JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 8913–8920 8913
DOI:10.1021/jf901598r

Collaborative Trial Validation Studies of Real-Time PCR-Based GMO Screening Methods for Detection of the *bar* Gene and the *ctp2-cp4epsps* Construct

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Polymerase Chain Reaction (PCR)-based screening methods targeting genetic elements commonly used in genetically modified (GM) plants are important tools for the detection of GM materials in food, feed, and seed samples. To expand and harmonize the screening capability of enforcement laboratories, the German Federal Office of Consumer Protection and Food Safety conducted collaborative trials for interlaboratory validation of real-time PCR methods for detection of the phosphinothricin acetyltransferase (*bar*) gene from *Streptomyces hygroscopicus* and a construct containing the 5-enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium tumefaciens* sp. strain CP4 (*ctp2-cp4epsps*), respectively. To assess the limit of detection, precision, and accuracy of the methods, laboratories had to analyze two sets of 18 coded genomic DNA samples of events LLRice62 and MS8 with the *bar* method and NK603 and GT73 with the *ctp2-cp4epsps* method at analyte levels of 0, 0.02, and 0.1% GM content, respectively. In addition, standard DNAs were provided to the laboratories to generate calibration curves for copy number quantification of the *bar* and *ctp2-cp4epsps* target sequences present in the test samples. The study design and the results obtained are discussed with respect to the difficult issue of developing general guidelines and concepts for the collaborative trial validation of qualitative PCR screening methods.

KEYWORDS: Genetically modified organism (GMO); screening; real-time PCR; *bar* gene; *ctp2-cp4epsps* construct; collaborative trial; validation; detection

INTRODUCTION

Screening tests are frequently applied to rapidly assess whether or not a sample under investigation is likely to contain materials derived from genetically modified (GM) plants. For DNA-based analyses this is typically done by Polymerase Chain Reaction (PCR) detection of genetic elements that are present in many GM plants. The most widely used targets are the 35S promoter (P-35S) sequence from cauliflower mosaic virus (CaMV) or derivatives of this promoter and the terminator sequence derived from the nopaline synthase (T-nos) gene of *Agrobacterium tumefaciens* (*1*, *2*). According to a 2003 report, which systematically surveyed the genetic elements being used in GM crops worldwide, the P-35S and T-nos elements or derivatives are present in 43 and 37 events, respectively (*3*). This survey identified also other target genes with significant numbers of applications in GM plants, for example, herbicide tolerance genes such as the *cp4epsps* gene derived from *A. tumefaciens* sp. strain CP4 and the phosphinothricin acetyltransferase (*bar*) gene from *Streptomyces hygroscopicus* or from *Streptomyces viridochromogenes* (*pat*). Methods for the detection of these genes using real-time PCR in combination with fluorescence-labeled hybridization probes have already been developed (*4*, *5*).

For national and international harmonization of analytical methods it is necessary to assess the method performance data, especially the interlaboratory repeatability and reproducibility confirming that these methods are fit-for-purpose and transferable to multiple laboratories (*6*, *7*). Furthermore, the availability of qualitative genetically modified organism (GMO) screening methods that are validated for their performance characteristics in collaborative trial studies will help to harmonize the GMO detection approaches at least at the national level. However, the existing harmonized guidelines for collaborative trial validation studies give detailed instructions only for evaluating quantitative methods. At the European level, for example, the guidance document of the European Network of GMO Laboratories (ENGL) provides practical recommendations of how quantitative event-specific PCR methods shall be evaluated in the context of the approval of GM food or feed products according to Regulation (EC) 1829/2003 (*7*, *8*). For qualitative PCR methods, however, appropriate guidelines are hard to find. The Association

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Published on Web 09/03/2009

of Analytical Communities (AOAC) has published a guideline for the validation of official methods for the detection of microorganisms in food (*9*). Recently, a draft Codex Alimentarius document has been published that proposes guidelines concerning the validation and acceptance criteria of qualitative PCR-based methods for the detection and identification of GM plants (*10*). For the validation of screening methods an additional requirement is of course that many existing target and non-target GM events have to be tested and specificity tests with materials of upcoming GM plants have to be continuously added.

Within the framework of the German working group "Development of methods for identifying foodstuffs produced by means of genetic engineering techniques", real-time PCR-based methods for the detection of the *bar* gene and the *ctp2-cp4epsps* gene construct were chosen for validation in two collaborative trial studies. The present work describes the results concerning the reproducibility of the methods regarding sensitivity, accuracy, and precision. The general procedure and validation scheme used are discussed.

MATERIALS AND METHODS

Reference Materials. Reference materials (flours) of the following GM lines were purchased from IRMM (Geel, Belgium): GM maize lines Bt11, Bt176, MIR604, MON810, MON863, NK603, GA 21, TC1507, 3272, 59122; sugar beet GM line H7-1; cotton GM line 3006-210-23x281-24-236; and soybean GM lines GTS40-3-2, DP305423, and DP356043.

Reference materials (flours) from maize GM lines MON88017, potato GM line EH92-527-1, rapeseed GM line GT73, cotton GM lines LL25, MON1445, MON531, and MON15985, and soybean GM lines MON89788, A2704-12, and A5547-127 were obtained from AOCS (Champaign-Urbana, IL). Reference materials (genomic DNA) from rice GM lines LL601 and LL62, rapeseed GM lines MS1, RF1, RF2, MS1xRF1, MS8, RF3, T45, and TOPAS19/2, and maize GM line T25 were purchased from Bayer CropScience (Gent, Belgium).

Genomic DNA from maize GM line CBH351 and rapeseed GM line OXY235 were purchased from Fluka (Buchs, Switzerland). Plant materials (seeds or leaves) from sugar beet GM line GTSB77, rapeseed GM lines Falcon GS40/90, Liberator pHoe6/Ac, LPAAT/Trierucin, and Laurat pCGN3828, and maize GM line T14 were obtained from deliberate field trials. Bt63 rice reference material was obtained from CRL-GMFF (Ispra, Italy). Detailed descriptions of commercial reference materials (catalog numbers, GM content, status of the materials) are given in a publicly available list (*11*).

DNA was extracted from these materials by using the Plant Mini Kit (Qiagen, Hilden, Germany) starting with a CTAB extraction (*12*).

Sample Preparation. For preparation of DNA test samples containing the *bar* target sequence, genomic DNA reference materials derived from leaves of the GM event MS8 and of the GM event LLRice62 were used (Bayer CropScience, Gent, Belgium). Conventional rice grains (local market) and rapeseeds (kind gift of KWS Saat AG, Einbeck, Germany) were ground, and genomic DNA was extracted using the Genomic-tip 100/G kit system (Qiagen, Hilden, Germany). DNA concentrations were determined by the PicoGreen method (*13*). DNA extracts from non-GM rice grains were tested by published PCR methods (*14*−*16*) and found negative for P-35S, *T-nos*, 35S-bar, LLRice601, and LLRice62 before use for preparation of DNA test samples. DNA extracts from non-GM rapeseeds were found to be negative in PCR tests (*4*, *15*) for *T-nos*, *pat*, *epsps*, and *nptII* (personal communication from M. Foth, Eurofins GeneScan, Freiburg, Germany).

DNA test samples containing the *ctp2-cp4epsps* target sequence were prepared from certified reference materials (CRM) of GM maize line NK603 (BF-415b with 1 g/kg NK603; IRMM, Geel, Belgium) and GM rapeseed line GT73 (AOCS 0304-B with 991.9 g/kg GT73; AOCS, Champaign-Urbana, IL). Conventional maize flour (local market) and ground rapeseed material (AOCS 0304-A with <0.5 g/kg GT73) were used as source for non-GM materials. Before use of the ground rapeseeds (AOCS 0304-A) as non-GM sample materials, five separate DNA extractions were checked in *ctp2-cp4epeps* PCR tests, and no positive signals for this target sequence were obtained. DNA from the materials was extracted using the Plant Mini DNA extraction kit (Qiagen, Hilden,Germany), and DNA concentrations were determined photometrically (*12*).

Table 1. Description of DNA Test Samples Used in the Collaborative Trial Studies

method	sample DNA and relative GM content [GM DNA/non-GM DNA]	DNA concn [ng/μL]	GM target copies per PCR[a]	test sample type
bar	0.1% LLRice62	20	200	blind triplicate
	0.02% LLRice62	20	40	
	0.1% MS8	40	75	
	0.02% MS8	40	15	
	non-GM rice	20	0	
	non-GM rapeseed	40	0	
	5% LLRice62	5	2500	standard DNA[b]
ctp2-cp4epsps	0.1% NK603	27	50	blind triplicate
	0.02% NK603	27	10	
	0.1% GT73	13	50	
	0.02% GT73	13	10	
	non-GM maize	27	0	
	non-GM rapeseed	13	0	
	4.91% NK603	28	2500	standard DNA[b]

[a] The number of GM target sequence copies (cp) per PCR was calculated on the basis of the genome sizes, the zygosity levels, and the number of integrations per haploid genome (see Materials and Methods). [b] DNA stock solution used as starting calibration standard (S-2500) and for preparation of four dilutions.

Standard DNAs were prepared either from NK603 CRM (ERM-BF415f with 49.1 g/kg, IRMM, Gel/Belgium) by DNA extraction using the Plant Mini DNA extraction kit (Qiagen) or by dilution of LLRice62 genomic DNA (Bayer CropScience) with DNA extracted from conventional rice grains to obtain a DNA solution with a relative GM content of 5% LLRice62 DNA.

Copy numbers were calculated on the basis of the genome sizes (*17*), the zygosity status of the GM plant materials, and the number of integrated copies of the target sequence. The DNA quantity was determined according to the PicoGreen method (*13*). NK603 CRM is prepared from heterozygous maize kernels. Two copies of the *ctp2-cp4epsps* construct are present in the NK603 genome (*18*), whereas the GT73 rape CRM is prepared from homozygous material with one copy per haploid genome (*19*). In LLRice62 and MS8 genomic DNA, a single copy of the *bar* gene is assumed for the homozygous LLRice62 event (*20*) and the hemizygous MS8 event (*21*), respectively. Correspondingly the absolute copy numbers of the target sequences per PCR given in **Table 1** were calculated by dividing the DNA weight (nanograms per PCR) by the published average 1C value for rice (0.5 pg), oilseed rape (1.33 pg), and maize (2.73 pg), respectively (*17*). Taking the hemizygous status of event MS8 into account, the calculated copy number for MS8 was divided by a factor of 2.

Collaborative Trials. Two collaborative trials were organized by the Federal Office of Consumer Protection and Food Safety (Berlin, Germany) and the German working group "Development of methods for identifying foodstuffs produced by means of genetic engineering techniques". Fifteen laboratories participated in the validation study of the *bar* gene and 11 laboratories in the study of the *ctp2-cp4epsps* gene construct. DNA test samples and standard DNAs provided to the participants are described in **Table 1**. Sample coding was done in a randomized manner. The standard DNA had to be used by the participants as starting calibration DNA standard and for preparation of serial dilutions with 0.2× TE buffer [2 mM Tris-HCl and 0.2 mM ethylenediaminetetraacetic acid (EDTA), adjusted to pH 8.0] to obtain four additional calibration DNAs, respectively. In addition, each laboratory received appropriate amounts of undissolved primers and probes and real-time PCR reagents. DNA test samples were shipped on dry ice and stored at −18 to −25 °C until the analysis.

Real-Time PCR. Real-time PCR was performed using the primers and probes and appropriate concentrations as described in **Table 2**. Primers RapB-F1 and Rap-B-R1 and probe RapB-S1 were used to

Table 2. Primers and Probes Used for Real-Time PCR

method (amplicon length)	name	sequence (5′−3′)	final conc in PCR [nmol/L] plastic vials	glass capillaries	ref
bar (60 bp)	RapB-F1	ACA AgC ACg gTC AAC TTC C	140	340	this work
	RapB-R1	gAg gTC gTC CgT CCA CTC	140	340	
	RapB-S1	FAM-TAC CgA gCC gCA ggA ACC-TAMRA	100	340	
ctp2-cp4epsps (88 bp)	GT73-TmF	ggg ATg ACg TTA ATT ggC TCT g	375	375	*4*
	GT73-TmR	ggC TgC TTg CAC CgT gAA g	375	375	
	GT73-TmP	FAM-CAC gCC gTg gAA ACA gAA gAC ATg ACC-TAMRA	150	150	

amplify and detect the *bar* target sequence; primers GT73-TMF and GT73-TMR and probe GT73-TMP were used to amplify and detect the *ctp2-cp4epsps* construct (*5*). Primers and probes were designed on the basis of a selection of a suitable region within the targeted genetic elements by alignments of sequence data available from a public gene database [NCBI GenBank (*22*)] and by consideration of the criteria recommended by Applied Biosystems (Foster City, CA) for selection and use of Primer Express software version 2. Laboratories equipped with real-time PCR instruments of Applied Biosystems (ABI 7500, 7700, or 7900 of Applied Biosystems, Darmstadt, Germany) performed all reactions in 1× TaqMan Universal Mastermix (Applied Biosystems, Darmstadt, Germany). Laboratories equipped with other real-time PCR devices adapted for plastic vials (iCycler of Bio-Rad, Munich, Germany; realplex2 of Eppendorf, Hamburg, Germany; Rotorgene 3000 of Qiagen, Hilden, Germany;) used 1× QuantiTect Probe PCR Mastermix (Qiagen) for all reactions. If real-time PCR equipment adapted for glass capillaries (LightCycler of Roche, Penzberg, Germany) was used, reactions were carried out in 1× QuantiTect Multiplex PCR No-Rox Mastermix (Qiagen). After the addition of 5 μL of undiluted test sample DNA or of 5 μL standard DNA solutions, the final PCR volume was 25 μL.

For amplification of the *bar* and *ctp2-cp4epsps* target sequence in plastic vials, the thermal cycling program used was 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s; for the amplification of *bar* in glass capillaries the conditions were set to 95 °C for 15 min followed by 45 cycles of 95 °C for 10 s and 60 °C for 15 s, whereas for *ctp2-cp4epsps* amplification cycling conditions of 95 °C for 15 min followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s were used. Fluorescence data were collected during the annealing/elongation step at 60 °C.

RESULTS

Study Design. The collaborative trials for validation of the *bar* and *ctp2-cp4epsps* methods were carried out in parallel during the year 2008. They were designed and conducted according to the principles described in the Harmonized ISO/AOAC/IUPAC Protocol (*6*). Both studies were designed as blind triplicate collaborative trials. The blind samples for the *bar* and *ctp2-cp4epsps* studies were prepared by two different laboratories not involved in the respective ring trial. A total of 15 laboratories participated in the bar trial, whereas in the *ctp2-cp4epsps* study only 11 laboratories participated. A set of 18 coded samples for each study was sent to the laboratories. Each set comprised 12 GM-positive and 6 GM-negative DNA samples. GM-positive samples were composed of triplicates at two analyte levels (0.02 and 0.1%) of GM events MS8 and LLRice62 (*bar*) and GT73 and NK603 (*ctp2-cp4epsps*), respectively (**Table 1**). In addition, participants received genomic standard DNA extracted from 4.91% NK603 CRM or LLRice62 genomic DNA adjusted to 5%.

The standard DNAs had to be used by the laboratories to prepare five calibration standards by serial dilution with 0.2× TE buffer to obtain DNA solutions with theoretical concentrations of 2500, 500, 150, 50, and 10 copies (cp) of the *bar* gene or *ctp2-cp4epsps* target sequence per reaction. A further dilution with a calculated concentration of 5 cp was prepared to generate a sensitivity sample at the LOD. The calibrants were analyzed as duplicates in parallel with the unknown DNA samples (single PCR determinations) in the same real-time PCR run. Calculations of slope and correlation coefficients of the standard curve had to be done by using an Excel spreadsheet provided to all participants. Thereafter, the copy number in the unknown sample DNAs was estimated by interpolation from the standard curves (see **Tables 5** and **7**).

All laboratories participating in the collaborative trial were real-time PCR experienced and in most cases ISO/IEC 17025 (*23*) accredited. Results had to be returned within 4 weeks. None of the participating laboratory reported specific problems in the preparation of the standard curves or in the performance of the PCR analyses with the test samples or the DNA calibrants.

Validation of the *bar* Method. All 15 participating laboratories returned results of their analyses. Because no deviations from the test protocol were reported by the participants, all 270 results were accepted for evaluation of the false-positive and false-negative rates (**Table 3**). All 180 *bar*-positive and 90 *bar*-negative DNA samples were correctly assigned by the laboratories. Accordingly, the resulting total false-positive and false-negative rates are 0%.

Characteristics of the standard curves and PCR instruments used by the participating laboratories are summarized in **Table 4**. Quantitative results and precision data for the analyses of *bar*-positive samples obtained by interpolations from the standard curves generated with 5% LLRice62 DNA standard solutions are shown in **Table 5**. Statistical tests for the rejection of outliers were not performed because the analyzed samples were in the low copy range and the expected high reproducibility variance should not be underestimated by suppression of these significant data. The relative standard deviations under reproducibility conditions (RSD_R) for the 0.02% LLRice62 and MS8 samples are 25 and 31%, respectively. For the 0.1% LLRice62 and MS8 samples the RSD_R values are 24 and 17%, respectively. These precision results conform to the performance requirements for the quantification of GM target sequences defined in ISO 24276:2006 (*24*).

Validation of the *ctp2-cp4epsps* Method. In **Table 6** are summarized the results of the *ctp2-cp4epsps* collaborative study. Eleven laboratories participated and returned their results. All reported results were accepted for the evaluation of the false-positive and false-negative rates. An unexpected high false-positive rate of 19.6% was observed, whereas the false-negative rate was 0%. When expressed in absolute numbers, in 66 independent single determinations a total of 12 false-positive

Table 3. 2008 Collaborative Trial Study Results Obtained for the *bar* Gene-Specific Detection Method

number of laboratories	15
number of laboratories submitting results	15
number of samples per laboratory	18
number of accepted results	270
number of samples containing the *bar* gene	180
number of samples not containing the *bar* gene	90
false-positive results	0 (0%)
false-negative results	0 (0%)

Table 4. Slopes and PCR Efficiences of the Standard Curves of Participating Laboratories

		bar method			*ctp2-cp4epsps* method		
lab code	PCR instrument (*bar*/*ctp2-cp4epsps*)	slope	R^2	PCR efficiency (%)	slope	R^2	PCR efficiency (%)
A	ABI 7500	−3.48	1.00	93.8	−3.36	1.00	98.4
B	ABI 7700/ABI 7900	−3.33	0.99	99.7	−3.52	1.00	92.3
C	ABI 7500	−3.77	0.99	84.2	−3.60	1.00	89.6
D	ABI 7700	−3.40	1.00	96.8	−3.41	1.00	96.5
E	Mx3005p/ABI 7500	−3.77	0.99	84.2	−3.60	1.00	89.6
F	ABI 7500/realplex2	−3.53	1.00	92.0	−3.38	0.98	97.6
G	LightCycler 1.5/Rotorgene 3000	−3.09	0.98	110.7	−3.47	0.99	94.2
H	iCycler	−3.63	0.99	88.6	−4.30	0.99	70.8
I	ABI 7500				−2.52	0.97	149.4
J	ABI 7500	−3.51	1.00	92.7	−3.50	1.00	93.1
K	ABI 7900	−3.15	0.99	107.7	−3.79	1.00	83.6
L	ABI 7500	−3.47	0.99	94.2			
M	ABI 7500	−3.65	1.00	87.9			
N	ABI 7500	−3.55	0.99	91.3			
O	ABI 7500	−3.65	1.00	87.9			
P	ABI 7900	−3.89	1.00	80.7			

Table 5. Quantitative Results Obtained in the Collaborative Trial for the *bar* Gene-Specific Detection Method

relative GM content of sample [GM DNA/non-GM DNA]	ratio of positive results/ determinations	mean copy number[a]	RSD_R (%)	relative content (%) detected[a]
0.1% LLRrice62	45/45	218	24	0.11
0.02% LLRice62	45/45	46	25	0.02
0.1% MS8	45/45	86	17	(0.11)[b]
0.02% MS8	45/45	21	31	(0.03)[b]
non-GM rice	0/45			
non-GM rapeseed	0/45			

[a] Mean values calculated on the assumption that 2×10^5 haploid rice genome copies (100 ng of rice DNA) or 2×10^5 haploid rapeseed genome copies (200 ng rapeseed DNA) were present per reaction, respectively. [b] Data given only for information. The standard curve was generated using LLRice62 DNA, and therefore the accuracy of these calculations cannot be assessed.

Table 6. 2008 Collaborative Trial Study Results Obtained for the *ctp2-cp4epsps* Construct-Specific Detection Method

number of laboratories	11
number of laboratories submitting results	11
number of samples per laboratory	18
number of accepted results	198
number of samples containing the *ctp2-cp4epsps* construct	132
number of samples not containing the *ctp2-cp4epsps* construct	66
false-positive results	13 (19.6%)[a]
false-negative results	0 (0%)

[a] It is noted that 12 false-positive results were caused by minimal contamination with *ctp2-cp4epsps* material, which supposedly occurred during preparation of the test samples.

results were reported by 7 laboratories for the non-GM DNA maize samples. These false-positive signals appeared at Ct values of 37 and higher (mean Ct = 38.5). For the non-GM rapeseed DNA a single positive result (Ct = 39.4) was reported among the 33 non-GM rapeseed DNA samples. By prolongation of the standard curves and interpolation it is assumed that fewer than 5 copies of the *ctp2-cp4epsps* target sequence are detected at Ct values of > 38 (data not shown). Notably, for the non-GM DNA solution no amplifications had been observed in *ctp2-cp4epsps* PCR tests before the test sample aliquots were prepared and distributed to the laboratories. Therefore, minimal contamination by *ctp2-cp4epsps* material supposedly occurred during preparation of the test samples, and it is highly unlikely that unspecific cross-reactivity of the *ctp2-cp4epsps* PCR system caused the false-positive results. Experiences gained by in-house validation and by using different instruments and different master mix reagents in the interlaboratory study indicate that the appearance of false-positives for *ctp2-cp4epsps* cannot be correlated to the real-time PCR equipment or chemicals.

Details on the characteristics of the *ctp2-cp4epsps* standard curves and PCR instruments used by the participating laboratories are summarized in **Table 4**. Quantitative results and precision data for the *ctp2-cp4epsps* method are shown in **Table 7**. RSD_R values for the 0.1% level of NK603 and GT73 DNA were 35 and 32%, respectively. These precision data are marginally above the requirements described in ISO 24276 specifying RSD_R values of 25% or less for the limit of quantification (LOQ) and of 33% for the LOD (*17*). The RSD_R values of 41 and 50% for the 0.02% NK603 and GT73 DNA samples can be attributed to low target copy numbers (9−11 cp) present in these determinations, respectively. It is noteworthy that, again, the precision data are calculated without elimination of potential outliers, so as not to suppress the reproducibility variance.

Specificity. According to the BATS report (*3*), the *bar* gene from *S. hygroscopicus* and the *cp4epsps* gene from *A. tumefaciens* sp. strain CP4 are found in 15 and 12 transgenic crops, respectively. **Table 8** illustrates the theoretical presence of the target sequences and the specificity experiments performed with DNAs derived from mainly publicly available reference materials. All GM crop events carrying the *bar* or *ctp2-cp4epsps* genes reacted positively and with comparable sensitivity when tested with the respective real-time PCR method. In addition, specificity experiments were conducted with several nontarget GM crop events that could potentially cross-react with the *bar* and *ctp2-cp4epsps* PCR primers or probes (**Table 9**). Specificity tests were done particularly with DNAs extracted from GM events carrying the *pat* gene, which is another phosphinothricin acetyltransferase gene derived from the related bacterium *Streptomyces viridochromogenes*, and with DNAs extracted from GM crops containing *epsps* genes with modified nucleotide sequences or derived from *A. tumefaciens* strains other than strain CP4. All tests performed showed no cross-reactivity and confirmed the specificity of the *bar* and *ctp2-cp4epsps* PCR systems (**Tables 8** and **9**). It has to be remarked that event MON40-3-2 (Roundup Ready soybean) is not detected because in this event a chloroplast transit peptide (CTP4) coding sequence from *Petunia hybrida* was used in the *cp4epsps* construct (*25*), whereas the CTP2 sequence targeted in the *ctp2-cp4epsps* PCR originates from *Arabidopsis thaliana*. Therefore, the *ctp2-cp4epsps* method is not suitable to screen for soybean event MON40-3-2.

Currently available reference materials are certified only for the relative absence of the GM event of interest (e.g., < 0.04% for NK603 maize material BF415a of IRMM), but not for the absence of other GM events and derived sequences (*26*). These reference materials are not intended for use in specificity testing, and therefore it is noteworthy that for interlaboratory and in-house method validation studies or for use as negative DNA target controls the available 0% GM maize, soybean and rapeseed reference materials at the IRMM and the AOCS are not suitable non-GM materials for this purpose.

Limit of Detection (LOD) and Limit of Quantification (LOQ). The participating laboratories had to prepare a dilution of the LLRice62 and NK603 maize standard DNAs with calculated concentrations of 5, 10, and 50 cp for the *bar* and *ctp2-cp4epsps* target sequences. These DNA test samples were essentially useful to obtain reproducibility data on the methods' sensitivity at the LOD and LOQ. All laboratories reported positive results in all duplicate PCR tests with the 5 cp test sample with a mean Ct of 35.9 for the *bar* method and a mean Ct of 37.4 for the *ctp2-cp4epsps* method (data not shown). On the basis of the results from the collaborative trials, the absolute LOD for each of the real-time PCR methods is here reported to be at least 5 copies.

Table 7. Quantitative Results Obtained in the Collaborative Trial for the *ctp2-cp4epsps* Construct-Specific Detection Method

relative GM content of sample [GM DNA/ non-GM DNA]	ratio of positive results/ determinations	mean copy number[a]	RSD_R (%)	relative content (%) detected[a]
0.1% NK603	33/33	50	35	0.11
0.02% NK603	33/33	11	41	0.02
0.1% GT73	33/33	36	32	(0.072)[b]
0.02% GT73	33/33	9	50	(0.018)[b]
non-GM maize	12[c]/33			
non-GM rapeseed	1/33			

[a] Mean values calculated on the assumption that 5×10^4 haploid maize or rapeseed genome copies were present per reaction. [b] Data given only for information. The standard curve was generated using NK603 maize DNA, and therefore the accuracy of these calculations cannot be assessed. [c] Laboratories with positive results for the non-GM maize reported Ct values of 37 and higher (mean Ct = 38.5) for these samples. It is supposed that minimal contaminations with ctp2-cp4epeps materials occurred during preparation of the non-GM maize test samples (see also text).

The LOD and LOQ are defined as the lowest concentrations of the analyte that can be reliably detected and quantified, respectively. According to the ENGL and Codex documents (*7*, *10*), the LOD refers to the amount of analyte at which the method detects the presence of the analyte at least 95% of the time (≤5% false-negative results). **Tables 3** and **6** show that for both the *bar* and *ctp2-cp4epsps* PCR methods no false-negative results were reported. The LOQ of a method has been defined as the lowest amount or concentration of an analyte that can be quantified with an acceptable level of precision and accuracy. Such LOQ precision values have been given in terms of the relative reproducibility standard deviation (RSD_R) and according to ENGL and Codex (*7*, *10*) the RSD_R should be ≤50% at relative GM target concentrations of < 0.2%. As presented in **Tables 5** and **7** the relative LOD and LOQ values are at least 0.02% and 0.1% for both methods.

DISCUSSION

Additional Targets for GMO Screening. Today, many European laboratories mainly use P-35S and T-nos as PCR screening tests to detect the presence of GM materials (*2*). To avoid time and cost-intensive multiple GM event-specific tests, expanding the repertoire of targeted genetic elements for more efficient screening is particularly useful. As can be seen in a recently published table (*27*, *28*), the combination of five real-time PCR-based screening tests, including the *bar* and *ctp2-cp4epsps* methods presented here, covers the detection of 67 GM events of several crop plants (potato, maize, papaya, rapeseed, rice, soya, tomato, sugar beet). In addition, this approach provides the advantage of detecting also GM events that are not authorized according to European food and feed regulations, an emerging risk reflected by the recent cases of unauthorized GM rice events found on the European Union market (*29*, *30*). The results reported in this study show that the *bar* and *ctp2-cp4epsps* real-time PCR methods are fit-for-purpose for screening of GM materials in the context of food, feed, and seed inspections and can now be used especially for official enforcement purposes. In combination with the ring-trial validated P-35S and T-nos real-time PCR methods (*14*, *15*, *31*), an expanded set of targets may now be applicable for efficient GMO screening.

Table 8. Theoretical and Experimental Specificity of the *bar* and *ctp2-cp4epsps* Real-Time PCR Methods for Target GM Events

	GM crop (event name)						
method	canola	cotton	maize	potato	rice	soybean	sugar beet
bar	MS1 (+)[a] RF1 (+)[a] RF2 (+)[a] MS1xRF1 (+)[a] MS1xRF2[b] MS8 (+)[a] RF3 (+)[a] MS8xRF3[b] PHY23[b] PHY36[b]	LL25 (+)[a]	B16 (DLL26)[b] Bt176 (+)[a] CBH-351 (+)[a] TC6275[b] DBT418 (+)[a] MS3[b] MS6[b]	−/−	LLRice62 (+)[a] LLRice06[b] LLRice601 (+)[a] LLRice604[b]	W62, W98[b]	none
ctp2-cp4epsps	GT200[b] GT73 (+)[a]	MON1445 (+)[a] MON1698[b]	MON80100[b] MON802[b] MON809 (+)[a] MON832[b] MON88017 (+)[a] NK603 (+)[a]	RBMT22−082[b] RBMT22−186[b] RBMT22−238 [b] RBMT22−262 [b]	−/−	MON89788 (+)[a] MON87754−1 [b]	GTSB77 (+)[a] H7−1 (+)[a]

[a] Experimentally verified using available reference materials. [b] Not experimentally verified, but theoretically contains the respective element according to the BATS report (*3*) and the agbios database (*25*).

Table 9. Experimental Specificity Tests with DNA Samples of Nontarget GM Events[a]

	GM crops (event name)						
method	canola	cotton	maize	potato	rice	soybean	sugar beet
bar	Liberator pHoe6/Ac GT73 Falcon GS40/90 TOPAS19/2 (HCN92) OXY235 T45 (HCN 28) LPAAT/Trierucin (pRESS) Laurat pCGN3828	MON1445 MON531 MON15985 3006-210-23x281-24-236	GA21 Bt11 MON809 MON810 MON863 MON 88017 NK603 DAS1507 DAS59122 MIR604 3272 T14 T25	EH92-527-1	Bt63	MON40-3-2 A2704-12 A5547-127 DP305423, DP356043 MON89788	GTSB77 H7-1
ctp2-cp4epsps	Liberator pHoe6/Ac Falcon GS40/90 Laurat (pCGN3828) TOPAS19/2 MS1xRF1 MS8 T45 (HCN 28)	−/−	3272 DAS59122 Bt176 MON810 T14 T25 DAS1507 GA21	EH92-527-1	LLRice62 LLRice601	DP305423 DP356043 MON40-3-2 A2704-12 A5547-127	−/−

[a] The indicated events showed no reactivity in PCR experiments using the *bar* and *ctp2-cp4epsps* real-time PCR methods.

Collaborative Trial Study Design. Several interlaboratory studies of quantitative real-time PCR methods for GMO analysis have been conducted, and the results have been published (*14*, *32*−*35*). However, the main application of these methods is to quantify the copy number of the target sequence in a sample, either of GM-specific targets (*14*, *32*, *33*) or of plant species-specific reference genes (*34*, *35*). Because the realization of interlaboratory studies requires considerable resources due to complex sets of blind test samples and the large number of participating laboratories, we intended to practically prove the suitability of a smart study design for interlaboratory studies of qualitative real-time PCR-based methods for GMO screening purposes. In general, a collaborative study for this method type generates qualitative data concerning accuracy and precision in terms of the frequencies of false-negative and/or false-positive results at the detection limit under reproducibility conditions. According to the concept for the validation of qualitative PCR-based detection methods proposed in the draft Codex document (*10*), the limit of detection to be achieved for these methods is defined as the concentration at which a positive sample yields a positive result at least 95% of the time, which results in a rate of false negatives of ≤5%. Test samples at the LOD intended to be used in a collaborative study must therefore be adjusted to analyte concentrations that allow correct identification also under reproducibility conditions. In addition, the relatively high false-positive rate obtained in the *ctp2-cp4epsps* collaborative validation shows that particular precautions have to be taken for the preparation of non-GM test samples to minimize the number of misclassified known negative samples. This indicates also that GMO testing laboratories have to evaluate with great care all samples that are PCR-positive at the LOD in the GM screening. Such samples require clear confirmatory PCR signals in the subsequent tests for GM line identification before the final analytical report is released.

A study design for qualitative PCR methods potentially applied for screening purposes has to consider that the presence and/or absence of the target sequence is correctly and reproducibly detected in as many as possible different GM events and respective crops. The collaborative trial studies described here are designed in a way that available CRMs of two GM events in two different crops were selected as basic test sample materials. To meet the sensitivity requirements for PCR-based qualitative screening methods, analyte levels of 0.1 and 0.02% relative target sequence contents were selected. To take advantage of the feasibility of real-time PCR methods to quantify the amplified target sequences, the data obtained in the interlaboratory study were used to calculate the precision of the *bar* and *ctp2-cp4epsps* PCR methods in terms of the reproducibility standard deviations in the low copy range at the LOD/LOQ. Taking into account that for GMO screening methods the sensitivity and not the quantification of the GM content is most important, a high variance of Ct values is acceptable in the low copy range. Therefore, it was decided to calculate the reproducibility standard deviation (RSD_R) values without rejecting outliers by statistical tests. A similar approach has been recommended in an interlaboratory study of a P-35S real-time PCR screening method (*14*). The authors of this study show that the classical calculation with the data from all laboratories will not lead to underestimation of the precision.

Test Samples for Determination of LOD and Specificity. A representative number of six negative test samples per laboratory was included in the study design. However, the production of true negative test samples appeared to be a particularly critical issue in collaborative trial validations of qualitative screening methods. A highly sensitive test method with an inherent broad specificity for several GMOs increases the risk dramatically that even minimal contaminations will result in unexpected (false-positive) detection in the test samples. This problem has already been experienced by several researchers in the GMO analysis field and is also reflected by the difficulty of purchasing certified non-GM reference material for soy, maize, and rapeseed that are PCR-negative in the low copy range at the LOD of the method (*26*, *31*).

It is expected that further work has to be done to confirm the sensitivity and specificity of the *bar* and *ctp2-cp4epsps* methods using reference materials of additional GM crops containing the two target sequences. In Germany, it is planned to add results obtained in these tests to a publicly available table for GMO screening (*28*). The draft Codex document (*10*) recommends that

before full validation of a method in a collaborative study the analytical method should be prevalidated for its performance characteristics (e.g., specificity and sensitivity) by involving at least two laboratories for interlaboratory comparison. We here propose that the sample DNA for the experimental verification of the negative reactivity in such a prevalidation study should ideally contain a high amount of amplifiable nontarget genomic DNA (e.g., $\geq$2500 cp per PCR) and that the analyses have to be negative in at least two replicates. To verify the positive reactivity of the PCR screening method, a low amount of amplifiable genomic DNA of the event to be tested (usually 10−50 cp) should be present per reaction, and all tests have to be positive in at least five replicates.

Modular Approach. For simplification of the study it was decided to follow the concept of the "modular approach" (*36*) that is applied also in the validation studies of event-specific methods of the European Community Reference Laboratory for GM food and feed (*16*). In this approach, it is assumed that the nucleic acid extraction is done by suitable methods fully characterized in terms of DNA yield and integrity prior to the PCR analysis and therefore applicable to any DNA template containing a given genetic element. However, in the routine analysis of enforcement laboratories the results obtained depend also on the quantity and quality of the template DNA extracted from a sample under investigation. In routine application of the *bar* and *ctp2-cp4epsps* PCR methods, the performance characteristics reported here demonstrate that even at analyte levels of 0.02% the targeted homozygous genetic elements should be detectable, if at least 25000 cp (LOD = 5 copies/25000 copies species DNA = 0.02%) of genomic rice, maize, or rapeseed DNA are used in PCR tests, respectively.

If the study design would include a DNA extraction from the test samples, it would be not possible, however, to distinguish which analytical module (extraction or PCR) affects the performance characteristics, for example, the achievable sensitivity and precision. It is therefore noted that for GMO screening purposes different DNA extraction methods suitable for various food and feed matrices should be available and ideally be validated separately in collaborative trials to assess performance data of the extraction module and its contribution to the measurement uncertainty of the whole analytical procedure.

ABBREVIATIONS USED

cp, copies; CRM, certified reference material; LOD, limit of detection; LOQ, limit of quantification; RSD_R, relative standard deviation under reproducibility conditions;

ACKNOWLEDGMENT

We are very grateful to Britta Boernsen (CVUA, Freiburg, Germany) and to Gülperi Kul and Marianne Voetz (CVUA, Münster/Germany) for their excellent technical help and to Almuth Spiegelberg (BfR, Berlin, Germany) for her help in preparing rapeseed DNA. We thank KWS Saat AG, Einbeck, Germany, for their kind gift of conventional rapeseeds. We thank Dr. Joachim Bendiek (BVL, Berlin/Germany) for carefully reading the manuscript.

We are especially grateful to all laboratories and participants of the collaborative trials. The participants of the *bar* collaborative trial were Claudia Brünen-Nieweler, Chemisches und Veterinäruntersuchungsamt Münsterland-Emscher-Lippe (Münster, Germany); Sabine Burkhardt, Institut für Lebensmittel, Arzneimittel und Tierseuchen im BBGES (Berlin, Germany); Norbert Graf, Landesuntersuchungsamt Rheinland-Pfalz, Institut für Lebensmittelchemie (Trier, Germany); Gerda Hempel, Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen (Dresden, Germany); Norbert Hess, Institut für Hygiene und Umwelt (Hamburg, Germany); Rupert Hochegger, Agentur für Gesundheit und Ernährungssicherheit (Wien, Austria); Gabi Mücher, GEN-IAL GmbH (Troisdorf, Germany); Frank Narendja, Umweltbundesamt (Wien, Austria); Ralf Reiting, Landesbetrieb Hessisches Landeslabor (Kassel, Germany); Hermann Rüggeberg, Impetus GmbH (Bremerhaven, Germany); Manuela Schulze, Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Braunschweig, Germany); Daniel Suter, Bundesamt für Gesundheit, Abt. Lebensmittelwissenschaft (Bern, Switzerland); Hans-Ulrich Waiblinger, Chemisches und Veterinäruntersuchungsamt (Freiburg, Germany); Annelis Winterstein, Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz (Bad Langensalza, Germany); and Karl Woll, Landesamt für Soziales, Gesundheit und Verbraucherschutz (Saarbrücken, Germany).

The participants of the *ctp2-cp4epsps* collaborative trial were Claudia Brünen-Nieweler, Chemisches und Veterinäruntersuchungsamt Münsterland-Emscher-Lippe (Münster, Germany); Ulrich Busch, Bayerisches Landesamt für Gesundheit (Oberschleissheim, Germany); Norbert Graf, Landesuntersuchungsamt Rheinland-Pfalz, Institut für Lebensmittelchemie (Trier, Germany); Gerda Hempel, Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen (Dresden, Germany); Rupert Hochegger, Agentur für Gesundheit und Ernährungssicherheit (Wien, Austria); Matthias Kuhn, Congen GmbH (Berlin, Germany); Gabi Mücher, GEN-IAL GmbH (Troisdorf, Germany); Frank Narendja, Umweltbundesamt (Wien, Austria); Manuela Schulze, Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Braunschweig, Germany); Daniel Suter, Bundesamt für Gesundheit, Abt. Lebensmittelwissenschaft (Bern, Switzerland); Karl Woll, Landesamt für Soziales, Gesundheit und Verbraucherschutz (Saarbrücken, Germany).

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Received May 14, 2009. Revised manuscript received July 22, 2009. Accepted August 20, 2009.