FOOD COMPOSITION AND ADDITIVES

Validation of PCR Methods for Quantitation of Genetically Modified Plants in Food

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For enforcement of the recently introduced labeling threshold for genetically modified organisms (GMOs) in food ingredients, quantitative detection methods such as quantitative competitive (QC-PCR) and real-time PCR are applied by official food control laboratories. The experiences of 3 European food control laboratories in validating such methods were compared to describe realistic performance characteristics of quantitative PCR detection methods. The limit of quantitation (LOQ) of GMO-specific, real-time PCR was experimentally determined to reach 30–50 target molecules, which is close to theoretical prediction. Starting PCR with 200 ng genomic plant DNA, the LOQ depends primarily on the genome size of the target plant and ranges from 0.02% for rice to 0.7% for wheat. The precision of quantitative PCR detection methods, expressed as relative standard deviation (RSD), varied from 10 to 30%. Using Bt176 corn containing test samples and applying Bt176 specific QC-PCR, mean values deviated from true values by –7 to 18%, with an average of 2 ± 10%. Ruggedness of real-time PCR detection methods was assessed in an interlaboratory study analyzing commercial, homogeneous food samples. Roundup Ready soybean DNA contents were determined in the range of 0.3 to 36%, relative to soybean DNA, with RSDs of about 25%. Taking the precision of quantitative PCR detection methods into account, suitable sample plans and sample sizes for GMO analysis are suggested. Because quantitative GMO detection methods measure GMO contents of samples in relation to reference material (calibrants), high priority must be given to international agreements and standardization on certified reference materials.

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The use of genetically modified (GM) plants for food and feed has become increasingly important worldwide. The global area of transgenic crops increased from 1.7 million hectares (Mio ha) in 1996 to 39.9 Mio ha in 1999. Three countries, the United States (72%), Argentina (17%), and Canada (10%), together covered 99% of the global area of transgenic crops. The most important GM crops grown in 1999 were herbicide-tolerant soybean, 54% of global area; insect-resistant corn, 19%; and herbicide-tolerant rape, 9% (1).

For the production of food, the European Commission (EC) has approved products from GM soybeans, corn, and rape according to novel foods legislation (2). In Switzerland, approval of GM plants for food production is regulated by Ordinance VBGVO (3). Switzerland and the EC have adopted labeling regulations for foods and food ingredients derived from GM plants (2, 4, 5) to guarantee consumers a choice between GM and non-GM products. The principle of substantial equivalence is decisive for GM food labeling in the United States, whereas the criterion for food labeling in the EC is the presence of proteins or DNA resulting from genetic modification. However, when the presence of GM material (DNA or protein) is adventitious and represents only a small amount, e.g., as a result of commingling during cultivation, harvesting, transport, or processing, labeling becomes noninformative for the consumer. Therefore, de minimis threshold values have recently been introduced to distinguish adventitious contamination of GM materials from food produced from GM material. In Switzerland and in the EC, the threshold value was set by the legislative bodies to 1% of GM material on the basis of ingredients (4, 6).

Methods based on polymerase chain reaction (PCR) are suitable for specific and sensitive detection of DNA from GM plants as described for Roundup Ready soybean (7, 8), Bt176 corn (9–11), Bt11 corn (12), BtMON810 corn (13), Flavr Savr tomato (14), Zeneca tomato (15), and the Liberty Link rape (16). Interlaboratory studies were undertaken to validate the methods for detection of GM soybeans (Roundup Ready soybean) and GM corn (Bt176; 17, 18).

The introduction of a threshold value per ingredient imposed new challenges for GM food analysis. For the enforcement of threshold values, quantitative detection methods for

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Table 1. Specificity testing of maize-specific real-time PCR detection system targeted to the maize invertase system

DNA-source[a]	English name	Ct-value	Factor[b]
Zea mays (conventional) 1	Corn	21.14	1.0
Zea mays (conventional) 2	Corn	21.27	0.9
Zea mays (Bt11 maize) 1	Corn	22.19	0.5
Zea mays (Bt11 maize) 2	Corn	20.96	1.1
Zea mays (Bt176 maize) 1	Corn	20.89	1.2
Zea mays (Bt176 maize) 2	Corn	21.06	1.1
Zea mays (BtMON810 maize)	Corn	21.07	1.1
Zea mays (T25 maize)	Corn	20.55	1.5
Sorghum halepense	Indian millet	26.56	2.3E-2
Pennisetum americanum 1	Pearl millet	33.12	2.5E-4
Pennisetum americanum 2	Pearl millet	30.18	1.9E-3
Pennisetum americanum 3	Pearl millet	30.31	1.7E-3
Oryza sativa 1	Rice	38.12	7.7E-6
Oryza sativa 2	Rice	37.62	1.1E-5
Oryza sativa 3	Rice	38.24	7.1E-6
Oryza sativa 4 (parboiled)	Rice	>40	<2.1E-6
Avena sativa	Oat	>40	<2.1E-6
Glycine max	Soybean	>40	<2.1E-6
Hordeum vulgare	Barley	>40	<2.1E-6
Secale cereale	Rye	>40	<2.1E-6
Triticum aestivum	Wheat	>40	<2.1E-6

[a] Numbers indicate different sources of plant cultivars.

[b] The factor is calculated by the formula: 2 exp (mean Ct*corn*-Ct[variety]. The mean Ct-value for the 8 tested corn varieties is 21.14 ± 0.47.

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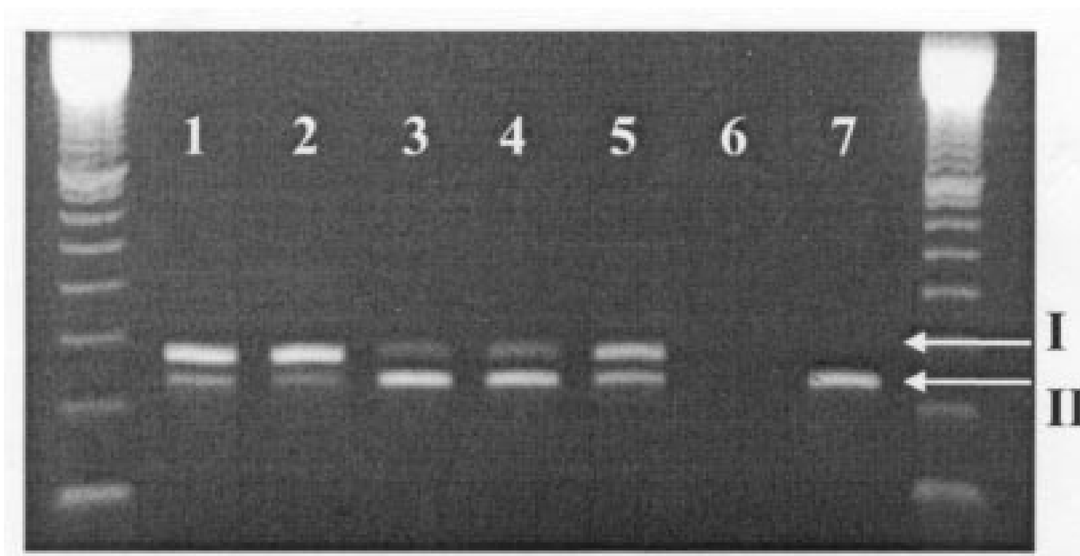

Figure 1. Variation of zein copy number between different maize cultivars. The zein QC-PCR system was equilibrated with 200 ng DNA isolated from corn flour (Fluka, No. 17111). For each PCR, 200 ng corn DNA from different cultivars were used. Lane 1: conventional corn (measured ratio of the intensities of target band vs competitor band: 3.2); lane 2: Bt11 corn (4.6); lane 3: BtMON810 corn (0.27); lane 4: T25 corn (0.37); lane 5: corn flour (No. 17111; 1.5); lane 6: mastermix control; lane 7: 250 000 copies of the competitor plasmid; as DNA length marker, a 100 bp ladder was used. I: Target band; II: competitor band.

Table 2. Influence of plant genome size on theoretical limit of quantatition

Common name	Scientific name	Genome size[a] (in Mia bp)	Genome copies (per 200 ng)	1%	0.1%	0.01%	Limit of quantitation, %[b]
Barley	*Hordeum vulgare*	9.8	19000	190	19	2	0.2
Cassava, manioc	*Manihot esculenta*	1.5	120000	1200	120	12	0.033
Corn	*Zea mays*	5.0	36000	360	36	4	0.1
Cotton	*Gossypium hirsutum*	4.5	40000	400	40	4	0.1
Oats	*Avena sativa*	22.6	8000	80	8	—	0.4
Pea	*Pisum sativum*	8.3	22000	220	22	2	0.2
Potato	*Solanum tuberosum*	3.5	53000	530	53	5	0.07
Rape	*Brassica napus*	2.4	77000	770	77	8	0.05
Rice	*Oryza sativa*	0.9	210000	2100	210	21	0.02
Sorghum	*Sorghum bicolor*	1.5	120000	1200	120	12	0.03
Soybean	*Glycine max*	2.2	82000	820	82	8	0.04
Sugarbeet	*Beta vulgaris*	1.5	120000	1200	120	12	0.03
Sugarcane	*Saccharum robustum*	6.3	29000	290	29	3	0.1
Sunflower	*Helianthus annuus*	6.1	30000	300	30	3	0.1
Tobacco	*Nicotiana tabacum*	8.9	20000	200	20	2	0.2
Tomato	*Lycopersicon esculentum*	1.9	96000	960	96	10	0.04
Wheat	*Triticum aestivum*	31.9	6000	60	6	—	0.6

[a] Published genome sizes (per 2C) were taken from Arumuganathan and Earle (30).

[b] The theoretical limit of quantitation is expressed as the fraction (%) of 36 copies divided by number of copies of the corresponding plant species within 200 ng DNA.

materials derived from GM plants are necessary. In the last 2 years, methods for the quantitation of GM plants have been published, based either on competitive (12, 19–21) or real-time PCR (21–24; Figure 1C). In addition, first interlaboratory results applying quantitative PCR detection methods were obtained (25). For the assessment of the results obtained by quantitative PCR methods, validation data concerning sensitivity, specificity, precision, accuracy, and reproducibility must be available. The suitability of genetically modified organism (GMO) detection methods for the surveillance of threshold values depends strongly on these validation data.

The practical experiences of 3 official food control laboratories working in 2 European countries were reviewed to describe the performance characteristics of modern GM detection methods in terms of validation parameters. Based on these experiences, recommendations for the validation of quantitative PCR methods are presented.

Experimental

Samples

Plant material (e.g., seeds, grains, tubers) from defined non-GMO or GMO plant species (e.g., MON810 corn, Bt11 corn) was used to test the specificity of PCR. Certified reference materials (CRM; Institute of Reference Materials and Measurements [IRMM], Geel, Belgium), consisting of soybean or corn flour with defined contents of GMO (Roundup Ready soybeans and Bt176 corn, respectively) were used for the validation experiments to determine accuracy and limits of quantitation (LOQ) and detection (LOD). Mixtures of reference materials treated as unknown samples as well as commercially available foodstuffs served as samples for reproducibility testing in interlaboratory studies.

DNA Extraction

DNA was extracted from samples in accordance with the Swiss Food Manual (24). DNA concentrations were determined by UV-spectrophotometry (24). DNA isolated from soybean flour (Fluka No. 53198, Buchs, Switzerland) was mixed with DNA isolated from corn (Fluka No. 63195) to yield DNA solutions with a concentration of 20 ng plant DNA/μL containing 100, 50, 25, 10, 5, 1, and 0.1% (w/w) soya DNA. These DNA mixtures were used for validation experiments to determine the range of quantitation of a soybean lectin-specific, real-time PCR.

Quantitative PCR

The specificity testing of species-specific PCR systems was performed by amplifying a corn-specific DNA-sequence (invertase or zein gene; 10, 26). The accuracy of quantitative competitive PCR (QC-PCR) was determined by quantitation of a Bt176 corn-specific PCR fragment (19). To determine the accuracy and reproducibility of real-time PCR using ABI Prism SDS 5700 or 7700 (Applied Biosystems, Rotkreuz,

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Table 3. Experimental determination of limits of quantitation and detection for real-time PCR-based GMO detection systems

A[a]

Target DNA		Measurement			
Name	Copies	Mean	RSD, %	CI 95%	*n*
Lectin	50000	54100	10.6	13.2	5
	10000	9100	6.4	8.0	5
	2000	1900	10.8	17.2	4
	670	720	10.0	12.5	5
	110	100	14.9	18.4	5
	55	62	19.4	23.0	5
RRS	2500	2700	13.6	16.9	5
	500	460	10.4	12.9	5
	100	110	15.5	24.6	4
	33	36	13.9	17.6	5
	6	5	40.0	46.3	5

B[b]

Target DNA	Limit of quantitation	Limit of detection
Lectin	50 copies	20 copies
RRS	30 copies	5 copies
RRS/lectin	30/50000copies (0.06%)	5/50000 copies (0.01%)

[a] DNA isolated from RRS standard (Fluka) was photometrically quantitated and adjusted to indicate concentration levels by dilution with buffer. At each concentration level 5, replicates were measured using real-time PCR. The mean values, RSDs, and relative 95% confidence intervals (CI 95%) are indicated for detection of soybean-specific lectin gene (lectin) and the transgene introduced into Roundup Ready soybean (RRS). The CI = 95% is calculated by:

$$\mathbf{CI95\%} = \frac{\sigma \times \boldsymbol{t} \times 100}{\sqrt{\boldsymbol{n}} \times \bar{x}}$$

whereby σ is the standard deviation, *t* is the Student factor ($t = 2.776$ for $n = 5$ and $t = 3.182$ for $n = 4$ at $P = 95\%$), *n* is the number of replicates, and $\bar{x}$ is the mean value.

[b] Limits of quantitation and detection were inferred from the experimental data shown in A. The relative CI = 95% reached 30% at the limit of quantitation and 100% at the limit of detection, respectively.

Switzerland) or Light Cycler™ (Roche, Rotkreuz, Switzerland), an endogenous soybean gene (lectin), the RRS-specific gene construct and a sequence from the Cauliflower Mosaic Virus 35S promoter were amplified (23, 24).

Results

Principles and Design of Quantitative PCR Methods

QC-PCR coamplifies in the same reaction mixture the target sequence (e.g., part of a transgene) and a suitable competitor sequence. The competitor can be a cloned target sequence on a linearized plasmid having distinguishable modifications such as a DNA insertion or DNA deletion. A few GMO-specific QC-PCR detection systems are described (12, 19, 20). For QC-PCR GMO-specific PCR primers, quality-assured competitor DNA and the equipment for qualitative PCR—including a conventional thermocycler, gel electrophoresis, and a video documentation system with appropriate integration software—are required.

In real-time PCR, fluorescent-labeled oligonucleotides homologous to the internal part of the amplified target sequence are required in addition to the PCR primer pair. The fluorescence increases if the probes bind to the target sequence during amplification either due to hydrolysis of quenching molecules by *Taq* DNA polymerase (TaqMan® probes) or to Fluorescence Resonance Energy Transfer (FRET) between 2 probes hybridizing in close proximity (FRET-probes). The amount of measured fluorescence is proportional to the amount of amplified target DNA and can be detected in real-time (online) during the PCR-process. For real-time PCR,

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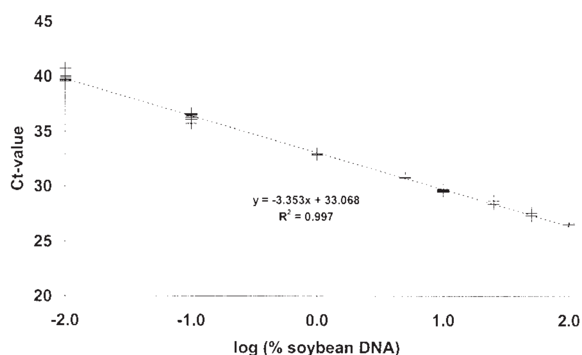

Figure 2. Range of quantitation for lectin-specific real-time PCR. For calibration of the lectin-specific real-time PCR method, duplicate DNA solutions containing 100, 50, 25, 10, 5, 1, and 0.1% soybean DNA, mixed with maize DNA, were measured. For determination of the precision, 6 replicates were determined at concentrations of 10, 0.1, and 0.01% soybean DNA in maize DNA. The mean % values and RSD values (in parenthesis) were 11 (5.3%), 0.11 (26%), and 0.009 (24%), respectively. Parameters of the regression line through all data points are indicated within the plot. The slope of the regression line (–3.353 ± 0.035) is close to the theoretical value of –3.322, representing 100% amplification efficiency from cycle to cycle.

GMO-specific PCR primers, GMO-specific probes (fluorescently labeled), and the equipment for real-time PCR, including a thermocycler with fluorescent detection system, are required.

For the design of new PCR detection methods, it is necessary to have the correct sequence information on the introduced transgene and reference material of a high degree of purity. Otherwise, the design of a PCR method and its subsequent validation are not feasible. For designing a PCR detection method, the following points have to be considered: The amplified fragment should be as small as possible to allow a sensitive detection in processed food, DNA is fragmented to pieces smaller than 500 base pairs (bp; 8). However, small amplicons are difficult to separate from primer dimers and competitor amplicons on agarose gels. In the case of QC-PCR, we found that fragments smaller than 100 bp cannot be separated and quantitated reliably with a video documentation. In addition, fragments smaller than 100 bp restrict the possibilities of positioning the specific PCR primers on the target sequence while respecting the rules of good PCR primer design (such as avoidance of hairpin, duplex, or other disturbing structures). To obtain GMO-specific PCR detection methods, the amplified target sequence should ideally span either the junction of elements used for construction of the transgene, such as promoter, intron, transit peptide gene, structural gene, or terminator, or the integration site of the transgene into the plant genome.

The PCR design must be tested using a primer design software program to avoid hairpin structures, primer duplexes, and unbalanced primer annealing temperatures (27). Moreover, the primer sequences should be run against a sequence database (e.g., EMBL database) to verify that only the target sequence and no other displays significant homology to the primer sequence. However, we emphasize that theoretical biocomputing tests will never replace the empirical testing of specificity and sensitivity of a given PCR detection method.

Validation Parameters for Quantitative PCR Methods

Before method validation, a written standard operating procedure (SOP) must be established, including all information needed to perform the entire analytical procedure (28). The analyte to be tested must be clearly identified and described. In the following sections, the availability of DNA, free of PCR inhibitors, and its sufficient quality and amount are assumed.

Specificity and Selectivity

Specificity is defined as the ability of a method to detect a substance or a class of substances without impairment by other components present in the sample and to identify the analyte unambiguously. Selectivity is the ability of a method to detect different components in parallel without reciprocal interference and to identify the analyte unambiguously (28). Thus, a selective method yields correct results for all interesting analytes, whereas a specific method produces correct results for one particular analyte while other analytes might interfere with each other. Quantitative PCR detection methods for GMO food analysis must be highly specific. If multiplex quantitative PCR detection methods are applied, they must also be selective.

The specificity testing of quantitative GMO-specific PCR detection methods (QC-PCR and real-time PCR) follow, in principle, the same guidelines used for qualitative GMO-specific PCR-methods. Specific detection systems for plant species such as wheat are difficult to achieve because of the presence of homologous DNA sequences in closely re-

Table 4. Experimental determination of accuracy for QC-PCR-based GMO detection systems

Description of samples		Bt176-specific QC-PCR[b]			
Sample ID	True value[a]	Mean[a]	RSD	abs. Δ	rel. Δ[c]
MA	1.4	1.7	19.5	0.3	18
MB	1.8	1.8	10.1	0.0	–1.1
MC	3.0	2.8	4.5	–0.2	–6.5
MD	0.7	0.7	10.7	0.0	1.4
ME	1.0	1.0	15.4	0.0	–3.9

[a] Values are given in % Bt176 corn within conventional corn.
[b] Bt176-specific QC-PCR, as described by Studer et al. (19); $n = 8$.
[c] Relative deviations are calculated by dividing absolute deviation by true value, and are given in %.

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Table 5. Experimental determination of reproducibility for PCR-based GMO detection systems

Sample ID	Parameter	Soybean-DNA, % lectin-DNA[a]	GMO content, % 35S (norm)[b]	GMO content, % RRS (norm)[c]
A	Mean	71	8.6	8.0
	Std. dev.	13	1.4	1.8
	RSD, %	19	17	23
B	Mean	69	11.4	12.1
	Std. dev.	11	3.0	3.3
	RSD, %	15	26	28
C	Mean	75	0.33	0.30
	Std. dev.	29	0.06	0.08
	RSD, %	39	18	27
D	Mean	70	34	36
	Std. dev.	7	9.4	13.1
	RSD, %	10	28	36

(The food samples were analyzed by 4 participant laboratories.)

[a] Lectin-specific real-time PCR (24).

[b] 35S-specific real-time PCR (24). The given values are normalized with the amount of soybean DNA present in the sample.

[c] Roundup Ready soybean specific real-time PCR (24). The given values are normalized with the amount of soybean DNA present in the sample.

lated plant species (e.g., barley, rye). Thus, we recommend testing the specificity of a detection system with DNA from closely related agricultural plants in addition to the most important plants in food production, such as wheat, rice, corn, potato, soybean, rye, barley, oat, and millet. The meaningful specificity testing detection methods must be adapted to the target organism as outlined in the following examples.

Like all cereals, corn (*Zea mays*) belongs to the family of *Gramineae*. Thus, the most important agricultural plants of the family *Gramineae* must be tested, and no target amplification should be detectable using plant genomic DNA originating from wheat (*Triticum aestivum*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), oat (*Avena sativa*), and rye (*Secale cereale*). In addition, specificity testing for soybean (*Glycine max*) and potato (*Tuberum solanacae*) is reasonable. The specificity testing of a *Zea mays*-specific real-time PCR system targeted to the maize invertase gene revealed that corn DNA gave a strong signal, whereas DNA from rice *(Oryza sativa*) and millet (*Sorghum halepense*, *Pennisetum americanum*) yielded detectable signals at least 50 times weaker than the signal from maize DNA (Table 1). A 50-fold less intensive signal might lead to a maximal error of 2% and will not cause any problems in routine analysis. Using qualitative PCR detection methods, such weak signals would most likely be left undiscovered. Nevertheless, for the assessment of an analytical result, it is important to know these relations to avoid potential misinterpretation.

Preferably, several varieties of *Zea mays* should be tested to verify that the copy number of the target sequence does not vary within the plant species of interest. This aspect is important for GMO testing because the analytically determined amount of corn DNA will be used to calculate the GMO content of the corn-derived ingredient. The gene coding for zein, a storage protein in *Zea mays,* was described for detecting the presence of maize DNA (10). A zein gene-specific QC-PCR was developed by constructing the corresponding competitor. Upon specificity testing of this QC-PCR system, we found that the measured ratio of the band intensities between the target band and the competitor band ranged from 4.6 (Bt11) to 0.27 (BtMON810), suggesting that the copy number of the zein gene can vary between different maize varieties up to 15-fold (Figure 1). Because this detection method would lead to incorrectly calculated GMO contents of corn-derived ingredients, the system was not suited for routine analysis and was abandoned. Likewise, many cultivated crop plants contain a high number of gene duplication as a result of the breeding processes. Thus, it might be difficult to find suitable target DNA sequences.

The specificity testing of a GMO maize-specific system (e.g., specific for Bt176 corn) must follow the guidelines just described. In addition, no signal should be obtained using genomic DNA from other commercial GM plants differing from the tested GM plant because similar or identical transgenic elements are used to construct different GM plants. For instance, a particular synthetic PAT-gene was used to construct Liberty corn, Liberty Link sugarbeet, and Liberty rape. In such cases, event-specific detection systems overlapping the integration sites must be applied. Even for official laboratories, it is still difficult to obtain commercialized, nonapproved GM plants from the corresponding agro-biotech companies.

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Table 6. Recommended sample sizes for analysis of GM plants

Plant	Kernel weight, mg[a]	Sample size, g[b]	Sample size, g[c]
Barley	37	370	140
Corn	285	2850	1000
Millet	23	230	81
Oat	32	320	112
Rape	4	40	14
Rice	27	270	95
Rye	30	300	105
Soybean	200	2000	700
Wheat	37	370	140

[a] The kernel weight was taken from Belitz and Grosch (32), except for soybean and rape kernels, which were determined in our laboratory.

[b] Recommended size of laboratory sample in case of inhomogeneous distribution of GM particles in the investigated lot (10 000 particles).

[c] Recommended size of laboratory sample in case of homogeneous distribution of GM particles in the investigated lot (3500 particles).

Limits of Quantitation and Detection

The sensitivity of quantitative PCR detection methods can be expressed in terms of LOQ and LOD. These 2 validation parameters depend on the amount of genomic DNA used for PCR, on the genome size of the investigated plant species, and on the number of transgenes per genome. The amount of genomic DNA template to be used in PCR should not surpass 500 ng (29). For GMO analysis, the amount of input DNA was standardized by the Swiss Food Manual to be maximally 200 ng (24). Depending on the assay volume (25, 50, or 100 μL), we routinely use either 100 or 200 ng genomic plant DNA for GMO analysis. The number of plant genomes present within 200 ng genomic DNA of a given plant species is compiled in Table 2. Apart from the copy number of the target DNA sequence, the detection limit depends also on the PCR primer quality, Mg^{2+} concentration, and annealing temperature (27).

A 200 ng sample of maize DNA contains about 40 000 genome copies. Thus, a GMO content of 0.1% corresponds to 40 copies of the transgenic genome. The minimal number of copies which still can be quantitated depends on the accepted analytical error and on the sensitivity of the PCR detection method. Following statistical considerations similar to those outlined for calculation of sample sizes (*see* below), 35 genome copies can be assumed to represent the theoretical limit of quantitation. Based on the assumption that 200 ng plant template DNA is used for PCR, the theoretical limits of quantitation were calculated and compiled (Table 2). For GMO food analysis, a minimal LOQ around 0.1% GMO is recommended; however, much lower LOQs are almost not feasible under the conditions specified above. Although the LOD of optimized PCR methods is in the range of 1–10 copies of the target sequence, no quantitation should be attempted below the LOQ. Applying soybean and Roundup Ready soybean-specific real-time PCR methods, the LOQ and LOD were experimentally determined and found to be close to the theoretical value of 35 copies (Table 3). Applying QC-PCR, the LOQ also depends on the used amount of competitor DNA and corresponds to about 10% of the target amount at the equivalence point. Using 200 ng genomic soybean DNA, LOQ was determined at 0.1% Roundup Ready soybean (data not shown).

Range of Quantitation

The range of analyte concentrations which lead to acceptable results determines the range of quantitation (working range) of a given method. QC-PCR can be used for quantitation in the range of 2 orders of magnitude: from one order of magnitude below the equivalence point to one order above the equivalence point. Changing the equivalence point by using different amounts of competitor DNA requires the experimental reassessement of the range of quantitation. Using real-time PCR, the lower limit of the working range corresponds to the LOQ described above. The upper limit of the range of quantitation corresponds to a GMO content of 100%, provided that the amount of genomic DNA used for real-time PCR is not inhibitory. A maize-specific real-time PCR detection method can be used for quantitation in the range of 3 orders of magnitude, whereas for plants with smaller genome sizes, such as rape or soybean, the range of quantitation for real-time PCR expands to 4 orders of magnitude (Figure 2).

Accuracy: Precision of Quantitative PCR Methods

The accuracy of an analytical method is determined by precision and trueness. Both validation parameters are experimentally determined by repeated measurements. For determination of repeatability, these measurements must be made within the same day by the same operator using the same thermocycler. The reproducibility can be tested by changing the day, the operator, or the thermocycler. For testing the accuracy (i.e., precision and trueness) of a method, the repeatability is chosen, whereas the reproducibility can be used for testing one important aspect of the ruggedness of a method. The precision of the tested methods is usually expressed in terms of the relative standard deviation (RSD), which is the ratio of the standard deviation of the mean divided by the mean value of the measurements.

For determination of the precision of QC-PCR detection systems, we recommend analysis of at least 5 replicates at 3 different concentration levels (at the equivalence point, one order of magnitude below the equivalence point, and one order of magnitude above the equivalence point). For determination of the precision of real-time PCR detection systems, at least 5 replicates should also be measured at 3–4 concentrations, separated by one order of magnitude (e.g., 0.1, 1, 10, and 100% GMO). We found that RSDs for QC-PCR (Table 4) and real-time PCR detection methods (Table 3) ranged from 5 to 20%. With QC-PCR, we sometimes encountered difficulties in evaluating the signals due to insufficient stability of the competitor DNA and formation of heteroduplexes. In the meantime, competitor DNAs were commercialized and the

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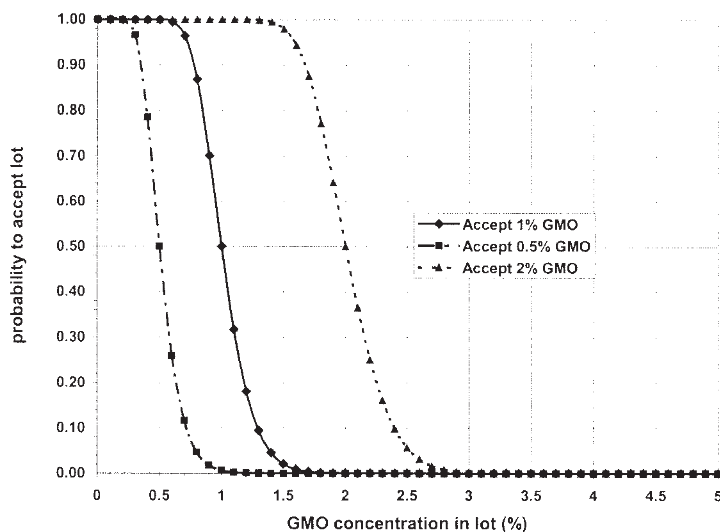

Figure 3. Probability of accepting lots with respect to their concentration of GM material. Thresholds were assumed to be at 0.5, 1, and 2% GM material. The sampling error was assumed to be twice the standard deviation of the expected mean. For details, *see* text.

stability of the products is indicated per batch under the recommended storage. With heteroduplexes, we sometimes experienced difficulties in avoiding their formation. Nevertheless, the interpretation of the signals is possible if the measured amount of heteroduplex is divided by 2 and each is added to the measured amounts of target and competitor DNA. For this kind of evaluation, the separation of DNA bands on agarose gels must be checked carefully.

Accuracy: Trueness of Measurements

Determination of the trueness of PCR-based GMO detection methods is a major challenge because of the difficulties in preparing homogenized test materials with known amounts of GM material without adversely affecting the quality of DNA present in the sample. One of our laboratories experienced many of these difficulties when quality testing reference material produced by IRMM. Thus, only limited data are available from an interlaboratory test using corn flours containing defined amounts of Bt176 corn. The results obtained in one of our laboratories using QC-PCR are summarized in Table 3. The relative deviations from the true values ranged from –7 to 18%, with an average of 1.7 ± 9.8%. Because of the lack of appropriate material needed to assess the trueness of quantitative PCR methods and the accompanying uncertainties, we strongly recommend the use of internationally standardized certified reference material produced by IRMM as calibrants for GMO analysis. The participation in proficiency testing such as that of Food Analysis Performance Assessment Scheme (FAPAS), Institute of Food Research (IFR), or others, is recommended for the assessment of the trueness of quantitative GMO detection.

Ruggedness

The testing of the ruggedness is challenging. Experiments that establish ruggedness include repeated analysis on several days, measurement of accuracy, and precision in fortified (spiked) samples or interlaboratory tests using well-defined samples. For PCR based detection methods, the influence of changing PCR parameters such as the PCR primer batch, Mg^{2+} concentration, and annealing temperature, or changing the operator, thermocycler, laboratory, or the analyzed matrix can be considered. Results of a small ring trial using homogeneous, commercial food samples are presented in Table 5.

Sampling, Sample Size, and Sampling Error

The probability of detecting small amounts of GM plant material within a bulk of conventional plant material depends on appropriate sampling procedures and on the chosen size of the laboratory sample. The sampling error will add to the overall analytical error of the GM detection method and should be minimized.

Assuming a homogeneous distribution of the GM material within the conventional material, the mean (μ) and the standard deviation of the mean (σ) can be calculated according to Poisson:

$$\mu = N \cdot p \qquad (1)$$

where μ = expected mean; N = number of particles in the sample; p = probability of GMO particles in the examined population.

$$\sigma = \sqrt{\mu} \qquad (2)$$

where σ = standard deviation of expected mean μ.

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Considering the precision of GMO detection methods described earlier, a relative sampling error of 20% was assumed to be acceptable. In statistical terms, this corresponds to the RSD (synonym is coefficient of variation [%CV]). Thus, Equation 3 can be postulated:

$$\mathrm{RSD} = \frac{\sigma}{\mu} = 0.2 \quad (3)$$

Equation 3 can be transformed to Equation 4.

$$\frac{\sqrt{\mu}}{\mu} = 0.2 \quad (4)$$

and solved with $\mu = 25$. Thus applying Poisson statistics our sample has to contain at least 25 GM particles in order to fulfill requirement (Equation 3). In order to have 25 or more GM particles with a security of $\alpha = 0.95$, the sample must actually contain an average of 35 GM particles, according to tabulated values (Equation 5; 31).

$$P(x>25 \mid \lambda = 35) = 0.95 \quad (5)$$

However, GM plant material can be distributed inhomogeneously in the investigated lot, particularly in raw materials containing large particles such as seeds and kernels. Assuming that 50% of the sampling error is attributed to this (incalculable) inhomogeneity (grouping error) and that 50% of the sampling error is attributed to the calculable distribution error (fundamental error), Equation 3 must be rewritten to get an overall sampling error of 20%:

$$\mathrm{RSD} = \frac{\sigma}{\mu} = 0.1 \quad (6)$$

Solving this equation leads to sample sizes containing 100 GM particles. Therefore, given the threshold value of 1% of GM material within conventional material, laboratory samples for GMO analysis should contain at least 10 000 particles to get an overall sampling error of 20%. The corresponding sample sizes of different crops and products were calculated and are compiled in Table 6. The probability of accepting lots with different concentrations of GM materials is displayed in Figure 3. Sampling errors of this magnitude can be considered acceptable and appropriate for the surveillance of a labeling issue.

The sampling procedure for large cargoes such as rail wagons, trucks, or ships consists of combining increments taken from different positions to form the bulk sample, and reducing this bulk sample to a laboratory sample, as described by the International Standards Organization (33). The optimal sampling strategy is always a compromise between cost and accepted sampling error, and must be adapted to the lot sizes to yield representative laboratory samples (34). For the correct interpretation of the analytical report, information on the sampling procedure must be provided.

Reference Material

Because quantitative PCR methods measure GMO contents of samples in relation to reference materials, the access to CRM is crucial for the calibration of quantitative GMO-specific PCR detection methods. For international reliability of GMO testing, internationally standardized reference materials are absolutely required. In the past, the processing of GM plant material to CRM impaired the amplification of the plant DNA (unpublished observations). Thus, it is important to ensure that the certified reference materials used are all produced under identical or at least comparable conditions.

CRMs for GMO testing should be classified as "Certified Reference Material for Calibrations" (calibrants) and used exclusively for calibration of quantitative GMO determinations. Every quantitative GMO analysis must be calibrated either with calibrants or with material that can be traced back to calibrants. This implies that calibrants must be consistent (e.g., material containing 2% GMO contains twice as much analyte as material with 1% GMO content). New production series of calibrants must be consistent with former production series. If needed, a conversion factor can be published for new production series. Important characterization of the raw material used for the production of calibrants includes homogeneity (i.e., absence of non-GMO particles, in the case of GM plant material), homozygosity for nonhybrid plants, polyploidy status for hybrid plants, source, keeping quality, and additives such as fungicides and colors used for seed treatment. The currently available calibrants are produced by the IRMM. Calibrants are distinguished from material used for evaluating performance characteristics of GMO detection methods such as precision and trueness. Processed material containing defined amounts of GMO such as protein isolates and lecithin is indispensable for assessment of possible matrix effects.

In the future, the "gold standard" for calibrants of PCR-based methods might be DNA solutions. However, because of the lack of knowledge concerning DNA solutions as calibrants, the introduction of such material on the market should not take place before rigorous testing of the production parameters and storage conditions.

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