JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 5521–5527 **5521**

Development of a Screening Method for Genetically Modified Soybean by Plasmid-Based Quantitative Competitive Polymerase Chain Reaction

Eri Shimizu,[†] Hisashi Kato,[‡] Yuki Nakagawa,[‡] Takashi Kodama,[§] Satoshi Futo,[†] Yasutaka Minegishi,[||] Takahiro Watanabe,[⊥] Hiroshi Akiyama,[⊥] Reiko Teshima,[⊥] Satoshi Furui,[#] Akihiro Hino,[#,▽] and Kazumi Kitta*[,#]

Fasmac Co., Ltd., 5-1-3 Midorigaoka, Atsugi, Kanagawa 243-0041, Showa Sangyo Co., Ltd., 2-20-2 Hinode, Funabashi, Chiba 273-0015, Food and Agricultural Materials Inspection Center, 2-1 Shintoshin, Chuo, Saitama 330-9731, Nippon Gene Co., Ltd., 1-8-7 Toiya-machi, Toyama 930-0834, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, and National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

A novel type of quantitative competitive polymerase chain reaction (QC-PCR) system for the detection and quantification of the Roundup Ready soybean (RRS) was developed. This system was designed based on the advantage of a fully validated real-time PCR method used for the quantification of RRS in Japan. A plasmid was constructed as a competitor plasmid for the detection and quantification of genetically modified soy, RRS. The plasmid contained the construct-specific sequence of RRS and the taxon-specific sequence of lectin1 (Le1), and both had 21 bp oligonucleotide insertion in the sequences. The plasmid DNA was used as a reference molecule instead of ground seeds, which enabled us to precisely and stably adjust the copy number of targets. The present study demonstrated that the novel plasmid-based QC-PCR method could be a simple and feasible alternative to the real-time PCR method used for the quantification of genetically modified organism contents.

KEYWORDS: Roundup Ready soybean (RRS); plasmid-based QC-PCR (PQC-PCR); genetically modified organism (GMO)

INTRODUCTION

Since the first commercialization of genetically modified (GM) crops developed through recombinant DNA technologies, GM crop acreage has kept on increasing over the world. Along with the expansion of GM crop production, the global area of approved GM crops reached 102 million hectares in 2006 (*1*). To enable consumers to make informed choices, the governments of some countries require food products and ingredients to be labeled if the contents of GM organisms (GMO) in food products exceed a certain threshold level of adventitious contamination, such as 0.9, 3, and 5% in the European Union (*2*), Korea (*3*), and Japan (*4*), respectively. For the affirmation of the labeling authenticity, the development of accurate, reliable, and rapid methods for the detection of GMOs has been highly demanded.

Methods based on polymerase chain reaction (PCR) are suitable for the specific and sensitive detection of DNA from GM crops as described for Roundup Ready soybean (RRS) and several lines of GM maize (*5–8*). For GMO screening, qualitative PCR methods are practical and can determine whether the content of GMO in a sample exceeds certain detectable limits (*7*). For the enforcement of threshold values, quantitative analytical methods are required to obtain more precise numerical information.

Recently, real-time PCR has been a first choice for the quantitative analysis of GMO due to its specificity and sensitivity (*5–13*). However, real-time PCR instruments and reagents are quite expensive and not always preferable for every laboratory. A quantitative competitive polymerase chain reaction (QC-PCR) method, in which a target DNA is coamplified together with a competitor DNA carrying the same primer binding sites, has proven to be a feasible method (*14*). The QC-PCR method has also been studied for the quantification of GMO in foods, and a single-QC-PCR was proposed as a simple quantitative screening method to survey the 1% threshold limit for GMOs in food (*15, 16*). Usually, a competitive plasmid used

* To whom correspondence should be addressed. Tel: +81-29-838-7369. Fax: +81-29-838-7996. E-mail: kaz@affrc.go.jp.
[†] Fasmac Co., Ltd.
[‡] Showa Sangyo Co., Ltd.
[§] Food and Agricultural Materials Inspection Center.
[||] Nippon Gene Co., Ltd.
[⊥] National Institute of Health Sciences.
[#] National Food Research Institute.
[▽] Current address: Food Safety Commission Secretariat, Cabinet Office, 2-13-10 Nagata-cho, Chiyoda, Tokyo 100-8989, Japan.

10.1021/jf073348n CCC: $40.75
Published on Web 06/18/2008

for QC-PCR contains an insertion or deletion in the sequence, large enough to allow separation from the target DNA. In the QC-PCR method, each sample is "titrated" with a competitor; that is, accretive known contents of a competitor are added to aliquots containing a constant content of target followed by PCR and electrophoresis. At an equivalence point where the same band densities are densitometrically acquired, the quantities of target and competitor are determined to be equal. After further modifications, dual competitive-PCR (DC-PCR) was developed for the quantitative analysis of heat-treated and/or processed foods (*13, 17, 18*). In the DC-PCR, target sequences, that is, endogenous and recombinant sequences, are coamplified with respective competitive plasmid DNAs, and the ratio of these two target sequences allows one to calculate the relative proportion of a GMO in foods. Furthermore, in a recently developed high-throughput double quantitative competitive PCR (HT-DCPCR), PCR products were detected by a biluminometric hybridization assay performed in a microtiter plate, instead of electrophoresis (*19*). In these above-mentioned methods, ground seeds were used as reference materials for the calibration of competitor plasmid to quantify the GMO contents. However, it is difficult to maintain the constant quality of reference materials because the reference materials are prepared from agricultural products in which the quality of DNA extract could be affected by many factors such as variety, growing area, production year, and so on.

The objective of this study was to develop a novel QC-PCR method using plasmid DNA as a reference molecule instead of ground seeds for the quantification of GMOs. In this report, we described the development of the method as compared with the data obtained by a fully validated real-time PCR method, which has not only been adopted as a Japanese official method but has also been adopted and published in the Annex of ISO 21570 (*7, 20*). In addition, analyses using two PCR machines and three kinds of analytical instrument systems were performed to assess the applicability of the proposed method.

MATERIALS AND METHODS

Materials. Genuine seeds of GM soy variety AG3301, a progeny of RRS developed by Monsanto (St. Louis, MO), were provided by the developer to the Ministry of Agriculture, Forestry and Fisheries (MAFF) to comply with the regulations under Food Sanitation Law and Japanese Agricultural Standard Law regarding mandatory labeling of GM foods. Certified reference materials (CRMs) produced by the Institute for Reference Materials and Measurement (IRMM) containing 1 and 5% RRS were purchased from Sigma-Aldrich (St. Louis, MO).

Oligonucleotide Primers and Probes. Oligonucleotide primers were synthesized and purified on a reversed-phase column by Fasmac (Kanagawa, Japan). Each oligonucleotide was diluted to an appropriate concentration with the appropriate volume of TE buffer. Primer sequences used to construct a competitor plasmid and to perform plasmid-based QC-PCR (PQC-PCR) are listed in **Table 1**. TaqMan probes for the quantification of RRS and soybean endogenous gene, lectin1 (Le1), have been described previously (*7*). These probes were labeled with 6-carboxy-fluorescein (FAM) and 6-carboxytetramethil-rhodamin (TAMRA) at the 5′- and 3′-ends, respectively, and were synthesized by Applied Biosystems (Foster City, CA).

Preparation of Samples and DNA Extraction. Dry seeds were ground with the P-14 speed rotor mill (Fritsch, Ibar-Oberstein, Germany). Ground seeds were lyophilized for 24 h in the FDU-540 freeze drier (Tokyo Rikakikai, Tokyo, Japan) and stored at −20 °C until use. The five levels of simulated GM-mixture samples containing 0.5, 1, 3, 5, and 10% (w/w) of RRS in non-GM soy were made from the ground seeds. DNA extraction was performed using DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany) with slight modifications as described previously (*7*). The DNA concentrations of solutions were determined by measuring UV absorbance at 260 nm, and the quality was evaluated by the absorbance ratios at 260/280 nm and 260/230 nm; the absorbance ratio at 260/280 nm was between 1.7 and 2.0, and that at 260/230 nm was >1.7. The homogeneity of these simulated GM-mixture samples was confirmed by a real-time PCR method as described elsewhere (*7*).

Table 1. Primer Sequences

no.	name	sequences (5′ −3′)
		for construction of competitor plasmid
1	QTso-F	ATAGGCGCCGCCCTCTACTCCACCCCCA
2	QTsi-1	ACATTCAGACGTCAGTGCTAATTTCTTTGTCCC
3	QTsi-2	TTAGCACTGACGTCTGAATGTCCGGTAGCGTTGCC
4	QTsi-3-3	GGGTTGACGCCCATCTGCAAGCCTTTTT
5	QTsi-4-1	AGATGGGCGTCAACCCTTTAGGATTTCAGCATCAGTGG
6	QTso-R	GCAGATGTCAAAGGATTGGGAGGACTTGTCGCCGGGAATG
7	QTsi-F	CATTCCCGGCGACAAGTCCTCCCAATCCTTTGACATCTGC
8	QTsi-R2	ATAGGCGCCTCGATTTCTCTCTTGGTGACAGG
		for PQC-PCR system
9	Le1n 02-5′	GCCCTCTACTCCACCCCCA
10	Le1n 02-3′	GCCCATCTGCAAGCCTTTTT
11	RRS 01-5′	CCTTTAGGATTTCAGCATCAGTGG
12	RRS 01-3′	GACTTGTCGCCGGGAATG

Real-Time PCR. The experimental procedure of real-time PCR followed the established method (*9*), also known as a Japanese official method for GM detection (*21*). One of the main features of this analysis method was to use plasmid DNAs as reference materials. Briefly, a 25 μL reaction solution contained 50 ng of sample DNA, 12.5 μL of Universal Master Mix (Applied Biosystems), 0.5 μM primer pair (Fasmac), and 0.2 μM probe (Applied Biosystems). The real-time PCR reactions were performed with the ABI PRISM 7700 (Applied Biosystems) according to the following step-cycle program: preincubation at 50 °C for 2 min and 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 30 s, annealing, and extension at 59 °C for 1 min. Calibration curves were drawn for RRS and Le1 using the GM Soy Detection Plasmid set-ColE1/TE- (Nippon Gene, Tokyo, Japan), which contains five different concentrations of reference molecule, that is, 20, 125, 1500, 20000, and 250000 copies per 2.5 μL. The copy number of each target sequence was obtained based on the calibration curve. Then, the GM contents of the test samples were calculated using a conversion factor (Cf) value described below (*7*).

Calculation of Cf and GM Contents (%). The ratio of the copy number of recombinant DNA to the taxon-specific sequence of genuine seed was calculated by formula 1 and defined as Cf. The GMO content (%) was calculated by formula 2.

$$\text{Cf} = \text{copy number of recombinant DNA sequence in the DNA extracted from genuine GM seed/copy number of taxon-specific sequence in the DNA extracted from genuine GM seed} \quad (1)$$

$$\text{GM content (\%)} = \text{copy number of recombinant DNA sequence in sample/copy number of taxon-specific sequence in sample/Cf} \times 100 \quad (2)$$

Construction of the Competitor Plasmid. The construction of a competitor plasmid for PQC-PCR system was performed with slight modifications of the overlap extension method described elsewhere (*7*) and followed the schematic diagram shown in **Figure 1**. PCR templates used in the reactions **A**−**D** in **Figure 1** were the following: **A**, reference molecule contained in GM Soy Detection Plasmid set-ColE1/TE-; **B**, same as for **A**; **C**, subcloned plasmid that contained 21 bp insertion in the RRS sequence; and **D**, reference molecule contained in GM Maize Detection Plasmid set-ColE1/TE- (Nippon Gene). The integrated fragment was ligated into pCR2.1 plasmid vector (Invitrogen, Carlsbad, CA) by using TOPO TA Cloning kit (Invitrogen). The cell of *Escherichia coli* strain TOP10 (Invitrogen) was transformed with the plasmid. The plasmid was extracted and purified with the GFX Micro Plasmid Prep Kit (GE Healthcare Bio-Sciences, Piscataway, NJ). The sequence of the inserted fragment was confirmed. After sequencing, the fragment was ligated into pUC19 plasmid vector. The sequence of

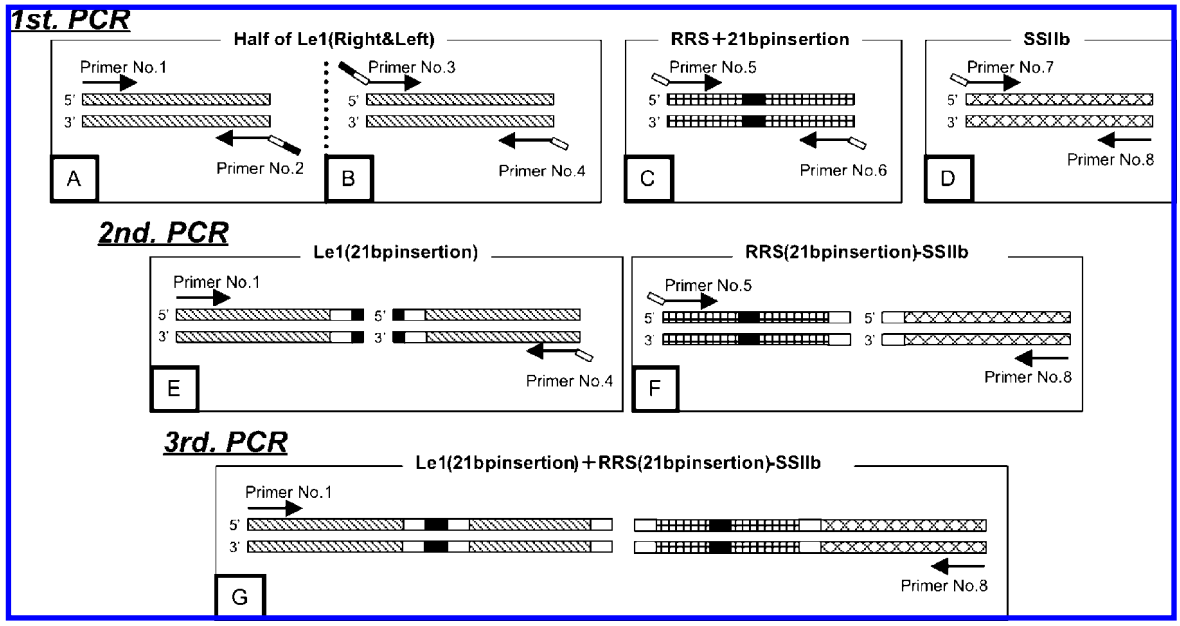

Figure 1. Scheme for the construction of competitor plasmid for PQC-PCR system. PCRs A, B, C, and D were performed using primer pair nos. 1 and 2, 3 and 4, 5 and 6, and 7 and 8, respectively, and of which primers 2−7 were tailed (**A**−**D**). The resultant amplicons from PCRs A and B and C and D were mixed and PCRs E and F were performed, respectively (**E** and **F**). The connected amplicons were used as templates for the third PCR (**G**). Finally, the integrated fragment was ligated into a plasmid vector.

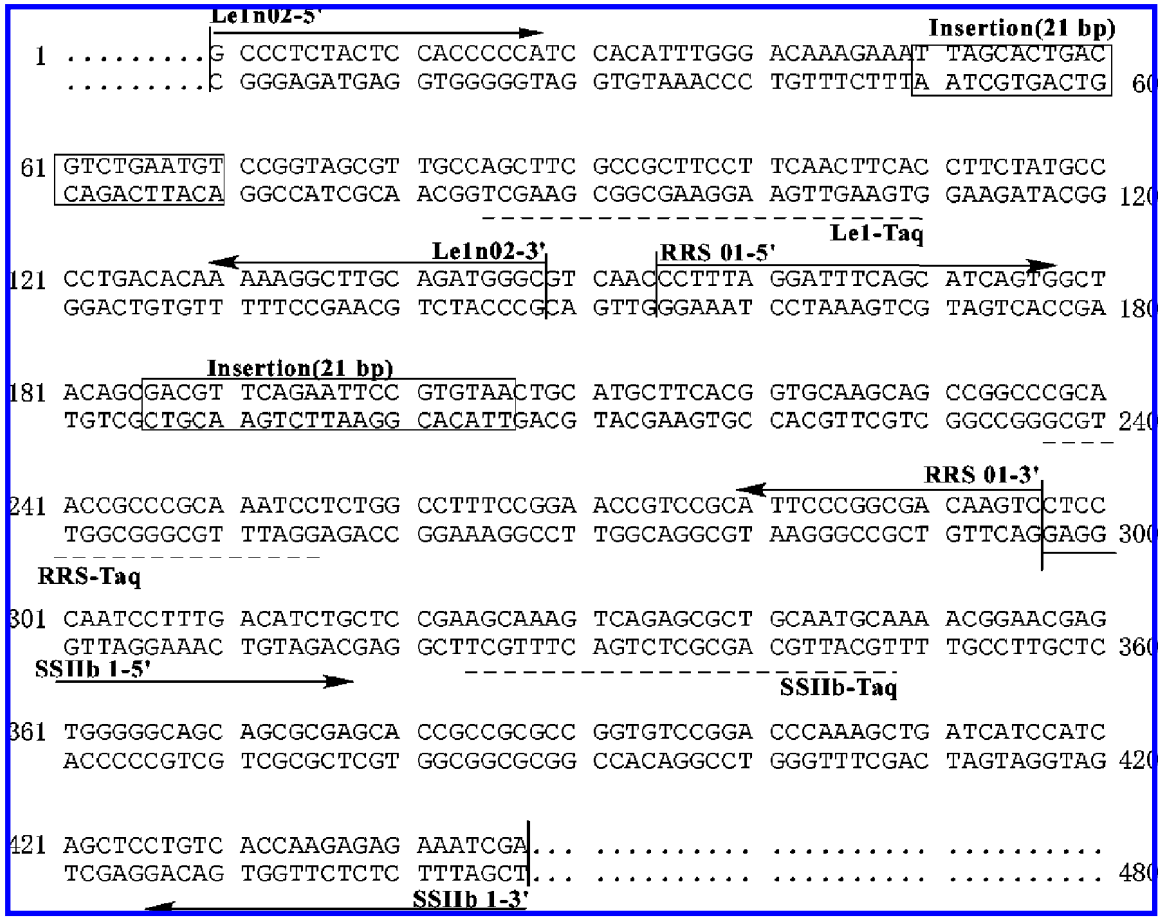

Figure 2. Sequence of integrated fragments inserted into pQCS. Arrowed lines indicate the sequences of primers for the detection of Le1, RRS, and SSIIb. The sequences of 21 bp insertions into Le1 and RRS are boxed, respectively. Dotted lines indicate the sites of TaqMan probes.

the competitor plasmid, pQCS, for GM-soy is shown in **Figure 2**.

Calibration of Competitor Plasmid. The plasmid, pQCS, was extracted and purified with the Qiagen plasmid Giga kit (Qiagen) and diluted with ColE1/TE buffer (Nippon Gene) to appropriate concentrations. To perform precise quantification, we made three sets of pQCS, that is, competitor plasmid sets H, M, and L, and each competitor plasmid set consisted of five different concentrations of pQCS (**Table 2**).

The copy numbers of the plasmid in each set were decided based on our experimental data, aiming to quantify samples containing RRS

Table 2. Competitor Plasmid Sets for PQC-PCR System

a. Competitor Plasmid Sets and Copy Numbers

competitor plasmid set	numbers of competitor plasmid/reaction tube				
H	56000	28000	14000	7000	3500
M	2800	1400	700	350	175
L	560	280	140	70	35

b. Combination of Competitor Plasmid Sets for Quantification of RRS

sample	Le1	RRS
100% RRS	H	H
3.0−10.0% RRS	H	M
0.5−3.0% RRS	H	L

Table 3. Cf of the PQC-PCR System[a]

	Gene Amp 9700			PTC-200		
analytical instrument	average	SD	RSD (%)	average	SD	RSD (%)
system 1	0.98	0.04	3.7	0.98	0.05	5.1
system 2	1.01	0.08	7.5	0.97	0.04	4.1
system 3	1.03	0.07	7.0	0.99	0.05	4.6

[a] System 1, Molecular Imager FX and Quantity One; system 2, Light-Capture (ATTO) and CS Analyzer; system 3, Printgraph (ATTO) and Image J; SD, standard deviation; and RSD, relative standard deviation.

Table 4. Quantification of RRS Content in Five Simulated Mixture Samples by a Real-Time PCR System[a]

		precision	
mixing level (%)	average	SD	RSD (%)
0.5	0.56	0.03	5.7
1	1.11	0.05	4.6
3	3.13	0.18	5.6
5	4.55	0.21	4.7
10	10.61	0.47	4.4

[a] Cf = 1.04; ABI PRISM 7700.

approximately 1 and 5%. The copy numbers of the competitor plasmid in each set were quantified and confirmed by real-time PCR. The sequence of Le1 constructed into the plasmid enabled us to quantify the copy numbers of the plasmid using the calibration curve obtained by the GM Soy Detection Plasmid set-ColE1/TE-, which also contains the sequence of Le1.

PQC-PCR. The genomic DNA extracted from ground seeds was coamplified with the competitor plasmid in a single PCR tube, and all reactions were conducted twice. Each reaction mixture contained 50 ng of sample DNA, 2 μL of the competitor plasmid (contents ranging from 35 to 56000 copy numbers per reaction as shown in **Table 2**), 2.5 μL of PCR bufferII (Applied Biosystems), 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.625 U of AmpliTaq Gold polymerase (Applied Biosystems), and 0.25 mM each primer pair (**Table 1**) for a final volume of 25 μL. To examine the applicability for PQC-PCR, we demonstrated the PCR reaction with two kinds of PCR equipment, that is, the GeneAmp 9700 (Applied Biosystems) and the PTC-200 DNA engine (MJ Research, Watertown, MA), with the following PCR step-cycle program: 10 min at 95 °C, 40 cycles, 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by a final extension at 72 °C for 7 min.

Analysis of PCR Products. *Electrophoresis.* After the PCR amplification, 4 μL of each PCR product was electrophoresed at a constant voltage of 100 V on 5% (w/v) agarose gel Takara LO3 (Takara Bio, Shiga, Japan) containing 0.5 mg/mL ethidium bromide (Sigma) in TAE [40 mM Tris-HCl, 40 mM acetic acid, and 1 mM EDTA (pH 8.0)]. A 100 bp ladder (New England Biolabs, Beverly, MA) was used for size control of the amplified fragments.

Analysis of Amplified Fragments. To examine the applicability for PQC-PCR, analytical instruments were used as follows: system 1, the Molecular Imager FX system (Bio-Rad Laboratories, Hercules, CA) and the Quantity One (Bio-Rad); system 2, the ATTO Light-Capture Cold CCD Camera System type AE6972 (ATTO, Tokyo, Japan) and the CS Analyzer (ATTO); and system 3, the CCD camera Printgraph-CX type AE6911 (ATTO) and the NIH Image (public domain image processing and analysis program). The relative band intensities were determined with these three kinds of image analysis software. The copy numbers of competitor plasmid and target should be the same at the equivalence point as depicted in **Figure 3**. Precisely, the decimal logarithm of the copy number of pQCS was plotted on the *x*-axis, and that of the intensity ratio of pQCS to target was plotted on the *y*-axis. The intersection between the calibration curve and the *y*-axis was defined to be the equivalence point, and the copy number of target was determined. The copy numbers of Le1 and RRS were determined, and Cf value was calculated with formula 1. The GM content in a sample was calculated with formula 2 using an obtained Cf value.

RESULTS AND DISCUSSION

Measurement of Cf. Initially, we obtained a conversion factor required to calculate GM contents for RRS. The 100% RRS sample was subjected to real-time PCR. To determine the Cf value, the copy numbers of recombinant DNA and taxon-specific sequence were separately measured, and all of the measurements were repeated three times. The resultant Cf was 1.04 ± 0.03, which was the average of triplicate PCR reactions with the ABI PRISM 7700, similar to the value calculated by our previous report (*22*) and also close to the theoretical Cf value of 1.00.

Because the measurement principle of real-time PCR is different from that of PQC-PCR, the Cf value for PQC-PCR was also independently measured. The same 100% RRS sample was subjected to PQC-PCR using a competitor plasmid set H. Competitor plasmid pQCS, having target sequences of Le1 and RRS together on the same plasmid, enabled us to ignore the dilution error caused by diluting the plasmid. If Le1 and RRS were constructed on separate plasmids, the dilution error of each plasmid had to be considered. The reaction was performed on the GeneAmp 9700 and the PTC-200 DNA engine. Then, the amplified fragments were analyzed using three types of analytical systems consisting of a photographic device and software, which resulted in six independent Cf values as shown in **Table 3**. These six independent Cf values were analyzed by one-way analysis of variance and the obtained *P* value was 0.74. This result demonstrated that there was no significant difference. In all measurements, the coefficients of determination (R^2) for standard curves were between 0.98 and 1.00. All of the obtained Cf values were close to the theoretical Cf value of 1.00 as well as that of real-time PCR. Therefore, both of the systems were suggested to work well to quantify the targets, although the measurement principles of real-time PCR and PQC-PCR are different. In addition, because Cf values for PQC-PCR, obtained irrespective of the combination of PCR machines and analytical systems, were all close to the theoretical Cf value, the variance of Cf values caused by machine and analytical system could be considered negligible. The average Cf value of 0.99 was used to calculate the following measurements.

Measurement of GM Contents (%). First, five samples of dried ground seeds with different mass fractions of RRS, that is, 0.5, 1, 3, 5, and 10% (w/w), were subjected to real-time PCR. The obtained Cf value (1.04) was used to calculate GM contents (%), and triplicate PCR reactions for each sample were performed with the ABI PRISM 7700 (**Table 4**).

Next, PQC-PCR was performed to determine the GM contents of the same simulated RRS samples. Competitor plasmid set L was used to measure the copy number of RRS in 0.5−3% of

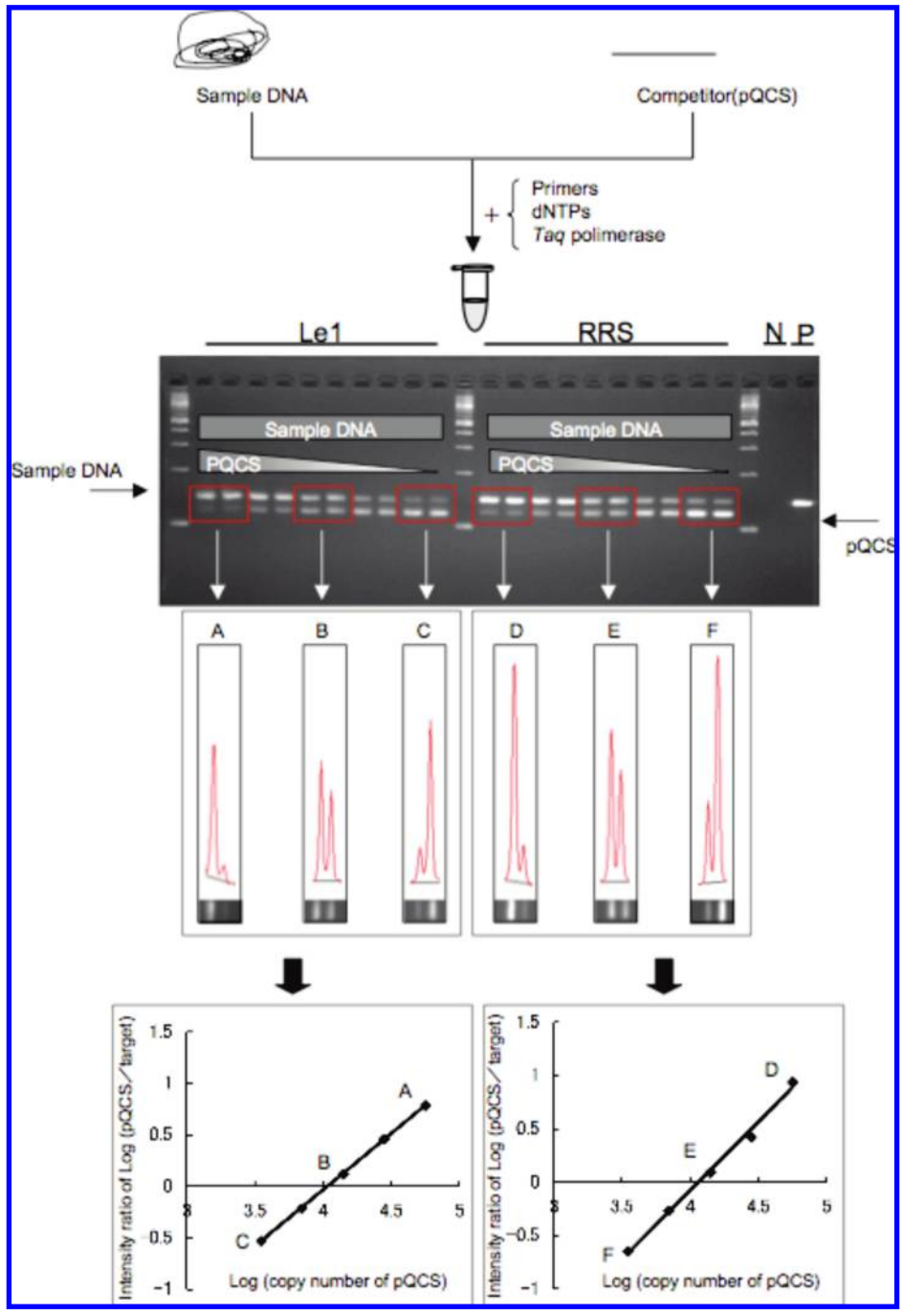

Figure 3. Principle of GMO quantification by the PQC-PCR system. Sample DNA and competitor were coamplified in the same reaction tube. The amount of DNA was constant in all PCR tubes. After PCR, the amplification products were separated by agarose gel electrophoresis on which the amplified competitor sequence could be distinguished from the amplified target sequence by size. At the equivalence point, the starting concentrations of target and competitor were equal. N, negative control (no primer); P, positive control (pQCS). Negative control (no DNA) was performed but not shown.

simulated RRS mixtures. Competitor plasmid set M was used for RRS in 3−10% samples, and competitor plasmid set H was used to determine the copy number of Le1 in all samples. The reaction was performed on the GeneAmp 9700 and the PTC-200 DNA engine in triplicate, and the amplified fragments were analyzed with three analytical systems as shown in **Table 5**. As intended, the samples in ranges of 0.5−3 and 3−10% were quantified well with each combination of PCR and analytical instruments with RSDs below 16.5%. The measurements of simulated samples obtained with PQC-PCR were compared with those obtained with real-time PCR (**Figure 4**). It was found that the GM contents obtained by both methods were almost equivalent, and the regression line between the methods was $y = 0.9810x + 0.0294$ ($R^2 = 0.9959$).

These results demonstrated that the PQC-PCR method would give almost equivalent values obtained with real-time PCR. Also, both of the real-time PCR and the PQC-PCR methods required about 2.5 h to complete the measurements. In addition, introduction of another technique, such as capillary electrophoresis into the PQC-PCR system, would reduce the analysis time.

PQC-PCR Measurement of IRMM-CRM. The CRMs containing 1 and 5% of RRS produced by IRMM were used to examine the PQC-PCR method. PQC-PCR was performed on

Table 5. Quantification of RRS Content in Five Simulated Mixture Samples by a PQC-PCR System[a]

	Gene Amp 9700			PTC-200		
mixing level (%)	average	SD	RSD (%)	average	SD	RSD (%)
			system 1			
0.50	0.54	0.08	14.3	0.55	0.02	4.2
1	1.10	0.11	10.4	1.06	0.05	5.2
3	3.17	0.02	0.8	2.95	0.24	8.0
3	2.64	0.31	3.14	0.49	11.6	15.5
5	4.24	0.22	5.1	4.59	0.07	1.6
10	10.15	1.29	12.7	10.16	0.47	4.6
			system 2			
0.50	0.64	0.08	13.0	0.57	0.05	8.7
1	1.16	0.04	3.0	1.13	0.08	7.4
3	3.08	0.22	7.3	3.11	0.34	10.9
3	2.70	0.33	12.1	3.37	0.22	6.5
5	4.59	0.42	9.1	4.71	0.16	3.4
10	10.19	0.33	3.2	10.86	0.57	5.3
			system 3			
0.50	0.60	0.10	16.5	0.60	0.05	8.4
1	1.06	0.14	12.8	1.10	0.06	5.5
3	3.14	0.15	4.8	3.14	0.31	9.8
3	2.85	0.13	4.4	3.54	0.12	3.2
5	4.61	0.44	9.5	4.78	0.32	6.6
10	10.29	0.53	5.2	10.85	0.29	2.7

[a] System 1, Molecular Imager FX and Quantity One; system 2, Light-Capture (ATTO) and CS Analyzer; and system 3, Printgraph (ATTO) & Image J; and Cf = 0.99.

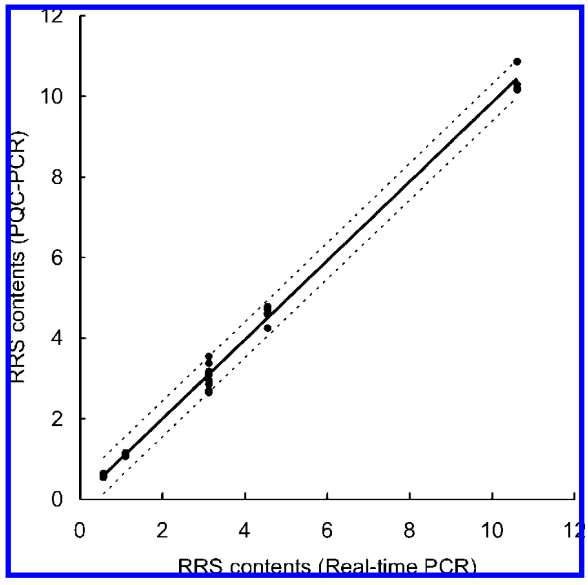

Figure 4. Correlation between RRS contents acquired by PQC-PCR and real-time PCR. RRS contents acquired by real-time PCR shown in **Table 4** are plotted on the *x*-axis, and those by PQC-PCR shown in **Table 5** are plotted on the *y*-axis. $y = 0.9810x + 0.0294$ ($R^2 = 0.9959$). The prediction interval for individual *y* is shown with dotted lines. The maximum and minimum values of standard uncertainties were calculated to be 0.23 and 0.22, respectively. For PQC-PCR, GeneAmp 9700 and PTC-200 DNA engine were used. For real-time PCR, ABI PRISM 7700 was used.

the GeneAmp 9700 and the PTC-200 DNA engine in triplicate, and the amplified fragments were analyzed with three analytical systems as shown in **Table 6**. The measured value for each of 1 and 5% RRS was in the range of the respective certified value. These results suggested that the PQC-PCR method could accurately measure GM contents and thus be useful for practical use.

Table 6. Quantification of RRS Contents in IRMM CRM by a PQC-PCR System[a]

		Gene Amp 9700			PTC-200		
sample (%)	analytical instrument	average	SD	RSD (%)	average	SD	RSD (%)
1 ± 0.16	system 1	0.92	0.01	0.6	1.00	0.08	8.4
	system 2	0.89	0.07	7.9	1.00	14.0	14.0
	system 3	0.92	0.05	5.1	1.04	0.17	16.0
5 ± 0.53	system 1	4.92	0.50	10.1	4.75	0.22	4.5
	system 2	5.07	0.23	4.6	4.74	0.38	8.0
	system 3	4.87	0.30	6.1	4.72	0.20	4.3

[a] System 1, Molecular Imager FX and Quantity One; system 2, Light-Capture (ATTO) and CS Analyzer; system 3, Printgraph (ATTO) and Image J; and Cf = 0.99.

ABBREVIATIONS USED

QC-PCR, quantitative competitive polymerase chain reaction; GM, genetically modified; RRS, Roundup Ready soybean; Le1, lectin 1; PQC-PCR, plasmid-based QC-PCR; GMO, genetically modified organism; MAFF, Ministry of Agriculture, Forestry and Fisheries; Cf, conversion factor; RSD, relative standard deviation; SD, standard deviation; CRM, certified reference materials; IRMM, Institute for Reference Materials and Measurements.

ACKNOWLEDGMENT

This study was supported in part by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan for the Research Project, "Assurance of Safe Use of Genetically Modified Organisms", and by a grant from the Ministry of Health, Labour and Welfare of Japan.

LITERATURE CITED

(1) James, C. Review—Global status of commercialized biotech/GM crops: 2006. *ISAAA Briefs* **2006**, *35*.
(2) Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off. J. Eur. Union* **2003**, L268/1−L268/23.
(3) Notification No. 2000-31; Ministry of Agriculture and Forestry of Korea: Seoul, Korea, 2000.
(4) Notification No. 1775; Food and Marketing Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan: Tokyo, Japan, 2000.
(5) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. A method of detecting recombinant DNAs from four lines of genetically modified maize. *J. Food Hyg. Soc. Jpn.* **2000**, *41*, 137–143.
(6) Matsuoka, T.; Kuribara, K.; Takubo, H.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *J. Agric. Food Chem.* **2002**, *50*, 2100–2109.
(7) Kuribara, H.; Shindo, Y.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Novel reference molecules for quantitation of genetically modified maize and soybean. *J. AOAC Int.* **2002**, *85*, 1077–1089.
(8) Shindo, Y.; Kuribara, H.; Matsuoka, T.; Futo, S.; Sawada, C.; Shono, J.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Validation of real-time PCR analyses for line-specific quantitation of genetically modifed maize and soybean using new reference molecules. *J. AOAC Int.* **2002**, *85*, 1119–1126.
(9) Terry, C. F.; Harris, N. Event-specific detection of Roundup Ready soya using two different real time PCR detection chemistries. *Eur. Food Res. Technol.* **2001**, *213*, 425–431.
(10) Permingeat, H. R.; Reggiardo, M. I.; Vallejos, R. H. Detection and quantification of transgenes in grains by multiplex and real-time PCR. *J. Agric. Food. Chem.* **2002**, *50*, 4431–4436.

(11) Terry, C. F.; Shanahan, D. J.; Ballam, L. D.; Harris, N.; McDowell, D. G.; Parkes, H. C. Real-time detection of genetically modified soya using lightcycler and ABI7700 platforms with TaqMan, Scorpion, and SYBR Green I chemistries. *J. AOAC Int.* **2002**, *85*, 938–944.

(12) Holst-Jensen, A.; Rønning, S. B.; Lovseth, A.; Berdal, K. G. PCR technology for screening and quantification of genetically modified organisms (GMOs). *Anal. Bioanal. Chem.* **2003**, *375*, 985–993.

(13) Ahmed, F. E. Detection of genetically modified organisms in foods. *Trends Biotechnol.* **2002**, *20*, 215–223.

(14) Gilliland, G.; Perrin, S.; Blanchard, K.; Bunn, F. H. Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 2725–2729.

(15) Studer, E.; Rhyner, C.; Lüthy, J.; Hübner, P. Quantitative competitive PCR for the detection of genetically modified soybean and maize. *Z. Lebensm.-Unters. Forsch. A* **1998**, *207*, 207–213.

(16) Hübner, P.; Studer, E.; Lüthy, J. Quantitative competitive PCR for the detection of genetically modified organisms in food. *Food Control.* **1999**, *10*, 353–358.

(17) García-Cañas, V.; Cifuentes, A.; González, R. Quantitation of Transgenic Bt Event-176 Maize Using Double Quantitative Competitive Polymerase Chain Reaction and Capillary Gel Electrophorsesis Laser-Induced Fluorescence. *Anal. Chem.* **2004**, *76*, 2306–2313.

(18) Hupfer, C.; Hotzel, H.; Sachse, K.; Moreano, F.; Engel, K.-H. PCR-based quantification of genetically modified Bt maize: Single-competitive versusu dual-competitive approach. *Eur. Food Res. Technol.* **2000**, *212*, 95–99.

(19) Mavropoulou, A. K.; Koraki, T.; Ioannou, P. C.; Iida, M.; Christopoulos, T. K. High-throughput double quantitative competitive polymerase chain reaction for determination of genetically modified organisms. *Anal. Chem.* **2005**, *77*, 4785–4791.

(20) ISO 21570:2005, Foodstuffs—Methods of analysis for the detection of genetically modified organisms and derived products—Quantitative nucleic acid based methods.

(21) Notification No. 110; Department of Food Safety, Ministry of Health, Labour and Welfare of Japan: Tokyo, Japan, 2001.

(22) Yoshimura, T.; Kuribara, H.; Matsuoka, T.; Kodama, T.; Iida, M.; Watanabe, T.; Akiyama, H.; Maitani, T.; Furui, S.; Hino, A. Applicability of the quantification of genetically modified organisms to foods processed from maize and soy. *J. Agric. Food Chem.* **2005**, *53*, 2052–2059.

Received for review November 16, 2007. Revised manuscript received April 1, 2008. Accepted April 12, 2008.

JF073348N