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ORIGINAL PAPER

Validation of a newly developed hexaplex real-time PCR assay for screening for presence of GMOs in food, feed and seed

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Abstract For years, an increasing number and diversity of genetically modified plants has been grown on a commercial scale. The need for detection and identification of these genetically modified organisms (GMOs) calls for broad and at the same time flexible high throughput testing methods. Here we describe the development and validation of a hexaplex real-time polymerase chain reaction (PCR) screening assay covering more than 100 approved GMOs containing at least one of the GMO targets of the assay. The assay comprises detection systems for Cauliflower Mosaic Virus 35S promoter, *Agrobacterium tumefaciens* NOS terminator, Figwort Mosaic Virus 34S promoter and two construct-specific sequences present in novel genetically modified soybean and maize that lack common screening elements. Additionally a detection system for an internal positive control (IPC) indicating the presence or absence of PCR inhibiting substances was included. The six real-time PCR systems were allocated to five detection channels showing no significant crosstalk between the detection channels. As part of an extensive validation, a limit of detection (LOD_{abs}) ≤ ten target copies was proven in hexaplex format. A sensitivity ≤ ten target copies of each GMO detection system was still shown in highly asymmetric target situations in the presence of 1,000 copies of all other GMO targets of each detection channel. Furthermore, the applicability to a broad sample spectrum and reliable indication of inhibition by the IPC system was demonstrated. The presented hexaplex assay offers sensitive and reliable detection of GMOs in processed and unprocessed food, feed and seed samples with high efficiency.

Keywords GMO · Multiplex · Hexaplex · Real-time PCR · Screening · Validation

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Introduction

Each year, an increasing number of different genetically modified (GM) plants are grown on commercial scale. The growing number of different genetically modified crops goes in parallel with an increase in the diversity of genetic modifications in commercialised genetically modified organisms (GMOs). Detection and identification—e.g. to verify compliance with food-labelling requirements—becomes more and more complex. Additionally, GM plants lacking common screening elements enter the food and feed chain and require revision of the common screening procedures. Accidental contamination of seed with 'non-approved' GMOs or illegal planting of new varieties (e.g. Bt63 rice) is another issue in this context. Thus broad and at the same time flexible new testing methods are needed. In combination with additional modular identification methods, this allows cost-efficient analysis and gives a comprehensive answer to the question if and which GMO is present in the sample.

Recent approaches consist of an initial amplification step with multiplex polymerase chain reaction (PCR) followed by a method for separation and detection of the PCR products, e.g. by agarose gel electrophoresis with ethidium bromide staining [1–6], capillary gel electrophoresis [7–10] or hybridization microarray technology [11–14].

Another approach is the use of real-time PCR offering a substantial advantage as compared to the former mentioned

methods—the lack of post-PCR manipulation. Integration of PCR and detection of the amplification products in a closed system minimises the risk of carryover contamination [15]. Furthermore the capability of real-time PCR for a high automatisation level can reduce hands-on time and consequently the costs per analytical result.

Multiplexing offers advantages as e.g. increased throughput and reduced turnaround times of GMO samples [16]. Furthermore multiplex PCR can increase the reliability by eliminating the risk of intertube variability (e.g. pipetting errors).

Several duplex real-time PCR assays, including cauliflower mosaic virus (CaMV) 35S promoter and/or *Agrobacterium tumefaciens* NOS terminator PCR detection system, have already been published [17–19]. Lately a quadruplex real-time PCR screening assay has been described comprising PCR systems for the detection of 35S promoter and NOS terminator in combination with reference systems for soy (lectin gene) and maize (alcohol dehydrogenase gene) [20].

A critical aspect linked to more complex real-time PCR assays and accordingly a higher number of different dyes involved is the increased likelihood of crosstalk. Crosstalk is a fluorescence increase in a detection channel caused by a dye which is not intended to be measured in a given detection channel. Therefore in a multiplex assay, the optimum combination of reporter dyes, which can vary from instrument to instrument, is of particular importance.

Over the past years, mainly new GM maize and GM soybean events have been approved. Here we describe the development and validation of a hexaplex real-time PCR assay covering more than 100 approved GMOs containing at least one of the GMO targets of the assay including all currently commercialised GM maize and GM soybean events. Combining three different screening methods, two modification-specific primer sets and an IPC system, the new assay was shown to be time and cost efficient as well as robust and fit for purpose under routine testing conditions. To prove performance, new validation approaches and controls had to be developed and implemented to account for the increased complexity of the multiplexed assay type.

Materials and methods

Reference material

For specificity testing, certified reference materials (CRMs) and DNA extracts were purchased from IRMM (Geel, Belgium), AOCS (Urbana, IL, USA) and Bayer BioScience (Gent, Belgium) respectively: soybean: GTS 40-3-2 (ERM-BF410gk), MON89788 (AOCS 0906-B), A5547-127 (AOCS 0707-C), A2704-12 (AOCS 0707-B), DP305423 (ERM-BF426d), DP356043 (ERM-BF425d); maize: Bt 176 (ERM-BF411F), GA21 (ERM-BF414d), MON810 (ERM-BF-413-3), Bt-11 (ERM-BF412F), NK603 (ERM-BF415F), MON863 (ERM-BF416D), TC1507 (ERM-BF418D), MIR604 (ERM-BF423D), MON88017 (AOCS 0406-D), 59122 (ERM-BF424D), 3272 (ERM-BF420C), T25 (AOCS 0306-H); rapeseed: T45 (AOCS0208-A), Ms8 (AOCS-0306-F), Rf3 (AOCS0306-G), RT73 (AOCS0304-B), Ms1, Rf1, Rf2, HCN92; cotton: MON1445 (AOCS 0804-B), 281-24-236 × 3006-210-23 (ERM-BF422B); MON531 (AOCS-0804-C), MON15985 × MON1445 (AOCS-0804-D), LLCotton25 (AOCS 0306-E); rice: LLRICE62 (AOCS0306-I); sugar beet: H7-1 (ERM-BF419b); potato: EH92-527-1 (AOCS 0806-D). At the time of development and validation, no CRM for maize LY038 has been available; thus non-certified material had to be used. Non-GM materials tested and used for preparation of DNA mixtures were cotton, soybean, canola, maize, rice, potato and sugar beet.

DNA extraction

DNA from GM and non-GM material was extracted by a protocol based on a cetyltrimethylammonium bromide (CTAB) extraction method [21] including an initial overnight incubation with RNase and Proteinase K. Subsequent purification was performed using gravity flow, anion exchange columns Genomic-tip 500/G (Qiagen, Hilden, Germany) following the instructions of the manufacturer.

Preparation of DNA samples for specificity testing

For specificity testing, 1% GM samples were prepared with a total DNA concentration of 20 ng/µl. Non-GM DNA samples were also normalised to a DNA concentration of 20 ng/µl.

Preparation of positive control material

Positive control material was prepared by cloning PCR products containing the target sequences each into a pCR®2.1 transformation vector (Invitrogen GmbH, Karlsruhe, Germany). Plasmids were then linearised using a restriction enzyme cutting opposite to the multiple cloning site. The plasmid DNA was quantified by fluorescence detection using the Quant-iT™ PicoGreen® dsDNA Reagent (Invitrogen, Karlsruhe, Germany) and diluted with 0.1× TE containing 10 ng/µl ssDNA from salmon testes (Sigma-Aldrich Chemie GmbH, Munich, Germany) to a stock solution of 10^5 copies plasmid DNA per microlitre, corresponding to 10^5 PCR target copies per microlitre. These stock solutions were diluted to a working solution of 1,000 copies and used for the

Table 1 Evaluation criteria in relation to the respective positive control as reference; cycle threshold (Ct) cut-off and fluorescence intensity (dR) limit$_{(Check)}$

System	35S	NOS	FMV	LY/SAMS	IPC
Ct cut-off: mean Ct($PosC_{GMO}$/NTC_{IPC})+Ct	8	7	7	7	4
dR limit: mean dR($PosC_{GMO}$/NTC_{IPC})	20%	20%	10%	15%	45%
dR $limit_{Check}$: mean dR($PosC_{FMV\ /\ LY/SAMS}$)	–	–	7.5%	10%	–

preparation of positive control reactions and spiking in development and validation experiments. Asymmetric target ratio samples were also diluted and mixed from these stock solutions.

Primers and probes

For two newly developed construct-specific real-time PCR systems, new primers and TaqMan™ probes have been designed using Primer Express® software v2.0 (Applied Biosystems, Foster City, USA). Primer sequences of the PCR systems: NOS PCR system: forward primer (ggcaa taaagtttcttaagattgaatcctg), reverse primer (catgcttaacgtaatt caacagaaatt), probe (HEX–ttgccggtcttgcgatgattatcat–BHQ-1); FMV PCR system: forward primer (aagacatccaccgaa gacttaaagttagtg), reverse primer (tctgcaccattccttttttgtctg), probe (CAL610–tgaaagtaatcttgtcaacatcgagcagctgg–BHQ-2); LY PCR system: forward primer (caatctgtgactggtagagg gaagg), reverse primer (gccgaagtgctctactccggtctt), probe (Cy5–ttccttggcagccatcactagtacaggttta–BHQ-2); SAMS PCR system: forward primer (gcttgttgtgcagtttttgaagtataacc), reverse primer (gaatcgggtggttctggaa), probe (Cy5–ccaca caacacaatggcggcca–BHQ-2). IPC PCR system: forward primer (agctctttgtgcgaaaggc), reverse primer (gtgaggattcg gacacgg), probe (ATTO425–tcgcctcccacgtctcaccga–DDQ-1). The CaMV 35S promoter specific real-time PCR system was taken from the ISO 21570:2005 "Screening method for the relative quantitation of the 35S-promoter DNA of soya bean line GTS40-3-2 using real-time PCR" [22], but the probe was labelled with FAM as a reporter dye and BHQ-1 as a quencher. 'In-silico' multiplex PCR was performed using Clone Manager Professional Version 9 (Scientific & Educational Software, Cary, USA) to evaluate potential oligonucleotide interactions leading to primer/probe dimerisation.

Real-time PCR

The real-time PCR experiments were performed on Stratagene Mx3005P QPCR system comprising an ATTO425, FAM, HEX, ROX and Cy5 filter set using MxPro–Mx3005p v4.00 Build 367, Schema 80 software (Agilent-Stratagene, Waldbronn, Germany).

Final concentration of the forward and reverse primer in all GMO detection systems was 300 nM and 150 nM for the probes. For the IPC system forward primer, reverse primer and probe were applied with 100 nM. In PCR reactions, a reagent mix from single components has been used containing the following components in specified final concentrations: 1× GeneAmp® PCR Buffer II, 2 units AmpliTaq Gold® DNA polymerase (both from Applied Biosystems, Foster City, USA), 0.01% Tween 20, 0.8% glycerol, 5.5 mM $MgCl_2$ (all from Sigma-Aldrich Chemie GmbH, Munich, Germany) and 200 µM dNTP (GE Healthcare, Munich, Germany).

The total reaction volume of 25 µl was made up with 20 µl mastermix containing all primers and probes and 50 copies of the $plasmid_{IPC}$ and 5 µl template solution.

All real-time PCR experiments were run with the following cycling parameters, 15 min at 95 °C followed by 45 cycles consisting of 15 s at 95 °C and 90 s at 60 °C.

The assay layout was as follows: four No Template Control (NTC) reactions with 5 µl 0.1× TE buffer instead of DNA template solution, however, containing 50 copies $plasmid_{IPC}$ and two positive control reactions (PosC) in duplicates each. All four PosC reactions comprised 50 copies of $plasmid_{35S}$, $plasmid_{NOS}$ and $plasmid_{FMV}$, while two reactions contained 50 copies of $plasmid_{LY}$ (PosC1), and the other two reactions contained 50 copies of $plasmid_{SAMS}$ (PosC2).

Table 2 Test reaction scoring in GMO detection systems (35S, NOS, FMV, LY/SAMS resp.)

Ct	dR	Result
Ct_{GMO} sample≤Ct_{GMO} cut-off	dR_{GMO} sample≥dR_{GMO} limit	Reaction positive
Ct_{GMO} sample≤Ct_{GMO} cut-off	dR_{GMO} sample<dR_{GMO} limit	Check amplification
$Ct_{FMV\ /\ LY/SAMS}$ sample>$Ct_{FMV\ /\ LY/SAMS}$ cut-off	$dR_{FMV/LY/SAMS}$ $limit_{Check}$≤$dR_{FMV/LY/SAMS}$ sample<$dR_{FMV/LY/SAMS}$ limit	Check amplification
Ct_{GMO} sample>Ct_{GMO} cut-off	dR_{GMO} sample≥dR_{GMO} limit	Reaction positive
$Ct_{35S/Nos}$ sample>$Ct_{35S/NOS}$ cut-off	$dR_{35S/Nos}$ sample<$dR_{35S/Nos}$ limit	Reaction negative
$Ct_{FMV\ /\ LY/SAMS}$ sample>$Ct_{FMV\ /\ LY/SAMS}$ cut-off	$dR_{FMV/LY/SAMS}$ sample<$dR_{FMV/LY/SAMS}$ $limit_{Check}$	Reaction negative
No Ct_{GMO}	–	Reaction negative

Table 3 Final result combining IPC and GMO screening results

IPC	GMO detection system	Final result
Valid	Positive	Positive
Valid	Negative	Negative
Invalid	Positive	Positive
Invalid	Negative	Inhibited

Data evaluation

For all real-time PCR experiments, adaptive baseline setting was used. Threshold was set individually for each run and each detection channel in the region of exponential amplification based on the respective positive control reactions. Data evaluation was performed using specific evaluation criteria, which were defined in relation to a positive control as reference: Positive control for the GMO detection systems were the respective PosC reactions (PosC1 and PosC2), while for the IPC system, NTC reactions served as positive control. As evaluation criteria cycle threshold (Ct) cut-offs, describing a maximum Ct shift relative to the mean Ct of the positive control reactions, and fluorescence intensity (dR) limits, describing a minimum percentage of the mean dR of the positive control reactions, were defined for each detection channel. For FMV and LY/SAMS detection channels, additionally dR $limit_{Check}$ criteria were defined (Table 1). The IPC in a test reaction was scored as valid if the Ct_{IPC} value of the sample was $\leq$ the Ct_{IPC} cut-off and the dR_{IPC} value of the sample $\geq$ dR_{IPC} limit. If one criterion or both were not fulfilled, the IPC reaction was scored as invalid. In case of the GMO detection systems, test reactions were scored as positive or negative or were indicated to be scored individually. The evaluation matrix describing all possible combinations of the evaluation criteria and the respective result is shown in Table 2. In case the result was 'Check amplification', this led to a check of the amplification plot of the reaction in the respective detection channel for presence or absence of a sigmoid PCR amplification signal and to a positive or negative scoring. The final result was generated by combining the IPC result and the result of the GMO detection systems (Table 3). The complete evaluation algorithm was integrated in a spreadsheet tool and used for all validation experiments.

Results

Selection of systems

The following targets were selected to be combined in a hexaplex real-time PCR assay:

- 35S: Cauliflower Mosaic Virus (CaMV) 35S promoter
- NOS: *Agrobacterium tumefaciens* NOS terminator
- FMV: Figwort Mosaic Virus (FMV) 34S promoter
- SAMS: Transition from *S*-adenosyl-L-methionine synthetase (SAMS) promoter to *Glycine max* acetolactate synthase (*gm-hra*) gene
- LY: Transition from *Zea mays*chloroplast transit peptide sequence for dihydrodipicolinate synthase to *Corynebacterium glutamicum* dihydrodipicolinate synthase (*cordapA*) gene encoding for a lysine-insensitive dihydropicolinate synthase enzyme
- IPC: Sequence of non-plant origin

The detection systems for CaMV 35S promoter, NOS terminator and FMV 34S promoter were selected because these regulatory elements are most frequently present in GMOs. However, these elements do no longer provide complete screening coverage for maize and soybean due to new varieties on the market, which lack these elements. Therefore two new construct-specific PCR systems were developed and included in the hexaplex assay.

The integrated IPC system, detecting a sequence of non-plant origin, enabled the verification of absence of PCR inhibitors.

Hexaplex assay development

A prerequisite of multiplexing with dye-labelled probes is an elaborate labelling strategy. Therefore a combination of

Table 4 Properties of reporter dyes and non-fluorescent quenchers used for probe labelling

Reporter dye				Non-fluorescent quencher	
Name	Excitation max. [nm]	Emission max. [nm]	Colour	Name	Absorption max. [nm]
ATTO425	436	484	Blue	DDQ-1	473
6-FAM	495	520	Yellow-green	BHQ-1®	534
HEX	535	556	Yellow	BHQ-1®	
CAL Fluor 610	590	610	Orange-red	BHQ-2®	579
Cy5	643	667	Red	BHQ-2®	

Table 5 Overview of real-time PCR detection systems and the respective detection channels

PCR system	Probe label	Filter set	Filter excitation max. [nm]	Filter emission max. [nm]
35S	6-FAM BHQ-1®	FAM	492	516
NOS	HEX BHQ-1®	HEX	535	555
FMV	CAL Fluor 610 BHQ-2®	ROX	585	610
LY	Cy5 BHQ-2®	Cy5	635	665
SAMS	Cy5 BHQ-2®			
IPC	ATTO425 DDQ-1	ATTO425	440	492

five reporter dyes was established, showing no significant crosstalk between the five detection channels on the real-time PCR platform. All reporter dyes were combined with a non-fluorescent quencher showing an absorption maximum similar to the emission maximum of the respective reporter dye (Table 4).

To allocate six real-time PCR systems to five detection channels, the frequency of appearance and distribution of the GMO targets was taken into account. Hence, Cy5 detection channel was chosen for double-use measuring signals from two PCR systems detecting less frequently occurring GMO targets (Table 5). For subsequent identification of a positive signal in the double-used Cy5 detection channel, duplex real-time PCR systems combining each GMO target with the IPC system were developed in parallel.

The primer and probe concentrations for all GMO detection systems were kept equal, while the IPC system was applied with lowered primer and probe concentrations.

In the context of a former assay development, it was shown that normalisation by a passive reference dye was not necessary for homogeneous performance on the used real-time PCR platform. No significant differences were observed in the standard deviation (SD) of the Ct values and relative standard deviation (rel. SD) of the fluorescence intensity values from 96 identical IPC reactions with and

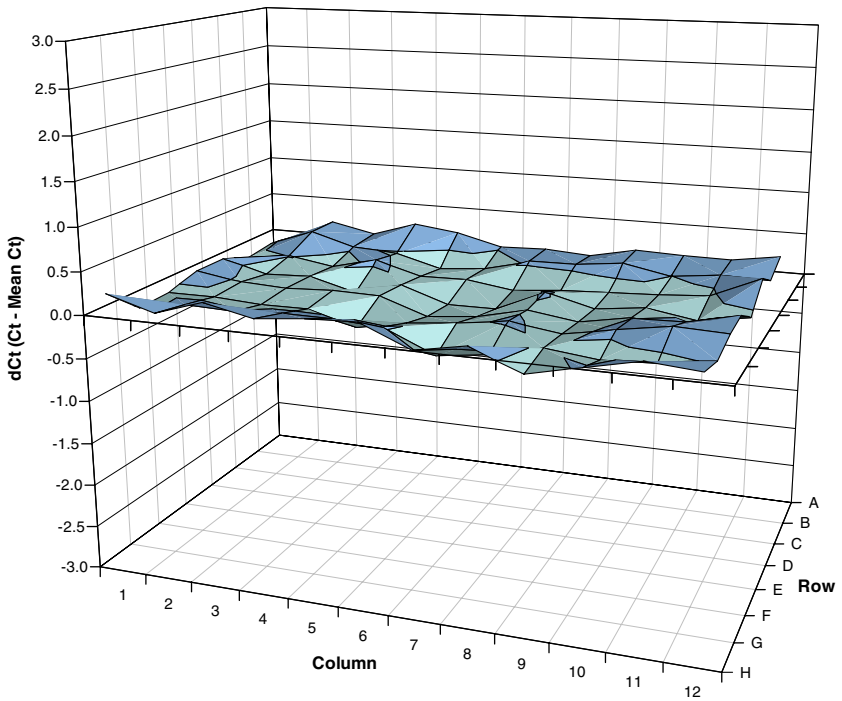

Fig. 1 Distribution of deltaCt (dCt) values of the NOS real-time PCR system in a multiplex assay homogeneity experiment. Ninety-six identical reactions in this experiment contained a mix spiked with 500 copies of $plasmid_{35S}$, $plasmid_{NOS}$, $plasmid_{FMV}$, $plasmid_{LY}$ and 50 copies $plasmid_{IPC}$. The mean Ct value of all 96 identical reactions was calculated for the NOS terminator detection system. dCt values were obtained by subtracting the mean Ct value from the Ct value of every single reaction in the NOS PCR system. These dCt values were then plotted in 96-well format showing a homogeneous distribution with a maximum dCt of 0.3

Table 6 Statistical indicators for uniform performance of the multiplexed real-time PCR systems

Target of Cy5 detection channel	Statistical indicators	35S system	NOS system	FMV system	LY system	SAMS system	IPC system
$plasmid_{LY}$	SD (Ct)	0.13	0.15	0.15	0.23	–	0.32
	rel. SD (dR)	7%	7%	5%	7%	–	8%
$plasmid_{SAMS}$	SD (Ct)	0.14	0.13	0.13	–	0.23	0.37
	rel. SD (dR)	6%	6%	6%	–	7%	7%

without normalisation by ROX reference dye (data not shown). To prove the homogeneous performance of the hexaplex assay, two test plates each consisting of 96 identical reactions were performed (Fig. 1). While in both experiments the reactions included 500 copies of $plasmid_{35S}$, $plasmid_{NOS}$, $plasmid_{FMV}$ and 50 copies $plasmid_{IPC}$, in the first experiment, the reactions contained 500 copies of $plasmid_{LY}$ and in the second 500 copies $plasmid_{SAMS}$ instead. The standard deviation of the Ct values and the relative standard deviation of the dR values from all identical reactions are shown in Table 6. The obtained SD of the Ct values ranged from 0.13 to 0.23, and the rel. SD of the dR values from 5% to 7% in the GMO detection systems. A SD of 0.32 and 0.37 and a rel. SD of 7% and 8% of the IPC system were in the range of obtained values from homogeneity experiments performed in the context of former assay developments (data not shown). The slightly elevated SD values of the IPC system compared to the GMO detection systems is likely due to the fact that it was developed as a less robust system with lowered primer and probe concentration to indicate the presence of inhibitors sensitively.

Validation

Different aspects were tested in an extensive validation proving that the developed hexaplex assay is fit for purpose:

- Specificity
- Limit of detection absolute (LOD_{abs})
- IPC reliability
- Competitive effects
- Matrix effects
- Stability

Specificity

The specificity testing performed in hexaplex assay format included DNA preparations from all commercially available GMs from soybean, maize, canola, cotton, rice, sugar beet, potato material and the respective non-GM plant DNAs as described above. All DNA preparations were tested in triplicates, and the obtained results for GM plant DNAs were in compliance with the expectations according to the theoretical presence of the tested GMO targets as recorded in publicly available GMO databases [23, 24]. Non-GM plant DNA preparations were tested negative in all GMO detection systems. To confirm specificity, amplicons of SAMS and LY PCR system were sequenced as well as amplicons of NOS and FMV PCR system, exemplarily.

Limit of detection absolute (LOD_{abs})

To investigate the LOD_{abs} of the GMO detection systems in hexaplex assay format, 12 reactions each theoretically containing 0.693 copies of the respective positive control DNA were tested. According to statistics, this means that 50% of all PCR reactions contained ≥ one DNA template molecule, whereas 50% of all PCR reactions did not contain any DNA template molecule. Consequently, provided single-copy sensitivity, the expected number of positive reactions was six out of 12 replicates—and the experimentally observed numbers of positive reactions ranged from five out of 12 to seven out of 12 in the multiplex situation (Table 7). In all validation experiments, a minimum of 122 (35S, NOS and FMV), respectively, 61 multiplex reactions (LY and SAMS) have been performed containing ten target copies. One hundred percent of these reactions were positive. Accordingly, a LOD_{abs} ≤ ten target copies for all GMO detection systems has been demonstrated.

IPC reliability

The following validation experiments demonstrated the sensitivity and reliability of the IPC system, indicating the presence of inhibiting substances in the DNA preparations.

Table 7 Number of positive reactions out of 12 reactions each containing 0.693 copies of the respective positive control DNA tested in multiplex format

35S	NOS	FMV	LY	SAMS
7/12	5/12	6/12	6/12	7/12

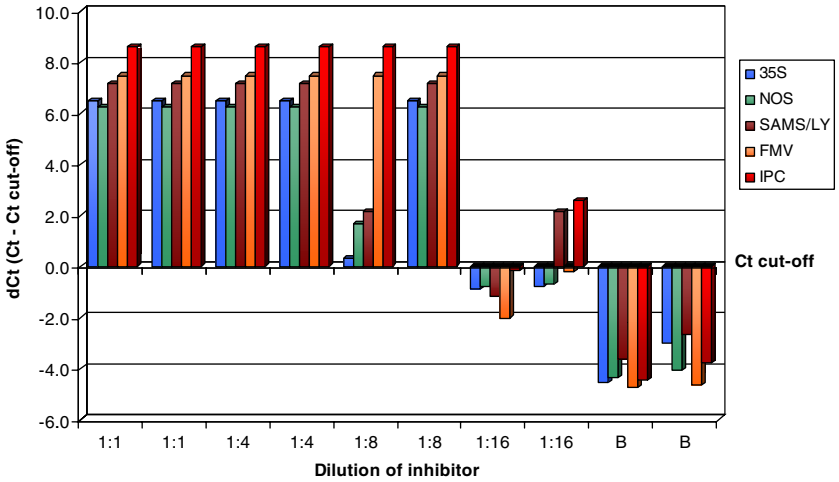

Fig. 2 Ct scoring of each detection channel in multiplex test reactions containing different dilutions of inhibitor. Duplicate reactions comprised ten copies of plasmid$_{35S}$, plasmid$_{NOS}$, plasmid$_{SAMS}$, plasmid$_{FMV}$ in the reaction mix and 5 µl of an inhibiting DNA extract in different dilutions or 5 µl 0.1× TE buffer (B). Depicted is the dCt of the obtained Ct values to the respective Ct cut-off values of each detection channel calculated based on the positive control reactions. A positive dCt means that the Ct value was above the Ct cut-off leading to a negative Ct scoring of the reaction in the GMO detection systems and a scoring as invalid of the IPC reaction. In all reactions where any of the GMO detection systems was above the Ct cut-off (1:1 to 1:8 and one of two 1:16 dilutions of the inhibitor), the IPC reaction was scored as invalid indicating inhibition

In the first experimental setup, reactions were spiked with ten copies of each positive control DNA. Due to the double use of the Cy5 detection channel, two test series were carried out, one with positive control material for SAMS system and the other with the positive control material for LY system. Four tests were performed with two inhibiting DNA preparations and two artificial inhibitors (CTAB and milk). These inhibitors were diluted and tested in duplicates. The highest inhibitor concentration resulted in a total inhibition of all PCR systems, while the lowest concentration showed only partial or no inhibition (Fig. 2). As a result, in no reaction the IPC was scored valid while in any of the GMO detection systems scored as negative, leading to a false-negative analytical result in the presence of ten copies GMO target.

In a second approach, the hexaplex assay was tested for IPC reliability in a experimental setup designed to simulate most realistically the application in GMO testing routine. Therefore sample material was used which showed (partial) inhibition in pre-testing and finally negative analytical results for 35S promoter and NOS terminator. Forty four sample materials were chosen representing food, feed and seed of a broad range of matrices for screening assay application. DNA preparations of these specifically challenging sample materials were then spiked with ten copies positive material for each GMO detection system in two series. The analysis of these samples in duplicates did not give any false-negative analytical result in any GMO detection system (Table 8). All reactions were scored as positive or inhibited according to the evaluation algorithm.

Competitive effects

To evaluate the sensitivity of the GMO detection systems in asymmetric target scenarios, various template mixes were tested with different target copy numbers. While competing targets were present in large excess, the target of the GMO detection system under investigation was limited. The tested ratios and corresponding results are summarised in Table 9. A reliable detection of ten copies of one GMO detection system was demonstrated in the presence of 50 copies IPC target and 1,000 target copies of all other GMO detection systems of each detection channel—corresponding to a target ratio of 1:100 (Fig. 3). Furthermore 12 reactions were tested each containing theoretically 0.693 copies of a GMO target in the presence of 64 copies of all other GMO targets of each detection channel and 50 copies IPC target. Between nine of 12 and three of 12 positive reactions were obtained for the GMO detection system under investigation. These results show a limit of detection ≤ ten target copies

Table 8 Results of validation experiments with spiked, (partially) inhibited DNA extracts from diverse sample matrices

Analytical result	False negative	Positive or inhibited
35S	0	88
NOS	0	88
FMV	0	88
SAMS	0	44
LY	0	44

Table 9 Performance of the hexaplex assay in asymmetric target scenarios with all reactions containing 50 copies IPC target

Target copy numbers					Number of positive reactions				
35S	NOS	FMV	LY	SAMS	35S	NOS	FMV	LY	SAMS
0.693	64	64	64	-	9/12	12/12	12/12	12/12	-
0.693	64	64	-	64	7/12	12/12	12/12	-	12/12
64	0.693	64	64	-	12/12	6/12	12/12	12/12	-
64	0.693	64	-	64	12/12	4/12	12/12	-	12/12
64	64	0.693	64	-	12/12	12/12	6/12	12/12	-
64	64	0.693	-	64	12/12	12/12	3/12	-	12/12
64	64	64	0.693	-	12/12	12/12	12/12	4/12	-
64	64	64	-	0.693	12/12	12/12	12/12	-	5/12
10	1000	1000	1000	-	3/3	3/3	3/3	3/3	-
10	1000	1000	-	1000	3/3	3/3	3/3	-	3/3
1000	10	1000	1000	-	3/3	3/3	3/3	3/3	-
1000	10	1000	-	1000	3/3	3/3	3/3	-	3/3
1000	1000	10	1000	-	3/3	3/3	3/3	3/3	-
1000	1000	10	-	1000	3/3	3/3	3/3	-	3/3
1000	1000	1000	10	-	3/3	3/3	3/3	3/3	-
1000	1000	1000	-	10	3/3	3/3	3/3	-	3/3

of each GMO detection system, even under highly asymmetric target conditions.

Matrix effects

To check for matrix-specific effects, 44 sample materials from various matrices, which were 35S and NOS negative and showed no inhibition in pre-testing, were tested with the hexaplex screening assay. As a result, neither matrix-specific inhibition nor unspecific signals in any multiplexed PCR system were observed (data not shown).

Stability

The proper functioning of the assay after storage of the mixes (reagent and oligonucleotide mix separately) under two storage conditions was validated in this experiment. For the first storage, variant mixes were kept for 3 days at

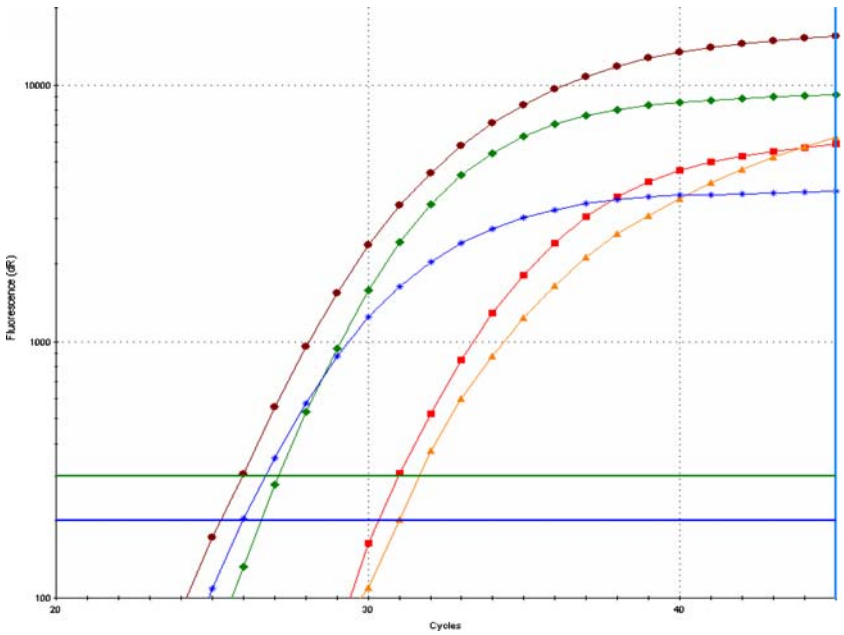

Fig. 3 PCR amplification curves of an asymmetric target ratio sample. The graph shows the amplification curves of SAMS (*brown*), NOS (*green*) and 35S (*blue*) PCR systems with a target copy number of 1,000 copies per reaction each, in comparison to the amplification curve of the FMV system (*orange*) with only ten target copies per reaction. The IPC system amplification in the presence of 50 target copies per reaction is shown in *red*

Table 10 Stability in assay performance after storage

System		35S	NOS	FMV	LY	SAMS	IPC
dCt (mean Ct_{VAR}-mean Ct_{REF})	5× freeze–thaw	−0.15	−0.3	0.0	0.1	0.4	−0.05
	3 days at RT	0.05	0.1	−0.05	0.5	0.6	−0.05
n		12	12	12	6	6	12

room temperature, and for a second storage, variant mixes were frozen and thawed five times, simulating potential strain. As reference, variant mixes were frozen and thawed once. Six reactions containing ten copies of all GMO targets and 50 copies of the IPC target were tested. The experiment was performed one time with $plasmid_{SAMS}$ and another time with $plasmid_{LY}$, due to the double use of the Cy5 channel. The mean Ct_{VAR} values of these reactions in both mix variants were not later than the mean Ct_{REF} value of the respective reference reactions plus maximum 0.6 Ct (Table 10). No unspecific signals were observed in any of six NTCs performed for each variant. These results indicate proper stability of the mixes after storage.

Discussion

Multiplex level

Several multiplex assays for GMO detection by conventional PCR have been described in the literature and succeeded for example in one case in a nonaplex PCR for detection and discrimination of nine GMO-related targets by agarose gel electrophoresis [6]. In real-time PCR, the number of targets for multiplexing is mainly restricted to the real-time PCR platform constraints. To our knowledge, the highest multiplex level of a published real-time PCR assay for GMO detection based on TaqMan™ technology is a quadruplex real-time PCR assay [20]. Therefore a major task in the development of a hexaplex assay was to overcome certain constraints of the real-time PCR platform. To achieve this, the real-time PCR platform needed to be specifically configured with a filter set to excite and detect ATTO425 dye-labelled probes, emitting in the blue spectrum. Furthermore the redundancy of a passive reference dye was demonstrated in homogeneity testing. All PCR systems of the hexaplex assay showed uniform performance without normalisation. The fluorophores for probe labelling (Table 4) for differential detection of the PCR products were chosen to match the filter sets of the real-time PCR platform (Table 5) while exhibiting a minimum overlap in the emission spectra. With this dye combination, all five detection channels of the real-time PCR platform were used, showing no significant crosstalk between the detection channels. For detection of a sixth real-time PCR system, one detection channel was chosen to be double-used.

The new generation of real-time PCR platforms extend the optical range in which dyes are excited and fluorescence is detected from UV to infrared wavelengths. This represents a significant advantage compared to laser based-real-time PCR platforms regarding the capability for multiplexing. In combination with newly developed dyes for oligonucleotide labelling, the multiplex level by means of real-time PCR has further potential to increase in the future.

Validation

The in-house validation data presented in this study shows that a hexaplex real-time PCR screening assay was developed, which proved to be fit for purpose for reliable detection of GM plants in food, feed and seed samples.

It was shown that multiplexing of real-time PCR systems is possible without a loss in sensitivity, presenting an assay with a limit of detection ≤ ten target copies in hexaplex format. There are critical issues in multiplex real-time PCR, which were taken into consideration in the development and successfully validated in this work.

One important aspect, which has been omitted in most validations on multiplex PCR assays for routine testing published to date, is competition between the multiplexed PCR systems. Especially for screening assays this is relevant, normally representing the first level of analysis in routine testing and thus dealing with various matrices and variable GMO contents. Such samples can have quite asymmetric target ratios. The validation of the hexaplex screening assay proved single-copy sensitivity for all GMO detection systems in the presence of 64 copies of all other GMO targets of each detection channel. Additionally the sensitivity and robustness of each GMO detection system was shown in a highly asymmetric target situation of ten target copies of one GMO target versus 1,000 copies of the GMO targets of each detection channel. This represents a broad working range for the analysis of food, feed and seed samples. To extend this range, single GMO detection systems were developed in parallel to the development of the hexaplex screening assay. All single GMO detection systems were duplexed with the IPC system used in the hexaplex screening assay and extensively validated for

LOD_{abs}, IPC reliability, matrix effects and stability, referring to this study. Hence, negative results can be reconfirmed with the respective single GMO detection systems in case that a sample is beyond the validated working range regarding asymmetry of targets. To indicate the occurrence of non-validated asymmetric target situations, a feedback signal was incorporated in the developed evaluation spreadsheet tool. This consequently leads to a re-analysis of the negative results by the single GMO detection systems.

Another critical factor in multiplex PCR is the increased risk for artefacts through interactions between the oligonucleotides, which can lead to unspecific signals and even to false-positive results. In the validation process of the assay, this was tested under challenging conditions in stability testing, and as a result, no significant increase of the fluorescence signals was observed in NTC reactions. Additionally the absence of unspecific signals with DNA extracts of different sample matrices was demonstrated.

The IPC reliability validation showed the multiplexed IPC system being sensitive enough to indicate PCR inhibiting substances in the DNA preparation before each of the GMO detection systems was significantly suppressed. Integrated in the developed evaluation algorithm, this enabled the reliable and elegant verification of absence or presence of PCR inhibition. This procedure alleviated the need for separate controls like spiked reactions in parallel and is consequently a time- and cost-saving strategy.

Conclusion

As a summary, multiplex real-time PCR screening assays, as the newly developed hexaplex screening assay presented in this study, offer a sensitive and reliable detection platform in GMO analysis with several advantages: low risk of contamination, decreased turnaround time and high throughput testing with improved process costs.

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