the 5′ end and a Black Hole Quencher at the 3′ end. For details on the primers, probe labelling and quenchers used, see Table 1. Each qPCR was performed in a final reaction volume of 25 µl containing 5 µl 10 ng/µl genomic DNA, 12.5 µl 2x QuantiTect Multiplex PCR NoROX Master Mix (QIAGEN; Hilden, Germany) or, for robustness experiments, Universal Mastermix D600 (Diagenode; Seraing, Belgium), 3.5 µl PCR grade water and 300 nM and 200 nM of each primer and probe, respectively. The reaction mixtures were distributed on 96-well Hard-Shell PCR plates (Bio-Rad Laboratories B.V., Veenendaal, Netherlands). All multiplex qPCR runs were performed on a Bio-Rad CFX96 qPCR machine (Bio-Rad Laboratories B.V., Veenendaal, Netherlands). The PCR reactions consisted of an initial activation step at 95 °C for 600 s to activate HotStarTaq DNA Polymerase by heating, followed by 45 cycles of a denaturation step for 15 s at 95 °C and an annealing/extension step for 60 s at 60 °C. The qPCR conditions were the same for all experiments and each sample was at least analysed in duplicate. Data were analysed using Bio-Rad CFX Maestro 1.0 software. Baselines of each PCR run were automatically calculated and only adjusted manually when necessary. Threshold fluorescence levels were set manually.

2.4. In-house validation of the two multiplex qPCR methods

Prior to implementation, the two qualitative mpPCR methods were validated in-house. The following performance parameters were determined: cross-talk, specificity, limit of detection (LOD), asymmetric limit of detection (LOD_{asym}), efficiency (E), correlation coefficient R^2 and robustness (Broeders et al., 2014; ENGL, 2021; 2017; 2015).

2.4.1. Cross-talk

To minimize the risk of spectral overlap and subsequent cross-talk due to wide fluorescence emission curves, fluorescent dyes that span a broad range of the fluorescent emission spectrum were selected. In addition, we used highly efficient Black Hole Quenchers (BHQs) that are able to reduce most of the background fluorescence because they emit heat instead of light and therefore have no native fluorescence. The quenchers were chosen for the individual PCR modules of mpPCR method 1 and 2 in accordance to recommendations on the LGC Biosearch Technologies website (LGC Biosearch Technologies, 2018). Details are shown in Table 1. Additional to the use of BHQs, potential cross-talk was assessed by performing experiments using 10–100% (w/w) target CRM DNA at 150 ng per reaction.

2.4.2. Specificity

The specificities of the individual event-specific methods were already established since all methods were fully validated previously. During the validation study of each individual event-specific method the primers and probes were checked for (the lack of) relevant homologies by means of bioinformatic analysis by the applicant, which was later confirmed by the EURL-GMFF.

To test for compatibility between primers and probes in each of the two mpPCR methods, all primer and probe combinations of mpPCR 1 and mpPCR 2 were analysed *in silico* for potential self-dimers and cross primer dimers using Multiple Primer Analyzer software (Thermo Fisher Scientific, 2020). The value of the sensitivity for dimer detection was set to 3 (default setting).

Table 1
List of GM events without common screening elements, including the plant actin endogenous gene target, with primer and probe details used in the two mpPCR methods.

Target	Name	Sequence (5′–3′)	Amplicon size (bp)	Final conc. (nM)	Multiplex set	Reference
Actin plant gene	Act-f	CAAGCAGCATGAAGATCAAGGT	103	300	1, 2	(EURL GMFF, 2023b)
	Act-r	CACATCTGTTGGAAAGTGCTGAG		300		
	Act-probe	**FAM**-CCTCCAATCCAGACACTGTACTTYCTCTC-**BHQ1**		200		
GHB614 cotton	SHA007	CAAATACACTTGGAACGACTTCGT	119	300	1	(EURL GMFF, 2023b)
	SHA008	GCAGGCATGCAAGCTTTTAAA		300		
	TM072 probe	**HEX**-CTCCATGGCGATCGCTACGTTCTAGAATT-**BHQ1**		200		
DP305423 soybean	DP305-f1	CGTGTTCTCTTTTTGGCTAGC	93	300	1	(EURL GMFF, 2023b)
	DP305-r5	GTGACCAATGAATACATAACACAAACTA		300		
	DP305-p	**Quasar705**-TGACACAAATGATTTTCATACAAAAGTCGAGA-**BHQ2**		200		
CV127 soybean	SE-127-f4	AACAGAAGTTTCCGTTGAGCTTTAAGAC	88	300	1	(EURL GMFF, 2023b)
	SE-127-r2	CATTCGTAGCTCGGATCGTGTAC		300		
	SE-127-p3	**6-ROX**-TTTGGGGAAGCTGTCCCATGCCC-**BHQ2**		200		
DAS40278 maize	DAS-40278–9_5'-f1	CACGAACCATTGAGTTACAATC	98	300	2	(EURL GMFF, 2023b)
	DAS-40278–9_5'-R3	TGGTTCATTGTATTCTGGCTTTG		300		
	DAS-40278–9_5′-S2	**CY5**-CGTAGCTAACCTTCATTGTATTCCG-**BHQ2**		200		
VCO1981 maize	VCO-01981–5 primer F	CCACTGAACGTCACCAAGAAGA	85	300	2	(EURL GMFF, 2023b)
	VCO-01981–5 primer R	GCCGCTACTCGAGGGATTTA		300		
	VCO-01981–5 probe	**HEX**-CAGTACTCAAACACTGATAG-**BHQ1**		200		
98140 maize	DP098-f6	GTGTGTATGTCTCTTTGCTTGGTCTT	80	300	2	(EURL GMFF, 2023b)
	DP098-r2	GATTGTCGTTTCCCGCCTTC		300		
	DP098-p5	**6-ROX**-CTCTATCGATCCCCCTCTTTGATAGTTTAAACT-**BHQ2**		200		

BHQ1: Black Hole Quencher 1, BHQ2: Black Hole Quencher 2, FAM: 6-Carboxyfluorescein, HEX: 6-Hexachloro-fluorescein, Quasar 705: an indocarbocyanine dye, CY5: Cyanine-5, ROX: 6-Carboxy-X-rhodamine.

Additionally, in order to determine potential cross-reactivity in a multiplex setting experimentally, the specificity of mpPCR 1 and mpPCR 2 was tested on different GM and non-GM CRMs with or without containing these events. The non-target GM reference materials contained a high GMO content (≥4.91% (w/w)) to ensure possible unspecific amplification or artefacts would be identified. For targets, 150 ng DNA per reaction, isolated from 10% to 100% (w/w) reference material was used. All tests were performed in duplicate (ENGL, 2021, 2015).

2.4.3. Sensitivity

The sensitivity of each assay was determined using serial dilutions, including dilutions containing only up to 20, 10, 5 and 1 copies target per PCR reaction, calculated using the haploid genome sizes (1 C-value) of cotton, soybean and maize (Arumuganathan and Earle, 1991). Ten replicates were performed. Performance criterion is that the LOD should be below 25 copies per reaction (ENGL, 2017, 2015).

Especially under highly asymmetrical target conditions, it is possible that the performance of individual PCR modules combined in a multiplex setting is lower compared to their singleplex PCR variants due to competitive effects. Therefore, next to the LOD, also the LOD_{asym} was determined for each target. In total, four dilutions (20, 10, 5 and 1 copies/reaction) of each of the six targets, actin excluded, were tested against 1000 copies per reaction of each of the other targets in the two multiplex qPCR assays. Ten replicates for each target were tested (ENGL, 2021, 2015).

2.4.4. Amplification Efficiency and correlation coefficient

Furthermore, the amplification efficiency and correlation coefficient R^2 were determined for each individual template of the two multiplex qPCR methods by preparing two dilution curves per target with five-fold serial dilutions and two replicates each. Acceptance criteria are that amplification efficiencies should be between 90% and 110% and correlation coefficients ≥ 0.98 (ENGL, 2021, 2017, 2015).

2.4.5. Robustness

To assess the capacity of the two mpPCR methods to remain unaffected by the introduction of small variations in the experimental conditions and materials used in the procedure, robustness tests were performed with the help of a multifactorial experimental design, first described by Plackett et al. in 1946 (Plackett and Burman, 1946). The following factors were tested: change of primer and probe concentrations (30% variation), thermal cycler, master mix brand (QuantiTect and Diagenode), reaction volume (± 1 µl) and annealing temperature (± 1 °C) (CEN, 2019). Concerning the thermal cyclers, only one instrument brand was available, albeit two consecutive models (Bio-Rad C1000 Thermal Cycler and C1000 Touch Thermal Cycler). Therefore, the robustness tests were performed on these two different thermal cyclers, both equipped with the CFX96 optical reaction modules. More information on the used mpPCR setup scheme is shown in Table 2. According to the minimum performance requirements the target amounts to be tested for qualitative modules should be three times the LOD and all replicates should give a positive result (CEN, 2019; ENGL, 2015). In our experiments, each combination was tested in fivefold using CRM dilutions containing target sequence copy numbers at three times the LOD, corresponding to 15–60 copies, depending on the target sequence. Each combination included a no-template control.

Table 2
Experimental set-up for robustness testing.

Factor	Combination							
	1	2	3	4	5	6	7	8
Thermocycler	A	A	A	A	B	B	B	B
Master mix	Q	Q	D	D	Q	Q	D	D
Primer concentration	St	-30%	St	-30%	St	-30%	St	-30%
Probe concentration	St	-30%	-30%	St	-30%	St	St	-30%
PCR reagent mix volume	19 µl	19 µl	21 µl	21 µl	21 µl	21 µl	19 µl	19 µl
Annealing temperature	+1 °C	-1 °C	+1 °C	-1 °C	-1 °C	+1 °C	-1 °C	+1 °C

A: Bio-Rad CFX96 qPCR machine 1, B: Bio-Rad CFX96 qPCR machine 2, Q: QuantiTect Multiplex PCR NoROX master mix, D: Diagenode D600 master mix, St: Standard conditions.

3. Results and discussion

3.1. Cross-talk

Initially, the composition of the two mpPCR methods was slightly different compared to the current configurations shown in Table 1. The mpPCR 1 method consisted of CV127 soybean, DP305423 soybean, GHB614 cotton, DAS40278 maize and actin and mpPCR 2 method consisted of the VCO1981 maize, 98140 maize and actin targets. The reasoning behind those compositions was to separate EU authorised (mpPCR 1) from unauthorised (mpPCR 2) event-specific methods. It was already observed that the partially overlapping cy5 and quasar 705 emission spectra could become problematic in practice. Initial experiments indeed showed fluorescence of the DP305423 probe in the Cy5 channel. Therefore, DAS40278 was shifted from mpPCR 1 to mpPCR 2, the mpPCR1 method now consisting of GHB614 cotton, DP305423 soybean, CV127 soybean and actin and the mpPCR 2 method consisting of only maize event-specific methods DAS40278 maize, VCO1981 maize, 98140 maize and actin. This dedicated mpPCR 2 for GM maize will be particularly useful in the case of analysing (non-GM) maize samples, since mpPCR 2 could now be performed in conjunction with a limited panel of screening elements to be able to cover all known (un) authorised maize events. During experiments with these final mpPCR compositions, no false positive signals due to fluorescence of a single probe apparent in adjacent channels were observed.

3.2. Specificity

To confirm that mpPCR 1 and mpPCR 2 only produce amplification products with the target sequences for which the methods were developed, both *in silico* and experimental analysis were performed. As stated in subsection 2.4.2, the specificity of each separate event-specific detection method was already established since these methods were fully validated previously and therefore limited practical experiments to assess specificity were sufficient.

In silico analysis using Multiple Primer Analyzer software showed potential formation of self-dimers and cross primer dimers between multiple primers and probes. In practise, the specificity, sensitivity and amplification efficiencies of mpPCR 1 and mpPCR 2 were in accordance with the minimum performance requirements (ENGL, 2015), indicating we did not encounter significant primer-dimer issues.

The experimental specificity of the two mpPCR methods was tested using a wide range of plant species. No false positive signals were observed and only GMO materials containing target sequences showed a positive signal as shown in Table 3.

Table 3
Results of specificity testing of mpPCR 1 and mpPCR 2 on target and non-target CRMs.

Reference materials	mpPCR 1	mpPCR 2
>99.99% GHB614 cotton	ND*	ND
10% DP305423 soybean	ND*	ND
96.32% CV127 soybean	ND*	ND
10% DAS40278 maize	ND	ND*
98.6% VCO1981 maize	ND	ND*
10% 98140 maize	ND	ND*
100% non-GM cotton	ND	ND
100% non-GM soybean	ND	ND
100% non-GM maize	ND	ND
100% non-GM pea	ND	ND
100% non-GM flax	ND	ND
10% GHB119 cotton	ND	ND
98.45% MON15985 cotton	ND	ND
10% T304-40 cotton	ND	ND
>97.9% 281-24-236 x 3006-210-23 cotton	ND	ND
10% GTS 40-3-2 soybean	ND	ND
>99.40% MON89788 soybean	ND	ND
100% A5547-127 soybean	ND	ND
>99.94% MON87701 soybean	ND	ND
9.86% TC1507 maize	ND	ND
4.91% NK603 maize	ND	ND
>99.88% 5307 maize	ND	ND
>99.94% MON88302 canola	ND	ND
>99.19% RT73 canola	ND	ND
100% EH92-527-1 potato	ND	ND
100% H7-1 sugar beet	ND	ND
Bt63 rice plasmid (2000 cp/µl)	ND	ND

ND: not detected. *: Only the targeted GM event showed a positive signal, the other GM events in this mpPCR method were negative, as expected.

3.3. Sensitivity

The LOD is the smallest copy number for which all PCR reactions are positive. Both mpPCR methods met the minimum performance requirements of < 25 copies per reaction (ENGL, 2015). The LOD of each previously interlaboratory validated singleplex PCR method shows equal or even slightly higher values compared to the LOD of the six individual modules of which the two multiplex methods consist.

The LOD_{asym} is defined as a performance parameter for the sensitivity of a mpPCR method when a target is present at a low concentration, compared to the other targets at high amounts and can be of special importance when screening for the potential low level presence of EU authorised and unauthorised GM events in feed, food and seed samples using mpPCR methods. The LOD_{asym} results of this study are comparable to the LOD values of the individual modules of both multiplex methods previously validated, except for those of GHB614 cotton and DAS40278 maize. These do meet the EURL minimum performance requirements, but are slightly higher at 10 and 20 copies per PCR reaction, respectively. All no-template controls were negative.

The LOD values for the previously interlaboratory validated singleplex modules as well as for the multiplex methods and the LOD_{asym} values for each target are summarized in Table 4.

3.4. Amplification Efficiency and correlation coefficient

The two mpPCRs described here are qualitative methods. Therefore, assessing the amplification efficiency (E) and the correlation coefficient *R2* performance characteristics is not obliged for validation, but gives a

Table 4
LOD and LOD_{asym} results of the singleplex and mpPCR 1 and mpPCR 2.

Target	LOD singleplex PCR (cp)*	LOD mpPCR 1 (cp)*	LOD_{asym} mpPCR 1 (cp)*	LOD mpPCR 2 (cp)*	LOD_{asym} mpPCR 2 (cp)*
CV127 soybean	10	10	5		
DP305423 soybean	20	10	10		
GHB614 cotton	5	5	10		
98140 maize	10			5	5
VCO1981 maize	20			20	10
DAS40278 maize	20			10	20

*Copies/25 µl qPCR reaction.

good indication of PCR performance. There are several parameters that could affect the amplification efficiency of a PCR method. Examples are PCR inhibitors in the DNA samples, amplicon length, suboptimal primer/probe design, reagent concentrations and reaction conditions (Debode et al., 2017; Svec et al., 2015). Amplification efficiencies may also be affected by competing effects between the different targets within each of the two multiplex assays. Therefore, the efficiencies of the PCR modules of each multiplex assay should not differ more than 15% (Huber et al., 2013). For the two multiplex assays, the amplification efficiencies and correlation coefficient R^2 of the individual qPCR modules met the minimum acceptance criteria of E = 90%− 110% (corresponding to a slope between −3.1 and −3.6) and R^2 value ≥ 0.98. Furthermore, the amplification efficiencies of the individual PCR modules within each multiplex assay did not differ more than 15%. Details on the results are shown in Table 5.

3.5. Robustness

In total, six experimental conditions were slightly altered to study the robustness of the mpPCR methods. Changing the PCR instrument, slight variations in master mix volume, primer or probe concentration and the annealing temperature did not result in significant changes in sensitivity at the level of three times the LOD for each target. All replicates were positive, indicating sufficient robustness of the two mpPCRs. Changing the master mix from the QuantiTect Multiplex PCR NoROX master mix, specifically developed for mpPCR assays, to the Diagenode D600 master mix, resulted in not reaching the three times the LOD values. When using the Diagenode mastermix, 199/240 reactions were positive. This demonstrates the importance of choosing a suitable master mix, which optimizes the PCR conditions significantly. The additional no-template controls, included in duplicate for each individual target of the two multiplex methods, were negative with no exception.

4. Conclusions

The results of this study show that the two mpPCR methods presented here meet the requirements for specificity, sensitivity and robustness, the latter indicating good reliability during normal usage when a mastermix designed for multiplex qPCR assays is used. In addition, no cross-talk was observed and the amplification efficiencies and the correlation coefficients of the targets within the two mpPCR methods all fall within the acceptance range of 90%− 110% and ≥ 0.98, respectively. To date, qPCR remains the preferred detection technique for GM plants. Due to the increasing number of EU-authorised GMOs since the commercialization of GM crops that started in 1994 with the FLAVR SAVR™ GM tomato, screening for the presence of these events becomes more costly and labour intensive every year. In addition, GM events without any common screening elements make routine GMO testing even more challenging. The combination of two event-specific mpPCR methods that were set-up and validated in-house are ready to use and will lead to a faster and more efficient routine GMO screening.

Table 5
Performance parameters of PCR standard curves of mpPCR 1 and mpPCR 2.

Target	Efficiency (E)	Slope	Correlation coefficient (R^2)
CV127 soybean	99%	-3.36	1.00
DP305423 soybean	94%	-3.47	1.00
GHB614 cotton	109%	-3.12	1.00
98140 maize	102%	-3.27	1.00
VCO1981 maize	110%	-3.10	0.98
DAS40278 maize	109%	-3.13	1.00

Funding

This research was funded by the Dutch Ministry of Agriculture, Nature and Food Quality, as part of the GMO method validation project 'Validatie en accreditatie van detectiemethoden voor genetisch gemodificeerde organismen' (WOT-02–004–005).

CRediT authorship contribution statement

Jorg A.B. Nijland: Conceptualization, Investigation, Visualisation, Validation, Writing- Original draft preparation. **Margriet W.J. Hokken:** Writing- Reviewing and Editing. **Theo W. Prins:** Conceptualization, Writing- Reviewing and Editing.

Declaration of Competing Interest

The authors declare that they have no relevant interest(s) to disclose.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Ingrid Scholtens for her valuable input and expertise and Merel Lonis for her practical work during this study.

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