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Quantitative competitive PCR for the detection of genetically modified organisms in food

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Abstract

Present polymerase chain reaction (PCR) detection methods only allow the qualitative detection of GMO in food without quantitation of the GMO content. Clearly, the availability of quantitative detection methods for GMO analysis is an important prerequisite for the introduction of threshold limits for GMOs in food. PCR is well known to be quantitative if internal DNA standards are co-amplified together with the target DNA. This quantitative competitive (QC) PCR was first described in the early nineties and is widely used nowadays.

We have developed and evaluated QC–PCR systems for the quantitative detection of Roundup Ready™ soybean (RRS) and Maximizer maize (MM) in food samples. Three DNA fragments differing from the GMO specific sequences by DNA insertions were constructed and used as internal standards in QC–PCR. These standards were calibrated by co-amplifying with mixtures containing defined amounts of RRS DNA and MM DNA, respectively. The calibrated QC–PCR systems were applied to several commercial food samples containing RRS and to three certified RRS flour mixtures (Fluka standards). Recently, quantitative methods for the detection of RRS were successfully tested in a collaborative study involving twelve European control laboratories. Thus, QC–PCR methods will allow to survey ''de minimis thresholds'' of GMOs in food.

Keywords: Genetically modified organism; Quantitative-competitive PCR; Food labeling

1. Introduction

The surveillance of food labeling with respect to genetically modified organisms (GMOs) requires DNA-based analytical techniques. The polymerase chain reaction (PCR) is a highly specific and sensitive method for the detection of nucleic acids (DNA or RNA) and a vast number of PCR systems for food control exists, such as for the detection of pathogenic microorganisms or of food components as well as for the identification of animal species in meat products (Allmann, Candrian, Höfelein & Lüthy, 1993; Meyer, Candrian & Lüthy, 1993; 1994; Allmann et al., 1995; Meyer, Höfelein, Lüthy & Candrian, 1995; Meyer & Candrian, 1996). PCR also provides a reliable tool for the identification of GMO containing food in order to control food labeling regulations concerning GMOs (Meyer, 1995; Ehlers et al., 1997; Hupfer, Hotzel, Sachse & Engel, 1997; Köppel, Stadler, Lüthy & Hübner, 1997; Meyer & Jaccaud, 1997; Pietsch, Waiblinger, Brodmann & Wurz, 1997; Studer, Dahinden, Lüthy & Hübner, 1997).

In Switzerland, the current detection method for GMO containing food is based on a qualitative PCR system specific for the 35S-promoter originating from the Cauliflower mosaic virus (Schweizerisches Lebensmittelbuch (Swiss Food Manual, 1998)). Food containing detectable amounts of 35S promoter DNA has to be labeled as ''GMO'' (Lebensmittelverordnung (Swiss Food Ordinance, 1995)).Thus, the detection limit of the 35S-PCR system represents the decisive factor for GMO labeling in Switzerland. The detection limits of the 35S-and NOS–PCR system were recently determined in a collaboratory study (Subcommission 29a, Swiss Food Manual, unpublished). This study revealed up to twenty-fold differences in sensitivity between different laboratories. Furthermore, the 35S-PCR was found to be more sensitive than the NOS–PCR. In addition to the 35S-PCR system, more sensitive, GMO-specific PCR systems (e.g. Köppel et al., 1997; Meyer & Jaccaud, 1997; Studer

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et al., 1997) are applied routinely by control or service laboratories in order to assure a sufficient detection sensitivity. Although current GMO detection methods are feasible for food control, the inter-laboratory reproducibility remains unsatisfactory. Clearly, the introduction of quantitative detection methods for GMO analysis will help to reduce such inter-laboratory differences. Furthermore, the control of threshold limits for GMOs in food requires quantitative detection methods.

2. Quantitative competitive PCR for GMO analysis

Quantitative competitive polymerase chain reaction (QC–PCR) using internal DNA standard provides means for determining relative amounts of target DNA (Gilliland, Perrin, Blanchard & Bunn, 1990; Piatak, Luk, Williams & Lifson, 1993; Siebert & Larrick, 1993; Riedy, Timm & Steward, 1995; Khan, Latif, Petrobelli, Yacoub & Dunn, 1996; Brichacek & Stevenson, 1997). The principle of QC–PCR is the co-amplification of standard DNA together with target DNA as illustrated in Fig. 1. We have developed QC–PCR systems for the detection and quantitation of Roundup Ready™ soybean (RRS) and Maximizer maize (MM) (Studer, Rhyner, Lüthy & Hübner, 1998). The internal DNA standards consisted of cloned PCR fragments of RRS- and MM–DNA carrying internal insertions of 21 and 22 bp, respectively. The standard procedure for QC–PCR comprised four steps: (1) DNA extraction, (2) determi-

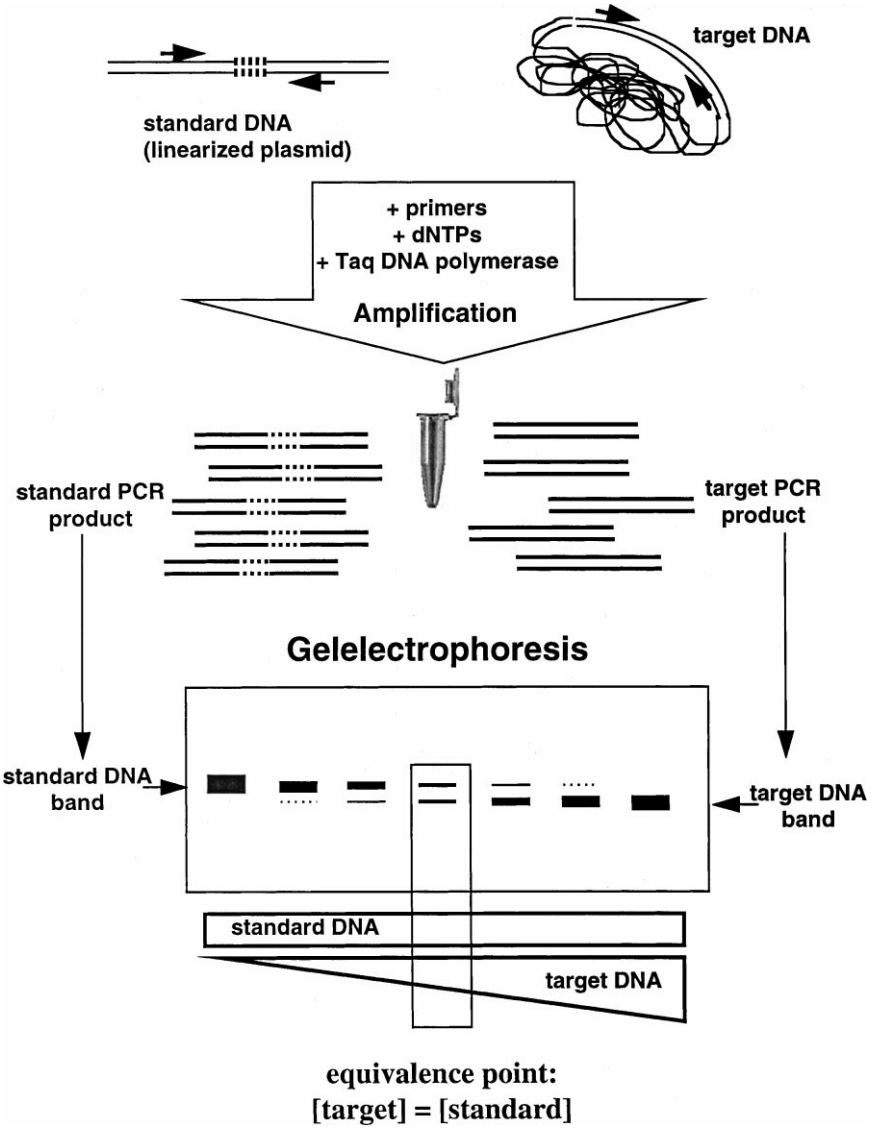

Fig. 1. *Principle of QC–PCR*. Standard DNA and target DNA are co-amplified in the same reaction tube. The standard DNA consists of linearized plasmid carrying a modified PCR amplicon. The modification can be a DNA insertion as shown above, a DNA deletion or a point mutation. After PCR, the products are separated by agarose gel electrophoresis whereby the amplified standard DNA can be distinguished from the amplified target DNA by size. At the equivalence point the starting concentrations of internal standard and of target are equal if the validation criteria are fulfilled (Raeymaekers, 1993).

nation of DNA concentration, (3) QC–PCR with defined internal DNA standard concentrations and (4) separation of PCR products by gel electrophoresis.

For semi-quantitative GMO analysis, QC–PCR was performed using 500 ng template DNA mixtures containing various percentages of RRS–DNA in the presence of constant concentrations of internal DNA standard. These standards were adjusted so that the equivalence point (i.e. the PCR signals derived from standard and from target DNA were equal) represented a GMO content of 1% (Fig. 2). The calibration of the QC–PCR system was controlled with certified RRS flour mixtures containing 0.1%, 0.5% and 2% RRS, respectively (Fluka, Buchs, Switzerland). The GMO contents of five commercial soy samples containing RRS were estimated and all tested samples contained RRS with the exception of sample #2 (Fig. 3).

In a second step, the GMO content of sample #4 was determined by means of QC–PCR using various amounts of internal DNA standards. The relative intensities of the PCR signals originating from the standard and from the target were measured by image processing software (Fig. 4). Sample #4 was found to contain 10% RRS. It is important to note that QC–PCR can only be used for determination of relative amounts of target and standard if the regression coefficient r^2 is better than 0.99 and the slope of the regression line is very close to unity (Raeymaekers, 1993; Hayward-Lester, Oefner, Sabatini & Doris, 1995).

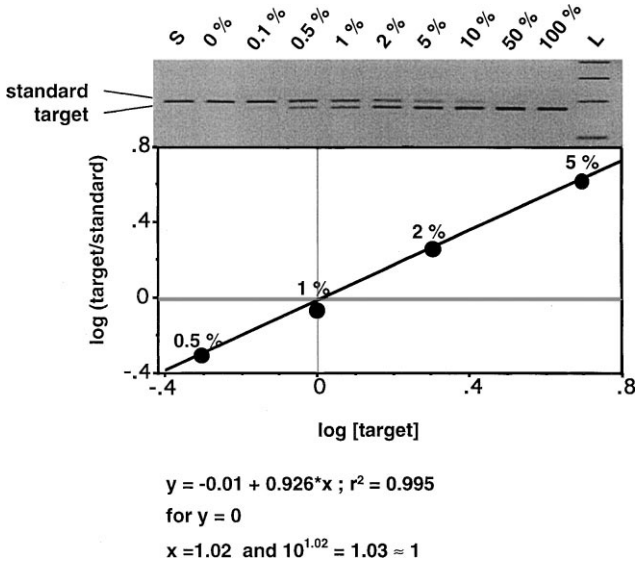

Fig. 2. *Calibration of QC–PCR.* The RRS-specific GM–QC–PCR system was calibrated by amplifying constant amounts of standard DNA (0.5 fg) together with constant amounts of soy DNA mixtures (500 ng) containing various percentages of RRS–DNA as indicated. After agarose gel electrophoresis the relative band intensities were determined by image processing software (ImageQuant, Molecular Dynamics, Sunnyvale, CA USA). The equivalence point was calculated by linear regression and found to be at 1% RRS (Studer et al., 1998).

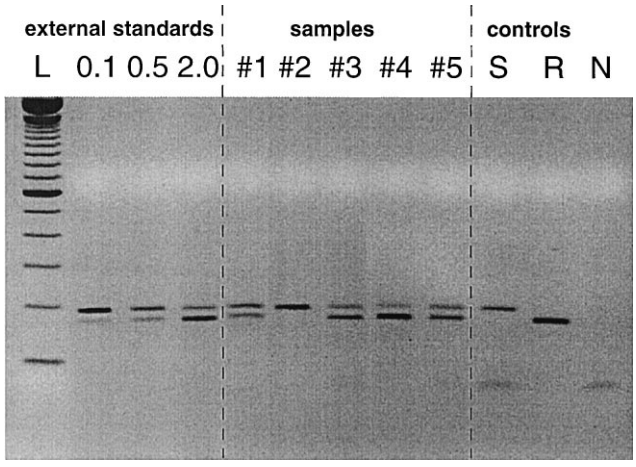

Fig. 3. *Semi-quantitative application of QC–PCR.* 500 ng DNA from food samples, were extracted according to the Swiss Food Manual (Chapter 52B) and analyzed by GM–QC–PCR using the internal standard amount equivalent to 1% RRS (see Fig. 2). The amplification products were analyzed on 1.5% MS agarose gels and the intensities of the corresponding PCR signals were compared visually with respect to the external standards containing the indicated amount of RRS (in %). The GMO content of the food samples was estimated as follows: sample #1 (lecithin): ≈1%; sample #2 (flour): <0.1%; sample #3 (protein): ≈2%; sample #4 (grist): >5% and sample #5 (grist): 1–2%. S: standard DNA alone; R: RRS DNA alone, N: PCR negative control (no DNA).

3. Collaborative study applying QC–PCR

Twelve European control laboratories participated from February to March 1998 in a collaborative study. Eight coded samples were tested for their GMO content applying four different QC–PCR systems. Each sample had to be processed in duplicate according to the Swiss Food Manual (Chapter 52B). In total, 81 PCR determinations were performed for each sample. The participants had to determine the GMO content of the investigated samples with respect to the supplied external GMO standards (Fluka, Buchs, Switzerland) containing 0.5% and 2% RRS, respectively. The PCR signals detected by agarose gel electrophoresis had to be classified as I (GMO content below 0.5%), as II (GMO content between 0.5% and 2%) or as III (GMO content above 2%), respectively. Two samples were uniformly found to contain less than 0.5% RRS, whereas all other samples were judged to contain RRS either between 0.5% and 2% or more than 2%. As an example, the classifications of a negative and a positive food sample is shown in Table 1. One of the four tested QC–PCR method (NOS–QC–PCR) was found to lack sufficient robustness and was omitted for the statistical evaluation. The other three QC–PCR methods were compared with each other

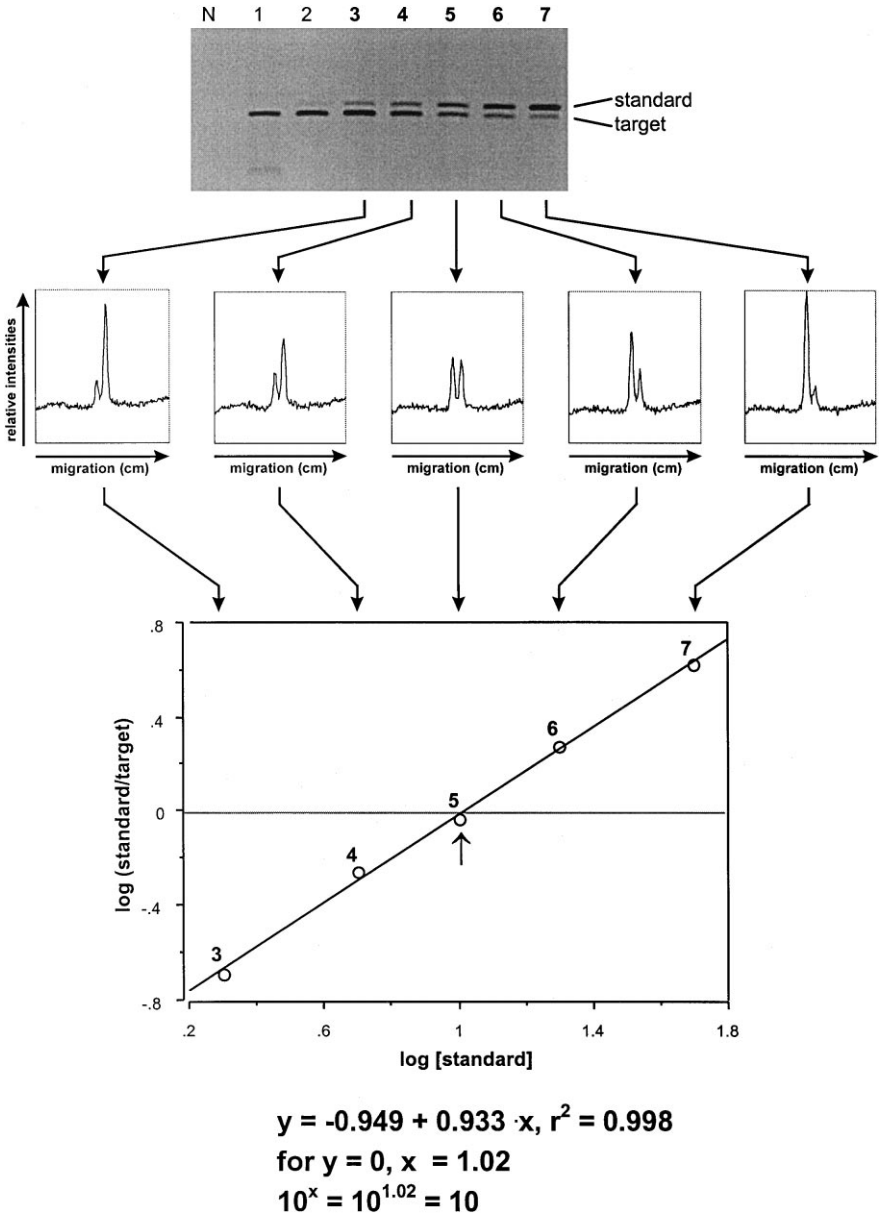

Fig. 4. *Quantitative application of QC–PCR.* The GMO content of sample #4 was determined by subjecting 500 ng extracted DNA to GM–QC–PCR together with various amounts of internal standard DNA equivalent to GMO contents of 0% (lane 1), 1% (2), 2% (3), 5% (4), 10% (5), 20% (6) and 50% (7). N: negative PCR control. The relative band intensities were determined after gel electrophoresis and used to calculate the linear regression (Studer et al., 1998).

by χ^2-testing revealing good correlations (Table 2). Most importantly, no sample was classified simultaneously as class I and as class III by two different methods. Inter-laboratory differences were mainly due to insufficient sample homogenization and differences in determination of DNA concentrations. Taking these points into account, the results of this collaborative study are very promising. Furthermore, inter-laboratory differences were reduced compared to qualitative PCR due to the introduction of internal standards. Interestingly, the highest inter-laboratory variations were caused by insufficient sample homogenization (data not shown).

4. Conclusion

The current GMO-analysis is based on qualitative PCR detection methods which allow a sufficiently sensitive detection of GMOs in food. However, inter-laboratory reproducibility and precision of the official 35S-PCR system were found to be unsatisfactory. Quantitative competitive PCR was successfully applied for quantitative GMO analysis. A recently performed collaborative study demonstrated that QC–PCR led to comparable determinations of the GMO content of food samples reducing existing inter-laboratory differences.

Table 1
GMO content of two test samples as determined in the collaborative study

QC–PCR-method[a]		GM	RRS	35S
Sample #6: soy flour				
Classification[b]	neg	22	21	19
	I	0	0	0
	II	0	1	0
	III	0	0	0
Sample #4: soy protein				
Classification	neg	0	0	0
	I	3	4	1
	II	19	18	18
	III	0	0	0

[a] The applied QC-PCR systems were: GM (Studer et al., 1998); RRS (Genescan, this volume) and 35S (P. Brodmann, Kantonales Laboratorium Basel-Stadt, unpublished).

[b] neg: no RRS-specific PCR product detectable; the categories I–III are explained in the text.

Table 2
Contingency tables of the applied QC–PCR detection methods in the collaborative study

	neg	I	II	III	Total
(a) GM vs RRS (*Contigency coefficient:* 0.810)					
neg	35	3	1	0	39
I	1	8	4	0	13
II	1	4	79	0	84
III	0	0	4	20	24
Total	37	15	88	20	160
(b) GM vs 35S (*Contigency coefficient:* 0.811)					
neg	32	1	0	0	33
I	1	6	4	0	11
II	0	0	67	7	74
III	0	0	2	16	18
Total	33	7	23	33	136
(c) RRS vs 35S (*Contigency coefficient:* 0.772)					
neg	33	2	1	0	36
I	1	5	2	0	8
II	2	11	70	0	83
III	0	0	10	15	25
Total	36	18	83	15	152

Furthermore, the calibration of QC-PCR can be controlled by certified reference standards which are commercially available. Although still existing inter-laboratory differences have to be further reduced, QC–PCR methods allow to survey threshold limits for GMOs in food, which are presently debated with respect to the European Novel Food Regulation.

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