JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Development and Validation of Duplex, Triplex, and Pentaplex Real-Time PCR Screening Assays for the Detection of Genetically Modified Organisms in Food and Feed

Ingrid Huber,*[,†] Annette Block,[†] Daniela Sebah,[†] Frédéric Debode,[‡] Dany Morisset,[§] Lutz Grohmann,[⊥] Gilbert Berben,[‡] Dejan Štebih,[§] Mojca Milavec,[§] Jana Žel,[§] and Ulrich Busch[†]

[†]Bavarian Health and Food Safety Authority (LGL), Veterinärstrasse 2, D-85764 Oberschleissheim, Germany

[‡]Walloon Agricultural Research Centre (CRA-W), Chaussée de Namur 24, B-5030 Gembloux, Belgium

[§]Department of Biotechnology and Systems Biology, National Institute of Biology (NIB), Vecna pot 111, SI-1000 Ljubljana, Slovenia

[⊥]Federal Office of Consumer Protection and Food Safety (BVL), Mauerstrasse 39-42, 10117 Berlin, Germany

Supporting Information

ABSTRACT: Worldwide, qualitative methods based on PCR are most commonly used as screening tools for genetically modified material in food and feed. However, the increasing number and diversity of genetically modified organisms (GMO) require effective methods for simultaneously detecting several genetic elements marking the presence of transgenic events. Herein we describe the development and validation of a pentaplex, as well as complementary triplex and duplex real-time PCR assays, for the detection of the most common screening elements found in commercialized GMOs: P-35S, T-nos, ctp2-cp4-*epsps*, *bar*, and *pat*. The use of these screening assays allows the coverage of many GMO events globally approved for commercialization. Each multiplex real-time PCR assay shows high specificity and sensitivity with an absolute limit of detection below 20 copies for the targeted sequences. We demonstrate by intra- and interlaboratory tests that the assays are robust as well as cost- and time-effective for GMO screening if applied in routine GMO analysis.

KEYWORDS: *real-time PCR, multiplex, GMO, screening, validation, LOD*

INTRODUCTION

During a typical genetically modified organism (GMO) analysis, a "screening" is performed as an initial step in the DNA analysis, in which a minimum set of PCR tests (targeting specific genetic elements) should allow conclusions to be drawn on the absence/presence of as many as possible genetically modified (GM) events. Only in the case of positive screening results, a second step using event-specific PCR assays will then specifically identify and, if required by the legislation, accurately determine the content of the individual event(s) present in the sample. The advantage of such a screening-based approach is that a minimum set of screening assays is sufficient to cover a maximum number of GM events.[1−3] In this way, there is no need to perform a high number of event-specific tests, and thus significant time and costs can be saved. With the growing number of GM events released on the market worldwide and their increasing genetic diversity,[4,5] the number of screening tests to be carried out needs to be increased accordingly. The use of multiplex real-time PCR can significantly facilitate future screening processes and should be regarded as a modern tool for time- and cost-saving GMO analysis.[1] There are already several studies published on the development of multiplex real-time PCR assays for GMO testing (reviewed in ref 3), but until now, there is still no evidence that they have been applied in routine analysis.

The aim of our study was to develop a pentaplex real-time PCR assay which can be easily implemented in routine analysis, covering as many GMOs as possible according to the screening table presented by Waiblinger et al.[6] Real-time (TaqMan) technology was used because of the added value in terms of sensitivity and specificity applying probes. To achieve the highest possible coverage of commercialized GMOs, we chose to target the 35S promoter region of the cauliflower mosaic virus (P-35S), the nopalin synthase gene terminator from *Agrobacterium tumefaciens* (T-nos), a construct containing a 6-enolpyruvylshikimate-3-phosphate-synthase gene from *Agrobacterium tumefaciens* strain cp4 with an upstream sequence of the ctp2 chloroplast transit peptide from *Arabidopsis thaliana* (ctp-cp4-*epsps*), and the two herbicide resistance genes for phosphinothricin: Basta resistance gene from *Streptomyces hygroscopicus* (*bar*) and phosphinothricine acetyltransferase gene from *Streptomyces viridochromogenes* (*pat*). Additionally, as many laboratories are not equipped with apparatus enabling simultaneous detection of five different fluorescent dyes, a duplex and a triplex assay covering the same genetic elements were developed to extend the range of instrument application.

The Bavarian Health and Food Safety Authority (LGL, Germany) developed the screening assays, which were transferred to the Walloon Agricultural Research Centre (CRA-W, Belgium) for interlaboratory robustness testing. The pentaplex method was also transferred to the National

Received: June 6, 2013
Revised: August 22, 2013
Accepted: August 24, 2013
Published: August 24, 2013

Table 1. Primer and Probe Sequences Used for the Multiplex Real-Time PCR Assays

name	target gene	sequence 5′ → 3′	reference
35S-F	P-35S	GCCTCTGCCGACAGTGGT	8
35S-R		AAGACGTGGTTGGAACGTCTTC	
35S-TM		CAAAGATGGACCCCCACCCACG	
180-F	T-nos	CATGTAATGCATGACGTTATTTATG	9
180-R		TTGTTTTCTATCGCGTATTAAATGT	
Tm-180		ATGGGTTTTTATGATTAGAGTCCCGCAA	
pat--F	*pat*	CGCGGTTTGTGATATCGTTAAC	10
pat--R		TCTTGCAACCTCTCTAGATCATCAA	
pat-P		AGGACAGAGCCACAAACACCACAAGAGTG	
RapB-F1	*bar*	ACAAGCACGGTCAACTTCC	11
RapB-R1		GAGGTCGTCCGTCCACTC	
RapB-S1		TACCGAGCCGCAGGAACC	
GT73-TmF	ctp2-cp4-*epsps*	GGGATGACGTTAATTGGCTCTG	11, 12
GT73-TmR		GGCTGCTTGCACCGTGAAG	
GT73-TM		CACGCCGTGGAAACAGAAGACATGACC	

Institute of Biology (NIB, Slovenia), where further robustness and specificity tests were performed and the method was evaluated in a proficiency test. The multiplex real-time PCR assays were implemented in routine analysis of food, feed, seed, and plant samples.

MATERIALS AND METHODS

Chemicals and Reagents. The DNA extraction kit GeneSpin Food was purchased from Eurofins (Eurofins GeneScan, Freiburg, Germany). All chemicals used for the CTAB extraction protocol, which was described previously, were obtained from Sigma-Aldrich (Steinheim, Germany). The PCR primers and probes were obtained from TIB Molbiol (Berlin, Germany), except the Atto425 labeled probe which was synthesized by Biomers (Ulm, Germany). As ready-to use Mastermix, the 2x QuantiTect Multiplex PCR NoROX Mastermix (Qiagen, Hilden, Germany) was applied. As background DNA for preparation of the dilution series, calf-thymus DNA from Sigma-Aldrich (Steinheim, Germany) was chosen. PCR-grade water was obtained from B. Braun Melsungen (Melsungen, Germany). The sources of GMO and non-GMO reference material are listed in Reference Material.

Reference Material. Certified reference material for GM and non-GM lines (ERM) were purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and the American Oil Chemists' Society (AOCS, Urbana, IL). Ground GM seed materials and genomic DNA (gDNA) were received from the German National Reference Laboratory of the Federal Office of Consumer Protection and Food Safety (BVL). Furthermore, a CaMV isolate was provided in inoculated cabbage tissue by Food and Environment Research Agency (FERA; York, UK). Additional details about the reference material used are available in the Supporting Information.

DNA Extraction. The DNAs extracted from reference material for method development and validation were obtained using a modified CTAB protocol as previously described.[7] The DNAs from food, feed, seed, flax, honey, and rapeseed samples as well as the proficiency test material (maize flour and soya flour) were extracted using the GeneSpin Food Kit (Eurofins GeneScan, Germany) as performed in the GMO routine analysis at LGL and NIB. The DNA quantity was determined by the PicoGreen method (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

Primers and Probes. The sequences of the used primers and probes, which have all been previously reported (for P-35S,[8] for T-nos,[9] for *pat*,[10] for *bar*,[11] for ctp2-cp4-*epsps*[12]) are listed in Table 1. For each of the five targets, the specific TaqMan probe was labeled with a different fluorescence dye (Table S1). The 6FAM-, HEX-, TEX-, and CY5-labeled probes were quenched on their 3′-end with a Blackberry quencher (BBQ, Berry and Associates Inc., Dexter, MI). The ATTO425-labeled probe GT73-TmP was quenched with a Deep Dark quencher (DDQ, Eurogentech, Belgium). For each primer and probe system, titration experiments have been performed to determine the optimum concentration for multiplexing without negative influence on the sensitivity, amplification efficiency, and specificity compared to the published singleplex assays.

Pentaplex, Triplex, and Duplex Real-Time PCR Assays. Real-time PCR reactions were performed on a Mx3005P cycler (Agilent Technologies, Santa Clara, CA) and a LC480 cycler (Roche Diagnostics, Germany) in a 25 μL volume containing 1x QuantiTect Multiplex PCR NoROX reagent (Qiagen, Hilden, Germany), optimized primer and probe concentrations (0.4 μM for the primers and 0.2 μM for the probes, for each detection system), and 5 μL of DNA. On both cyclers, the thermo profile comprised an initial denaturation and activation of the polymerase for 15 min at 95 °C followed by 40 cycles with 30 s at 95 °C (denaturation), 45 s at 60 °C (annealing), and 45 s at 72 °C (elongation).

For data analysis on the Mx3005P cycler, the software package MxPro (Agilent Technologies) was used. The fluorophores for the probe labeling were chosen to match the filter-sets of the Mx3005P. All fluorescence channels were analyzed separately. The threshold was set at 200 fluorescence units (FU) for Atto425, HEX, ROX/TEX, and CY5 channels and at 1000 FU for the FAM channel. No cross-talk was observed between the five channels of the Mx3005P cycler. All multiplex real-time PCR assays were transferred to the LC480 real-time PCR cycler (Roche Diagnostics, Germany) for which color compensation was performed to avoid cross-talk between the detection channels (Cyan500, 6FAM, HEX, ROX, CY5). The color compensation was performed once for each assay according to the Roche manual. For analysis on the LC480 cycler, the Fit points method was used. The noise band was manually set as low as possible without including any background noise. Once the background noise was removed, the threshold was set at 0.1 FU above the noise band for the CY5 and at 0.2 FU for the FAM, HEX, ROX, and Cyan500 channel. At NIB, reactions were performed on a LC480 cycler following a slightly modified protocol as described in Method Transfer to Other Laboratories.

Specificity Testing. All GMO events used for specificity tests at LGL were at concentration 1% transgene (mass/mass ratio) in a background of 20 000 taxon-specific reference gene copies. GM reference materials of the plant species maize, soybean, rape, oilseed rape, potato, rice, and sugar beet (Table 2) were used. If a reference material was not available at the 1% level, non-GM and GM genomic DNA was mixed in an appropriate ratio, keeping an absolute number of 20 000 copies of the taxon-specific reference gene.

Additionally, non-GMO reference materials of maize, rice, rape, soy, and sugar beet as well as one cauliflower mosaic virus sample were tested. Reference materials were analyzed with the duplex, triplex, and pentaplex real-time PCR assays with exception of the reference

Table 2. Specificity Test of the Three Multiplex Real-Time PCR Assays at LGL[a]

species	GM event	reference material	P-35S	T-nos	ctp2-cp4 -*epsps*	*pat*	*bar*
maize	DAS-59122-7	ERM BF424c	+	−	−	+	−
	Bt176	ERM BF411d	+	−	−	−	+
	CBH-351	BVL	+	+	−	−	+
	Bt11	ERM BF412d	+	+	−	+	−
	NK603	ERM BF415d	+	+	+	−	−
	GA21	ERM BF414d	−	+	−	−	−
	non-GMO	ERM BF414a	−	−	−		−
oilseed rape	MS8xRF3	[b]Fluka 55231	−	+	−	−	+
	GT73	[b]Fluka 55231	−	−	+	−	−
	GS40/90	[b]Fluka 55231	+	−	−	+	−
	T45	WIV-ISP,	+	−	−	+	−
	non-GMO	BVL	−	−	−	−	−
rice	LL Rice62	BVL	+	−	−	−	+
soybean	GTS40-3-2	ERM BF410gk	+	+	−	−	−
	A5547-127	BVL	+	−	−	+	−
	non-GMO	ERM BF410a	−	−	−	−	−
sugar beet	H7-1	ERM BF419a/b	−	−	+	−	−
	non-GMO	ERM BF419a	−	−	−	−	−

[a]+, presence of the element detected. −, presence of the element is not detected. [b]Fluka: Reference material purchased from Sigma-Aldrich Co. LLC.

materials tested at NIB, which were solely analyzed with the pentaplex assay (Table 3). The reference materials tested by NIB were of varying GMO content (4% to 100%, m/m). Approximately 10 ng of DNA extracted from the reference material was introduced in the 10 μL reaction volume of the pentaplex tests performed at NIB.

Efficiency of the Multiplex Real-Time PCR Assays. For each individual detection system (amplicon) of each multiplex real-time PCR assay, the PCR efficiency (E) and R^2 were calculated on dilution series, containing 5000, 1250, 250, and 50 genome copies, on the basis of the equations proposed in the ENGL document for "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing".[13] Acceptance criteria were the following: PCR efficiencies between 90% and 110% (corresponding to a slope of regression between −3.1 and −3.6) and R^2 value ≥0.98. [13] For the multiplex assays, it was assumed that the PCR efficiency of the different PCR assays within one multiplex real-time PCR did not differ more than 15%.

Determination of the Limits of Detection. The absolute limit of detection (LOD_{abs}) is defined as the amount or concentration of analyte in the sample, expressed in target copy numbers, at which the analytical method reliably detects the presence of the analyte. The LOD_{abs} was analyzed with two approaches according to AFNOR guidelines (LOD_6) [14] and to DIN 32645 ($LOD_{C.L.}$).[15] Additionally, the relative LOD ($LOD_{\%}$) was determined. The LOD% is the relative GM content, based on the GM copy (cp) numbers relative to the taxon-specific copy numbers (cp/cp). The gDNA standard was adjusted to GMO content levels from 0.5% to 0.010%, corresponding to 100 to 2.0 target copies per reaction in a background of 20 000 non-GM target copies. Finally, the performance of the multiplex real-time PCR in samples presenting asymmetric distribution of the targets was evaluated by determining the asymmetric LOD (LOD_{asym}). The LOD_{asym} represents the sensitivity of the multiplex assay when one target is at very low concentration in comparison with the other targets of the assay. Three standard dilution series were prepared to reach concentration levels from 1620 to 20 target copies in a background of 54 000 copies of all other multiplex targets.

Robustness of the Multiplex Real-Time PCR Assays. For the pentaplex, triplex, and duplex real-time PCR assays, robustness to slight deviations in the annealing temperature was tested on a Mx3005P cycler (Agilent Technologies). The change of annealing temperature (±1 °C) on the sensitivity of the PCR assays was also analyzed.

The effects of primer/probe concentration variation and robustness to instrument change were tested for pentaplex on the Light cycler LC480 instrument. A 20% variation of primer and probe final concentrations was tested on different low DNA concentrations, including one close to the determined LOD_6. For T-nos, *pat*, *bar*, and ctp2-cp4-*epsps*, approximately 45 copies and 15 copies per reaction were used, while for P-35S, approximately 115 and 10 copies per reaction were used due to the different estimated copy numbers of the different targets in the material used.

Analysis of GM Food, Feed, and Seed Samples. Different GMO-containing food samples and matrixes (n = 23) were analyzed with the pentaplex, triplex, and duplex real-time PCR assays. The sample collection included 11 chocolate bars (butterfinger), five honey samples, one flax seed sample, and five rapeseed samples as well as one GMO papaya (Table 4). All food samples were analyzed in parallel with the duplex, triplex, and pentaplex real-time PCR assays and with the accredited real-time PCR assays used in routine analysis at LGL (for P-35S and T-nos,[16] for *pat*,[10], for *bar*,[8] for ctp2-cp4-*epsps*[12]). Furthermore, within the method transfer, 27 GMO-containing feed samples were tested by NIB (Table 5). These feed samples were only tested with the pentaplex real-time PCR assay. In addition, four maize seed samples known to contain GMOs were tested with the pentaplex real-time PCR assay at NIB.

Method Transfer to Other Laboratories. The in-house validated duplex, triplex, and pentaplex real-time PCR assays were transferred from LGL to CRA-W and NIB. Two types of transfer were performed. One transfer was designed so that the transfer laboratory (CRA-W) tested the assays on a different instrument following exactly the validated protocol and using the samples provided by the developer (LGL). CRA-W received the same dilution series used during in-house validation and performed the duplex, triplex, and pentaplex real-time PCR assays on the LC480 cycler under the same PCR conditions as during the in-house validation at LGL. CRA-W determined the LOD_6 and $LOD_{C.I.}$, and the data were compared to the in-house validation data obtained at LGL.

In the transfer to NIB, the ability of the pentaplex real-time PCR assay to perform in smaller reaction volumes (for enhanced cost-efficiency) was tested. Furthermore, extended specificity and practicability tests were performed. During this transfer, the pentaplex PCR reactions were performed on a LC480 real-time PCR cycler following a slightly modified protocol: 10 μL reaction volume containing 1x QuantiTect Multiplex PCR NoROX reagent (Qiagen, Hilden, Germany), primer and probe concentrations for each system at 0.4 μM for the primers and 0.2 μM for the probes, and 2 μL of sample DNA were prepared, and the reaction took place in 384-well reaction plates. The thermo profile remained unchanged except 10 additional cycles for amplification. For data analysis, the Fit points method was used.

At NIB, LOD_6 was independently determined using different DNA dilution series than for the in-house validation at LGL prepared from maize instead of rapeseed reference materials. Three heterozygous CRMs for maize events Bt176, NK603, and DAS59122 were mixed to obtain DNA standard with 1702 copies of P-35S and 675 copies of T-nos, *pat*, *bar*, and ctp2-cp4-*epsps*. Standard dilutions of the DNA standard were prepared with 170, 17, 6, 2, 0.4, 0.2, and 0 copies of P-35S and 68, 7, 2, 0.7, 0.2, 0.05, and 0 copies of T-nos, *pat*, *bar*, and ctp2-cp4-*epsps* and analyzed by the transferred methods. NIB determined LOD_6 in two independent runs of the above-described

Table 3. Specificity Test of the Pentaplex Real-Time PCR Assay at NIB[a]

species	GM event	reference material	P-35S	T-nos	ctp2-cp4 -*epsps*	*pat*	*bar*
cotton	281-24-236 × 3006-210-23	ERM-BF422d	−	−	−	+	−
maize	3272	ERM-BF420c	−	+	−	−	−
	Bt11	ERM-BF412f	+	+	−	+	−
	Bt176	ERM-BF411f	+	−	−	−	+
	DAS-59122-7	ERM-BF424d	+	−	−	+	−
	GA21	ERM-BF414f	−	+	−	−	−
	MIR604	ERM-BF423d	−	+	−	−	−
	MON810	ERM-BF413f	+	−	−	−	−
	MON863	ERM-BF416d	+	+	−	−	−
	NK603	ERM-BF415f	+	+	+	−	−
	T25	AOCS 0306-H	+	−	−	+	−
	DAS1507	ERM-BF418d	+	−	−	+	−
	MON89034	AOCS 0906-E	+	+	−	−	−
	MON88017	AOCS 0406-D	+	+	+	−	−
	MIR162	AOCS 1208-A	−	+	−	−	−
	98140	ERM-BF427d	+	−	−	−	−
	40278	ERM-BF433d	−	−	−	−	−
oilseed rape	MS8	AOCS 0306-F2+	−	+	−	−	+
	RF3	AOCS 0306G	−	+	−	−	+
	GT73	AOCS 0304-B	−	−	+	−	−
	T45	AOCS 0208-A2+	+	−	−	+	−
potato	EH92-527-1	AOCS 0806-C	−	+	−	−	−
	AM04-1020	ERM-BF430e	−	+	−	−	−
rice	LL Rice62	AOCS 0306−12+	+	−	−	−	+
soybean	A2704-12	AOCS 0707-B2	+	−	−	+	−
	A5547-127	AOCS 0707-C2	+	−	−	+	−
	GTS40-3-2	ERM-BF410gk	+	+	−	−	−
	MON89788	AOCS 0906-B	−	−	+	−	−
	68416	ERM-BF432d	−	−	−	+	−
	DP-356043	ERM-BF425d	+	−	−	−	−
	DP-305423	ERM-BF426d	−	−	−	−	−
	FG72	AOCS 0610-A2	−	+	−	−	−
	CV127	AOCS 0911-C	−	−	−	−	−
	MON87701	AOCS 0809-A	−	−	−	−	−
sugar beet	H7-1	ERM-BF419b	−	−	+	−	−
CaMV	non-GMO	FERA	+	−	−	−	−

[a]+, presence of the element detected. −, presence of the element is not detected.

standard dilutions series analyzed in triplicates (six data points per concentration).

Performance of the Pentaplex Assay in a Proficiency Test. The modified pentaplex real-time PCR assay was performed by NIB on the samples from the USDA/GIPSA proficiency program testing for the presence of biotechnology events in corn and soybeans from April 2011. Based on the screening results from the pentaplex assay and following a matrix approach (modified from ref 6), prediction of the possible specific events present in the samples was made. Further experimental confirmation was done using real-time PCR assays specific to the GM events potentially present according to the proficiency test information. The following accredited event-specific assays validated in ring-trial studies by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) were used: T25, GA21, NK603, DAS1507, MON863, DAS-59122-7, MIR604, MON88017, GTS40-3-2, A2704-12, and MON89788 (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm), MON810,[17] and Bt11.[18]

RESULTS

Specificity. The specificity of the P-35S, T-nos, *pat*, *bar*, and ctp2-cp4-*epsps* systems was tested on different GM events reference materials with and without the target elements. Analytical results were compared with the information from diverse databases and publications.[6,19−24] All results obtained from the pentaplex, triplex, and duplex real-time PCR assays were in accordance with the official information. The pentaplex, triplex, and duplex real-time PCR assays are fully specific to their respective targets as demonstrated by the inclusive and exclusive specificity of 100% after having tested 54 samples (n = 49 GMOs − 34 individual GM events, n = 4 non-GMOs and n = 1 CaMV virus, see Table 2). The *pat* and the *bar* gene are both derived from the soil bacteria *Streptomyces* and show some similarities in the gene structure. However, the multiplex real-time PCR assays clearly distinguished the *pat* and the *bar* target sequence (from *S. viridochromogenes* and *S. hygroscopicus*, respectively) as it is shown for the GMO events Bt11, GS40/90, A5547-127, A2704-12, DAS-59122-7, DAS1507, T25, T45 (where the *pat* gene was detected) and Bt176, MS8 × RF3, CBH-351, and LL Rice62 (where the *bar* gene was detected, Table 2).

While the six GMO events MON89788, GTS40-3-2 soybean, GA21, NK603 maize, H7-1 sugar beet, and GT73 rapeseed are known to carry the herbicide resistance gene *epsps*, only NK603 maize, MON89788 soybean, GT73 rapeseed, and H7-1 sugar beet harbor the construct ctp2-cp4-*epsps*. Logically, the tested NK603 maize, H7-1 sugar beet, GT73 rapeseed, and

Table 4. GMO-Positive Food, Feed, and Seed Samples Screened by the Duplex, Triplex, and Pentaplex Real-Time PCR Assays at LGL[a]

GMO content	sample name	T-nos	ctp2-cp4-*epsps*	*bar*	*pat*	P-35S
DAS-59122-7, Bt11, GA21, MIR604, MON810, MON88017, TC1507	butterfinger 1	+	+	−	+	+
DAS-59122-7, MON810, MON863, MON88017, TC1507	butterfinger 2	+	+	−	+	+
DAS-59122-7, Bt11, GA21, MIR604, MON810, MON88017, NK603, TC1507	butterfinger 3	+	+	−	+	+
Bt11, GA21, MON810, MON863, MON88017, NK603, TC1507	butterfinger 4	+	+	−	+	+
Bt11, GA21, MON810, MON863, MON88017, NK603, TC1507	butterfinger 5	+	+	−	+	+
DAS-59122-7, Bt11, GA21, MIR604, MON810, MON88017, TC1507	butterfinger 6	+	+	−	+	+
DAS-59122-7, Bt11, GA21, MIR604, MON810, MON863, MON88017, NK603, TC1507	butterfinger 7	+	+	−	+	+
DAS-59122-7, Bt11, GA21, MIR604, MON810, MON88017, TC1507	butterfinger 8	+	+	−	+	+
DAS-59122-7, MIR604, MON810, MON863, MON88017, TC1507	butterfinger 9	+	+	−	+	+
DAS-59122-7, MIR604, MON810, MON863, MON88017, NK603, TC1507	butterfinger 10	+	+	−	+	+
DAS-59122-7, Bt11, GA21, MIR604, MON810, MON863, MON88017, NK603, TC1507	butterfinger 11	+	+	−	+	+
MS8 × RF3, GT73	honey 1	+	+	+	−	−
MS8 × RF3, GT73	honey 2	+	+	+	−	−
MS8 × RF3, GT73	honey 3	+	+	+	−	−
MS8 × RF3, GT73	honey 4	+	+	+	−	−
MS8 × RF3, GT73	honey 5	+	+	+	−	−
SunUp	papaya	+	−	−	−	+
GT73	rapeseed 1	+	+	−	−	+
GT73	rapeseed 2	+	+	−	−	+
GT73	rapeseed 3	+	+	−	−	+
GT73	rapeseed 4	+	+	−	−	+
GT73	rapeseed 5	+	+	−	−	+
FP967	flax seed	+	−	−	−	−

[a]+, presence of the element detected. −, presence of the element is not detected.

Table 5. GMO-Positive Feed, Seed, and Proficiency Test Samples Screened by Pentaplex Real-Time PCR Assay at NIB[a,b]

GMO content	sample name	T-nos	ctp2-cp4-*epsps*	*bar*	*pat*	P-35S
GTS40-3-2	feed 1 to 19	+	−	−	−	+
MON810	feed 20	−	−	−	−	+
GTS40-3-2, MON810	feed 21	+	−	−	−	+
GTS40-3-2, MON89788, A2704−12	feed 22	+	+	−	+	+
GT73, GTS40-3-2	feed 23	+	+	−	−	+
GTS40-3-2, MON89788	feed 24	+	+	−	−	+
GTS40-3-2, MON89788	feed 25	+	+	−	−	+
GTS40-3-2, MON89788	feed 26	+	+	−	−	+
GTS40-3-2, MON89788	feed 27	+	+	−	−	+
MON810	maize seed 1	−	−	−	−	+
TC1507	maize seed 2	−	−	−	+	+
MON810	maize seed 3	−	−	−	−	+
MON810	maize seed 4	−	−	−	−	+
GTS40-3-2, MON89788	USDA PT soya 1	+	+	−	−	+
A2704-12,	USDA PT soya 2	−	−	−	+	+
GTS40-3-2, A2704-12	USDA PT soya 3	+	−	−	+	+
MON810, MON863, 59122, event 3272, MON88017, MON89034	USDA PT corn 1	+	+	−	+	+
T25, CBH351[c], MON810, Bt176, Bt11, NK603, event 3272,	USDA PT corn 2	+	+	+	+	+
MON810, GA21, Bt11, NK603, MON863, MIR604, MON88017, MON89034	USDA PT corn 3	+	+	−	+	+
MON810, GA21, MIR604, event 3272,	USDA PT corn 4	+	−	−	−	+
T25, CBH351[c], MON810, GA21, Bt176, Bt11, NK603, TC1507, 59122, MIR604, event 3272,	USDA PT corn 5	+	+	+	+	+

[a]USDA PT: USDA/GIPSA proficiency test. GMO content: the presence of screening elements and GM events was confirmed using accredited screening and event-specific singleplex real-time PCR assays and, in the case of the USDA/GIPSA proficiency test, was documented in the proficiency test report. [b]+, presence of the element detected. −, presence of the element is not detected. The observed pattern of the positive screening elements matches with the expected presence of targets. [c]This event was not confirmed (not tested) by event-specific real-time PCR.

Mon89788 soybean events were experimentally found positive for the presence of the ctp2-cp4-*epsps* target sequence using the pentaplex and triplex assays (Table 2). The soybean GTS40-3-2 containing the cp4-*epsps* gene but without the sequence of the ctp2 (chloroplast transit peptide = ctp) and the GA21 maize event with the m-*epsps* sequence (variant gene originating from maize) both tested negatively for the ctp2-cp4-*epsps* target as expected (Table 2). In conclusion, the pattern of the screening elements detected during specificity testing perfectly matches with the theoretical presence of genetic elements in the tested

samples, thus demonstrating the specificity of the tested multiplex real-time PCR assays. However, it should be mentioned that screening for the presence of the cauliflower mosaic virus (CaMV) 35S promoter (P-35S) and the *nos* terminator sequences derived from *Agrobacterium tumefaciens* (T-nos) lacks specificity for the identification of the particular GM plant event. Positive screening results for P-35S and T-nos should therefore be verified using construct- and/or event-specific methods, if available.[6]

PCR Efficiency and Linearity. For all multiplex assays, the amplification efficiency (E) and coefficient of correlation (R^2) of the individual detection systems met the minimum acceptance criteria of 90−110% (E) and >0.98 (R^2). Also, for all three real-time PCR assays, the mean PCR efficiencies (E_{mean}) and mean R^2 (R^2_{mean}) calculated from three independent runs met the minimum acceptance criteria (Table S2, Supporting Information). All three assays fulfilled our criterion that the PCR efficiencies of the individual detection systems of the multiplex assays did not differ more than 15% (see Table S2, Supporting Information).

Determination of the Detection Limits. *Absolute Limit of Detection LOD_6 and $LOD_{C.I.}$.* For the pentaplex, triplex, and duplex real-time PCR assays, the absolute LOD_6 and $LOD_{C.I.}$ for each target are summarized in Table 6. For the pentaplex real-time PCR assay, both LOD_6 and $LOD_{C.I.}$ met the minimal acceptance criteria of 20 copies per PCR reaction. The most sensitive detection system of the pentaplex real-time PCR assay was the ctp2-cp4-*epsps* system while the least sensitive was the T-nos system. In the duplex, triplex, and pentaplex real-time PCR assays, all PCR systems showed a detection limit equal or below 20 target copies although the pentaplex real-time PCR assay appears slightly less sensitive than the triplex and duplex real-time PCR assays.

Table 6. LOD_{abs} of the Pentaplex, Triplex, and Duplex Assays[a]

target	Plex	LOD_6	$LOD_{C.I.}$
bar	5	10	10
	2	10	10
P-35S	5	5	2
	3	5	2
pat	5	5	5
	2	5	5
ctp2-cp4-*epsps*	5	5	2
	3	2	1
T-nos	5	20	10
	3	20	10

[a]All experiments were performed on the Mx3005P instrument (Agilent Technologies). The absolute LOD (LOD_{abs}) is expressed in target copy number. Two approaches (LOD_6 and $LOD_{C.I.}$) were used to determine the LOD_{abs}. Plex: degree of multiplexing.

Relative Limit of Detection ($LOD_{\%}$) of the Pentaplex Assay. The relative limit of detection ($LOD_{\%}$) was determined for the pentaplex real-time PCR assay (shown in Table S3, Supporting Information). The $LOD_{\%}$ for all detection systems of the pentaplex real-time PCR assay was determined below 0.01% corresponding to 2 gene copies, except for the *pat* detection system with 0.025% corresponding to 5 gene copies and the *bar* detection system with 0.05% corresponding to 10 gene copies. Given the excellent $LOD_{\%}$ of the pentaplex real-time PCR assays, we did not judge necessary to evaluate the $LOD_{\%}$ of the even more sensitive duplex and triplex real-time PCR assays for the $LOD_{\%}$.

Asymmetric Limit of Detection (LOD_{asym}). The LOD_{asym} was determined for the duplex, triplex, and pentaplex real-time PCR assays (Table S4, Supporting Information). In the triplex and the duplex real-time PCR assays, the LOD_{asym} for all PCR systems was found below 20 copies, meeting the acceptance criteria of the AFNOR XP V03-044 guidelines for singleplex methods.[14]

In the pentaplex assay, the LOD_{asym} was also detected below 20 copies for the targets P-35S, *pat*, and ctp2-cp4-*epsps*. The *bar* PCR system showed a slightly higher LOD_{asym} of 60 copies in a background of 20 000 copies of the other targets. Under these highly asymmetric target conditions, the T-nos PCR system had the lowest sensitivity with a LOD_{asym} of 1040 copies.

Robustness of the Multiplex Real-Time PCR Assays. The robustness to instrument change was measured via the comparison of the LOD_6 and $LOD_{C.I.}$. Changing the instrument did not significantly modify the sensitivity of the three multiplex real-time PCR assays, as all targets can be still detected with a high sensitivity of at least 20 copies (Table S5, Supporting Information). As observed in the Mx3005P instrument, the T-nos detection system is the least sensitive system in the pentaplex real-time PCR assay while the P-35S and the CTP2-CP4-epsps systems are the most sensitive ones. The robustness of the assays to the deviations in the annealing temperature was also tested via the absolute LOD determination at annealing temperature between 59 °C and 61 °C. No significant change in LOD_{abs} was observed, the absolute LOD still being found below or equal to 20 copies for all targets (data not shown). The mid- and long-term stability of the pentaplex, triplex, and duplex PCR reaction mixes (Supporting Information) did not show a significant trend. The stability of the mixes was proofed at −20 °C for least 6 months (data not shown).

Transfer of the Multiplex Real-Time PCR Assays to Other Laboratories. The three multiplex real-time PCR assays were transferred to two other laboratories. At CRA-W, the three multiplex assays were applied to the LC480 cycler where LOD_6 and $LOD_{C.I.}$ were determined using the same samples as for the in-house validation at LGL. The absolute LOD values determined at CRA-W were identical or even lower than the values observed at LGL (Table S5, Supporting Information), therefore meeting the minimum sensitivity requirements for the assays and proofing the robustness of the assays.

At NIB, the pentaplex real-time PCR assay was adapted to a smaller reaction volume and performed in 384 reaction plates. The transfer of the pentaplex assay to these more cost-efficient conditions was successful. The absolute LOD of the modified pentaplex was determined at 15 copies which correspond to the data obtained with the 20 μL reaction assay tested at LGL and CRA-W. The most sensitive systems appear to be P-35S and *bar* (LOD_{abs} = 5 copies) while the three other systems present a slightly lower sensitivity (15 copies for *pat*, T-nos, and the ctp2-cp4-*epsps*) (data not shown). A robustness test was also successfully performed by applying 20% variations in primers and probes concentrations.

Analysis of GM Food, Feed, and Seed Samples with the Multiplex Real-Time PCR Assays. Food, feed, and seed samples were analyzed by the pentaplex, triplex, and duplex assays, and the data were compared to the results of the LGL and NIB GM routine analyses for food, feed, and seed samples applying accredited singleplex event-specific real-time PCR

assays. For all tested samples, the GM screening elements were correctly detected by the applied multiplex real-time PCR assays. In total, 54 GMO samples (23 food, 27 feed, and 4 seed) were successfully tested in both laboratories (23 at LGL (Table 4) and 31 at NIB (Table 5). In all cases, the pattern of the screening elements detected during specificity testing matched perfectly with the theoretical presence of genetic elements based on the GM events identified in the samples.

In addition, the modified pentaplex assay was successfully used by NIB as a screening method in a proficiency test for maize and soya flour samples (Table 5). The results of further event-specific tests confirmed the theoretical prediction of the presence of possible events in samples, based on the results of the pentaplex assay. The absence of GMOs as predicted using the results of the pentaplex assay and the matrix-approach was confirmed by the official proficiency test results. At LGL and NIB, the pentaplex assay has been introduced in the quality management system and is regularly used for routine analyses of food, feed, and seed samples.

DISCUSSION

The constantly increasing number of GM plants worldwide demands more efficient, time- and cost-saving analytical strategies for GMO testing. Multiplex real-time PCR has previously shown its potential applicability to routine diagnostics.[25] The new generation of real-time PCR platforms extends the optical range in which dyes are excited, and fluorescence can be detected from UV to infrared wavelengths, which offers significant advantage for multiplexing. In combination with the newly developed dyes for oligonucleotide labeling, the multiplexing level in real-time PCR has further potential to increase in the future.

However, multiplex real-time PCR is still poorly employed in GMO testing laboratories, probably because of the limited use of the proposed assays (target choice and availability of suitable instruments) or the lack of proper validation. We herein describe the development, in-house validation, and transfer to other laboratory of a pentaplex real-time PCR assay for GMO routine screening of food, feed, plant, and seed samples. As in multiplex real-time PCR, the number of targets is mainly restricted to the real-time PCR platforms available; additional complementary duplex and triplex assays targeting the same genetic elements as those in the pentaplex assay were developed and optimized to meet the specifications of most real-time PCR cyclers available on the market.

Currently, many European laboratories still rely on the use of the sole P-35S- and T-nos-specific PCR screening tests to detect the presence of GM materials[26] although this screening strategy does not allow proper detection of the wide range of commercialized (or even EU-approved) GM events. This strategy is also poorly informative regarding the possible GMOs present in the sample. To avoid time- and cost-intensive multiple event-specific tests, expanding the repertoire of targeted genetic elements would allow more efficient screening during GMO testing. As presented in the paper of Waiblinger et al.,[6] the combination of just five real-time PCR-based screening tests can be used as a universal screening approach for most GM plant events described in publicly available databases. Compared to the approach proposed by Waiblinger et al.,[6] we chose the P-35S target instead of the P-35S-*pat* target for the proposed pentaplex, triplex, and duplex real-time PCR assays to cover even a broader range of GM events in several crop plants (potato, maize, papaya, rapeseed, rice, soya, tomato, sugar beet, cotton). Using these five genetic elements, the screening phase covers 92% (70/76) of the GM events approved for commercialization in the EU, under the so-called low-level presence regulation for feed,[27] tolerated after withdrawal, or unauthorized but already observed in the EU. At the European level, a guidance document of the European Network of GMO Laboratories[13] provides practical recommendations on how quantitative event-specific PCR methods shall be validated in the context of the approval of GM food or feed products according to regulation (EC) 1829/2003.[28] As previously mentioned,[11] appropriate guidelines for qualitative PCR methods are hard to find. The three multiplex qualitative real-time PCR assays presented in this study were in-house-validated, taking into account the validation guidelines proposed in national and international documents from the French National Organization for Standardization (AFNOR),[14] the Codex Alimentarius, [29] and the European Network of GMO Laboratories (ENGL)[13] although the criteria from the latter are defined for quantitative event-specific singleplex real-time assays. The specificity, efficiency, limits of detection (LOD), robustness of the multiplex real-time PCR assays, and the influence of matrix effects were tested during an extensive in-house validation process and transfer to other laboratories.

Foreseeing an update of the ENGL guideline[13] introducing more stringent sensitivity acceptance criteria and the need to assess performance parameters in both relative and absolute units, we validated the assays against more criteria than currently required in the literature. Moreover, our multiplex real-time PCR assays were developed by combining published singleplex assays that are nationally or internationally accepted, which should further ensure quality of the proposed multiplex tests.

The in-house validation data presented in this study show that all three multiplex real-time PCR assays are fit for purpose for reliable detection of GM food, feed, and seed samples. For all targets, multiplexing of real-time PCR systems was made possible without loss of sensitivity, presenting an assay with a limit of detection of 20 or less target copies in all multiplex formats. The sensitivities of the different systems in the multiplex assays are similar to the ones reported for the singleplex assays (15 copies for *bar*,[11] 10 copies for ctp2-cp4-*epsps*,[11] 5 to 10 copies for T-nos,[9] 10 copies for P-35S,[8] 20 copies for *pat*[10]) and the P-35S-T-nos duplex (10 copies for both targets[8]).

For each multiplex assay, the working range spanned over at least 3 orders of magnitude, the highest target concentration tested being 20 000 copies per reaction. This working range is broad enough for the analysis of food, feed, plant, and seed samples.

Screening assays normally represent the first level of analysis in routine testing dealing with various matrixes and variable GMO contents. Multiplex PCR assays are more sensitive to asymmetric levels of target copies than singleplex PCR assays due to the potential increased competition of the oligonucleotides for reagents. Therefore, the sensitivity was tested in asymmetric target concentrations. Pentaplex, triplex, and duplex PCR assays were tested close to the expected LOD of 20 target copies in a very high background of 20 000 copies of the other targets. The T-nos target in the pentaplex assay showed a significant lower sensitivity (LOD_{asym}) when the method was pushed to its limits. Such a high asymmetric grade of targets is not expected in routine analysis of food, feed, and seed samples. The *bar* target showed a slightly lower sensitivity only in the

very high background of 20 000 target copies of the other targets, which is not expected in routine analysis of GMO testing and not in lower background levels of the other targets of the pentaplex assay (10 000 target copies of the other targets and below). In the triplex assay no significant decrease of sensitivity was determined for T-nos, applying asymmetric target concentrations. Therefore, when using the pentaplex real-time PCR assay for GMO screening, we recommend analyzing the data with great care. In case some targets are observed at high concentrations while T-nos is negative, this target should be reanalyzed with an alternative assay (e.g., a singleplex assay for T-nos).

For national and international harmonization of analytical methods, it is recommended to assess the method performance data in an interlaboratory study, confirming that these methods are fit for purpose and transferable to multiplex laboratories prior to a collaborative ring-trial. All three multiplex real-time PCR assays were thus transferred to CRA-W and performed similarly. In addition to this conservative transfer, the pentaplex assay was down-scaled to smaller reaction volumes at NIB for more cost-efficient screening. This transfer and the adaptation to smaller reaction volume were successful, showing absolute LOD comparable with that observed at the developer and CRA-W in larger reaction volumes. As smaller volumes were used, the method may have been more prone to bias in the case of pipetting errors. Therefore, robustness of primers/probe variability was additionally tested and has shown that the method is robust for minor pipetting variations.

The results from the proficiency test confirmed that the pentaplex assay can be used as an accurate screening tool for maize and soybean samples and is suitable with the matrix-based approach. On the basis of the analysis results using the pentaplex assay, one can infer upon the absence and the potential presence of some GMO events in a sample, therefore reducing the cost and time of further identification and quantification steps. Finally, the pentaplex assay has already been implemented for routine GMO testing of food, feed, and seed samples at LGL and NIB, showing both the practicability and applicability of the assay.

In summary, the multiplex real-time PCR assays developed, validated, and applied in this study offer a sensitive and reliable screening platform in GMO analysis with several advantages such as a decreased turnaround time and improved process costs. The methods are fit for purpose and should be easily adopted by other laboratories for a more efficient GMO screening.

■ ASSOCIATED CONTENT

Supporting Information

Additional details on the materials and methods used. Table S1: Probe labeling strategy for the pentaplex, triplex, and duplex real-time PCR assays. Table S2: Comparison of the amplification efficiency and linearity of the pentaplex, triplex, and duplex assays. Table S3: $LOD_{\%}$ of the pentaplex real-time PCR assays. Table S4: Comparison of the LOD_{asym} of the pentaplex, triplex, and duplex assays. Table S5: Comparison of LOD_{abs} of the pentaplex, triplex, and duplex assays during the laboratory transfer testing. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 0049 9131 6808 5158. Fax: 0049 9131 6808 5458. E-mail: ingrid.huber@lgl.bayern.de.

Funding

This study was financially supported by the UK 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 as well as by FSA (contract FS244027) through the Project GMOval. The study performed at NIB was partially financed by the Slovenian Ministry of Environment and Spatial Planning (contract no. 2511-11-240009) and by the Metrology Institute of the Republic of Slovenia (contract no. 6401-18/2008/67).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are very grateful to Andrea Harwarth, Karola Grünwald, and Krimhilde Posthoff for their excellent technical help and support at LGL and Dr. Tanja Dreo for valuable discussions at NIB.

■ ABBREVIATIONS USED

AOCS, American Oil Chemists' Society; ERM, reference material from the European Commission Institute for Reference Materials and Measurements; CaMV, cauliflower mosaic virus; GMO, genetically modified organism

■ REFERENCES

(1) Querci, M.; Van den Bulcke, M.; Zel, J.; Van den Eede, G.; Broll, H. New approaches in GMO detection. *Anal. Bioanal. Chem.* **2010**, *396*, 1991−2002.

(2) Holst-Jensen, A.; Bertheau, Y.; De Loose, M.; Grohmann, L.; Hamels, S.; Hougs, L.; Morisset, D.; Pecoraro, S.; Pla, M.; den Bulcke, M. V.; Wulff, D. Detecting un-authorized genetically modified organisms (GMOs) and derived materials. *Biotechnol. Adv.* **2012**, *30*, 1318−1335.

(3) Zel, J.; Milavec, M.; Morisset, D.; Plan, D.; Van den Eede, G.; Gruden, K. *How to Reliably Test for GMOs*, 1st ed.; Springer: New York, 2012.

(4) Stein, A. J.; Rodriguez-Cerezo, E. International trade and the global pipeline of new GM crops. *Nat. Biotechnol.* **2010**, *28*, 23−25.

(5) Stein, A. J.; Rodriguez-Cerezo, E. *The global pipeline of new GM crops: Implications of asynchronous approval for international trade*; EUR 23486 EN; Office for Official Publications of the European Communities: Luxembourg, 2009.

(6) Waiblinger, H. U.; Grohmann, L.; Mankertz, J.; Engelbert, D.; Pietsch, K. A practical approach to screen for authorised and unauthorised genetically modified plants. *Anal. Bioanal. Chem.* **2010**, *396*, 2065−2072.

(7) Ausubel, F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. G.; Smith, J. A.; Struhl, K. *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, 4th ed.; Wiley: New York, 1999.

(8) Waiblinger, H. U.; Ernst, B.; Anderson, A.; Pietsch, K. Validation and collaborative study of a P-35S and T-nos duplex real-time PCR screening method to detect genetically modified organisms in food products. *Eur. Food Res. Technol.* **2008**, *226*, 1221−1228.

(9) Reiting, R.; Broll, H.; Waiblinger, H. U.; Grohmann, L. Collaborative Study of a T-nos Real-Time PCR Method for Screening of Genetically Modified Organisms in Food Products. *J. Verbraucherschutz Lebensmittelsicherh.* **2007**, *2*, 116−121.

(10) Zeitler, R.; Pietsch, K.; Waiblinger, H. U. Validation of real-time PCR methods for the quantification of transgenic contaminations in rape seed. *Eur. Food Res. Technol.* **2002**, *214*, 346−351.
(11) Grohmann, L.; Brunen-Nieweler, C.; Nemeth, A.; Waiblinger, H. U. Collaborative Trial Validation Studies of Real-Time PCR-Based GMO Screening Methods for Detection of the *bar* Gene and the ctp2-*cp4epsps* Construct. *J. Agric. Food Chem.* **2009**, *57*, 8913−8920.
(12) Waiblinger, H. U.; Ohmenhauser, M.; Pietsch, K.; Ritter, W.; Steegmuller, J.; Krech, A.; Horn, P.; Schroeder, A. The analysis of genetically modified rape in honey by real-time PCR. *Dtsch. Lebensm.-Rundsch.* **2005**, *101*, 543−549.
(13) EURL-GMFF. Definition of minimum Performance requirements for analytical methods of GMO testing. 2008. http://gmocrl.jrc.ec.europa.eu/doc/MinPerfRequirementsAnalyticalmethods.pdf.
(14) AFNOR. *Standard XP-V-03-044: Criteres de validation intra-laboratoire pour les méthodes de détection et quantification de séquences d'acides nucléiques spécifiques*; AFNOR: Saint-Denis La Plaine, 2008.
(15) Deutsches Institut für Normierung (DIN). *DIN 32645: Nachweis-, Erfassungs- und Bestimmungsgrenzen*; DIN 32645; DIN: Berlin, 1994.
(16) Kuribara, H.; Shindo, Y.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Novel reference molecules for quantitation of genetically modified maize and soybean. *J. AOAC Int.* **2002**, *85*, 1077−1089.
(17) Holck, A.; Vaitilingom, M.; Didierjean, L.; Rudi, K. 5′-Nuclease PCR for quantitative event-specific detection of the genetically modified Mon810 MaisGard maize. *Eur. Food Res. Technol.* **2002**, *214*, 449−454.
(18) Brodmann, P. DNA-Extraction: Important Step of Quantitative Detection of GMO in Food Matrices. *New Food* **2002**, *1*, 57−61.
(19) Bruderer, S.; Leitner, K. E. *Genetically Modified (GM) crops: molecular and regulatory details*; BATS, Centre for Biosafety and Sustainability: Zurich, Switzerland, 2003.
(20) Center for Environmental Risk Assesment. http://www.cera-gmc.org/?action=gm_crop_database (last accessed: Apr 16, 2013).
(21) Dong, W.; Yang, L.; Shen, K.; Kim, B.; Kleter, G. A.; Marvin, H. J.; Guo, R.; Liang, W.; Zhang, D. GMDD: a database of GMO detection methods. *BMC Bioinf.* **2008**, *9*, 260.
(22) European Food Safety Authority (EFSA). http://registerofquestions.efsa.europa.eu/roqFrontend/questionsListLoader?unit=GMO (last accessed: Apr 16, 2013).
(23) Gerdes, L.; Busch, U.; Pecoraro, S. GMO*finder* - A GMO Screening Database. *Food Anal. Methods* **2012**, 1−9.
(24) International Service for the Acquisition of Agri-biotech Applications (ISAAA). http://www.isaaa.org/gmapprovaldatabase/ (last accessed: Apr 16, 2013).
(25) Bahrdt, C.; Krech, A.; Wurz, A.; Wulff, D. Validation of a newly developed hexaplex real-time PCR assay for screening for presence of GMOs in food, feed and seed. *Anal. Bioanal. Chem.* **2010**, *396*, 2103−2112.
(26) Bonfini, L.; Moens, W.; Ben, E.; Querci, M.; Aygun, B.; Corbisier, P.; Morisset, D.; Zel, J.; Van den Eede, G. *Analytes and related PCR primers used for GMO detection and quantification*; EUR 23059-EN 2007; European Communities: Luxembourg, 2007.
(27) European Commission Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired. *Off. J. Eur. Union.* **2011**, *L166*, 9-15.
(28) European Commission Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off. J. Eur. Union.* **2003**, *L 268*, 1-23.
(29) Codex Committee On Methods Of Analysis And Sampling. *Guidelines On Performance Criteria And Validation Of Methods For Detection, Identification And Quantification Of Specific DNA Sequences And Specific Proteins In Foods*; CAC/GL 74-2010; Codex alimentarius commission; WHO: Rome, 2010.