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Quantification of Genetically Modified Soybean by Quenching Probe Polymerase Chain Reaction

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Quenching probe (QProbe) polymerase chain reaction (PCR) is a simple and cost-effective real-time PCR assay in comparison with other real-time PCR assays such as the TaqMan assay. We used QProbe-PCR to quantify genetically modified (GM) soybean (Roundup Ready soybean). We designed event-specific QProbes for *Le1* (soy endogenous gene) and RRS (recombinant gene), and we quantified certified reference materials containing 0.1, 0.5, 1, 2, and 5% GM soybean. The TaqMan assay was also applied to the same samples, and the results were compared. The accuracy of QProbe-PCR was similar to that of TaqMan assay. When GM soybean content was 0.5% or more, the relative standard deviations of QProbe-PCR were less than 20%. QProbe-PCR is sensitive enough to monitor labeling systems and has acceptable levels of accuracy and precision.

KEYWORDS: GMOs; fluorescence quenching; quenching probe; QProbe-PCR; real-time PCR; Roundup Ready soybean

INTRODUCTION

In several countries, foods or feeds containing genetically modified organisms (GMOs) are subject to compulsory labeling. The unintentional contamination by GM material of a non-GM background is difficult to avoid during field culture or seed transport. Hence, the threshold for unintentional mixing of a GMO that does not require labeling is defined as 0.9% in the EU (*1*−*4*) and 5% in Japan (*5*). Therefore, analytical methods to accurately quantify GMO contents are required.

Many analytical methods used to quantify GMOs have already been developed. Methods based on the polymerase chain reaction (PCR) have been most widely used, because of their sensitivity, specificity, and applicability. Competitive PCR was applied to the quantification of Roundup Ready soybean and Bt Maize (*6*−*10*). Real-time PCR was used for the quantification of Roundup Ready soybean (*11*−*21*), Bt11 (*17*, *19*, *22*, *23*), Bt176 (*11*, *16*, *17*, *19*, *22*, *23*), and MON810 (*17*, *19*, *20*, *22*, *24*, *25*). In particular, real-time PCR has engendered wider acceptance because of its rapidity, sensitivity, and reproducibility and the low risk of carry-over contamination. The TaqMan assay is one of the most commonly used real-time PCR methods for the determination of GMO content (*11*−*13*, *15*−*23*, *25*). The Japanese Government has adopted the TaqMan assay as the standard method to quantify GMOs (*26*). This assay requires the TaqMan probe, which is an oligonucleotide modified with two fluorescent dyes at both ends. The fluorescence of one dye in the TaqMan probe should be quenched by the fluorescence resonance energy transfer to the other dye, and the probe should be degraded by Taq polymerase during the elongation reaction in PCR. The evaluation of the performance of TaqMan probes is usually done by trial and error by performing real-time PCR, and this step is very time-consuming.

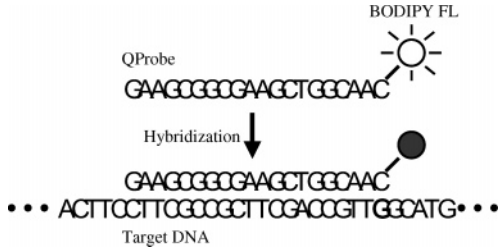

Figure 1. Fluorescence quenching of a QProbe. When a QProbe hybridizes with a target DNA, its fluorescence is quenched by the guanine in the target complementary to the modified cytosine, and the quench rate is proportional to the amount of target DNA (*27*).

Quenching probe (QProbe) PCR (*27*, *28*) is a simple real-time PCR assay which requires QProbe (**Figure 1**). A QProbe is an oligonucleotide with a fluorescent dye modified cytosine at its 3′ or 5′ end. This method utilizes the phenomenon whereby the fluorescence of the dye is quenched by an electron transfer to a guanine base at a particular position (*27*, *29*). The

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Table 1. Primers, Probes, and Complementary Oligonucleotides

target	name	orientation	sequence (5′–3′)	reference
Le1	Le1n02–5′	forward primer	GCCCTCTACTCCACCCCCA	17
	Le1n02–3′	reverse primer	GCCCATCTGCAAGCCTTTTT	17
	Le1-QP	QProbe	GAAGCGGCGAAGCTGGCAAC-(BODIPY FL)	this study
	Le1-comp	complementary DNA	GUACG*GUUGCCAGCUUCGCCGCUUC*[a]	this study
	Le1-Taq	TaqMan probe	(FAM)-AGCTTCGCCGCTTCCTTCAACTTCAC-(TAMRA)	17
RRS	RRS01–5′	forward primer	CCTTTAGGATTTCAGCATCAGTGG	17
	RRS01–3′	reverse primer	GACTTGTCGCCGGGAATG	17
	RRS-QP	QProbe	CGCAACCGCCCGCAAATCC-(BODIPY FL)	this study
	RRS-comp	complementary DNA	CCAGA*GGAUUUGCGGGCGGUUGCG*[b]	this study
	RRS-Taq	TaqMan probe	(FAM)-CGCAACCGCCCGCAAATCC-(TAMRA)	17

[a] Italicized sequence is complementary to Le1-QP. [b] Italicized sequence is complementary to RRS-QP.

performance of the QProbe can be estimated using a complementary oligonucleotide without performing real-time PCR, and therefore, the evaluation of the probe's performance is rapid and easy. QProbe needs only one dye, so its synthesis is simpler and more cost-effective than that of the TaqMan probe and other probes/primers which need two dyes (*30*−*33*). Moreover, in QProbe-PCR, a melting curve analysis can be performed after the PCR to measure the melting temperature (T_m) between the QProbe and PCR products. If the PCR products do not contain the sequence that perfectly matches the QProbe, the measured T_m is lower than the expected value, and therefore, T_m measurement is useful to ensure that the PCR products contain the target sequence and consequently eliminate pseudopositive results. In the TaqMan assay, a melting curve analysis after the PCR is impossible, and there is no easy way to eliminate pseudopositive results. Thus, QProbe-PCR has several advantages.

In this study, we used QProbe-PCR to determine GMO levels in soybean and compared the performance with the results of a TaqMan assay. QProbe-PCR showed enough sensitivity and accuracy for GMO quantification.

MATERIALS AND METHODS

Materials. The certified reference materials IRMM-410S consisting of 0.10 ± 0.05, 0.50 ± 0.10, 1.0 ± 0.2, 2.0 ± 0.3, and 5.0 ± 0.6% (w/w) Roundup Ready soybean produced by the Institute for Reference Materials and Measurements (IRMM) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Standard curves for measuring GM soybean by real-time PCR were constructed using GM soybean (RRS) Detection Plasmid Set -ColE1/TE- (Nippon Gene, Toyama, Japan). This plasmid set contained linearized plasmid DNAs possessing *Le1* and RRS sequences at concentrations of 40, 250, 3000, 40 000, and 500 000 copies per 5.0 μL.

Oligonucleotides. The sequences of the primers, probes, and complementary oligonucleotides used in this study are listed in **Table 1**. The primers and the complementary oligonucleotides were purchased from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). The QProbes and TaqMan probes were purchased from Espec Oligo Service (Ibaraki, Japan) and Nippon Gene, respectively. The QProbes were labeled at the 3′ end with BODIPY FL via an aminohexyl phosphate linker having a seven-carbon spacer. The TaqMan Probes were labeled at the 5′ end with the fluorescent reporter dye 6-carboxyfluorescein (FAM) and at the 3′ end with the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA).

DNA Extraction and Purification. Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) from 100 mg of soybean sample according to the manufacturer's manual. The concentration of the extracted DNA was calculated from the absorbance at 260 nm measured by a UV spectrometer DU-600 (Beckman Coulter Inc., Fullerton, CA). The extracted DNA was diluted to 20 ng/μL.

Melting Curve Analysis To Measure T_m and Quench Rate of QProbes. The reaction mixture (25 μL) contained 0.2 μM QProbe, 0.4 μM complementary oligonucleotide, 1 × PCR Buffer (10 × Gene Taq Universal Buffer; Nippon Gene), and 1 mM $MgCl_2$. As a negative control, sterilized distilled water was added instead of the complementary oligonucleotide. The reaction mixture was heated to 95 °C for 90 s, cooled to 50 °C, kept at 50 °C for 2 min, and then slowly heated back to 95 °C at a ramp rate of 2% with continuous fluorescence acquisition using an ABI PRISM 7900HT (Applied Biosystems, Foster, CA). T_m was calculated from peaks generated by plotting the negative derivative of the fluorescence intensity over temperature versus the temperature ($-dF/dT$ versus T). The fluorescence quench rate at each temperature was calculated by eq 1:

$$\text{fluorescence quench rate (\%)} = [(F_2 - F_1)/F_2] \times 100 \quad (1)$$

where F_1 and F_2 are the fluorescence intensities at each temperature of the reaction mixture with and without complementary oligonucleotide, respectively.

QProbe-PCR. QProbe-PCR was carried out using an ABI PRISM 7900HT. The reaction mixture (25 μL) contained 100 ng of sample DNA or 5 μL of GM soybean (RRS) Detection Plasmid Set -ColE1/TE- (Nippon Gene), 0.2 μM QProbe, 1 μM forward primer Le1n02−5′ plus 0.3 μM reverse primer Le1n02−3′ (for *Le1*) or 0.3 μM RRS01−5′ plus 1 μM RRS01−3′ (for RRS), 200 μM each dATP, dCTP, and dGTP, 600 μM dUTP (Roche Diagnostics, Mannheim, Germany), 1 × PCR Buffer (10 × Gene Taq Universal Buffer; Nippon Gene), 0.625 U of DNA polymerase (Gene Taq; Nippon Gene), 0.035 μM BD Taqstart Antibody (BD Biosciences Clontech, Palo Alto, CA), 0.25 U of uracil-DNA glycosylase (heat-labile; Roche Diagnostics), and 1 mM $MgCl_2$. Uracil-DNA glycosylase in the reaction mixture was used to prevent carry-over contamination of PCR products, and the glycosidase reaction was performed during the preparation of the reaction mixture at room temperature before PCR. PCR conditions were as follows: an initial denaturation at 95 °C for 2 min; 50 cycles of denaturation at 95 °C for 45 s, annealing at 59 °C for 1 min, and extension at 72 °C for 45 s; and a final extension at 72 °C for 2 min. The fluorescence intensity was measured after denaturation and annealing steps in each cycle, and the fluorescence quench rate at each cycle was calculated according to a previous report (*27*). The cycle at which the quench rate plot crosses the threshold is defined as Ct (cycle of threshold), and the standard curve was constructed from the mean Ct values of triplicate determinations. The copy number of *Le1* and RRS in each sample was calculated from the Ct value of the sample using the standard curve. GMO contents (%) were calculated using eq 2:

$$\text{GMO content (\%)} = [N_1/(N_2 C_V)] \times 100 \quad (2)$$

where N_1 and N_2 are the initial copy numbers of RRS and *Le1* in the samples, respectively, and C_V (coefficient value) is the ratio of copy numbers of RRS and *Le1* in genuine GM seeds. Roundup Ready soybean includes a single copy of RRS per genome, so the theoretical C_V is 1.0. Therefore, in QProbe-PCR, we used 1.0 as the C_V. After PCR, a melting curve analysis was performed.

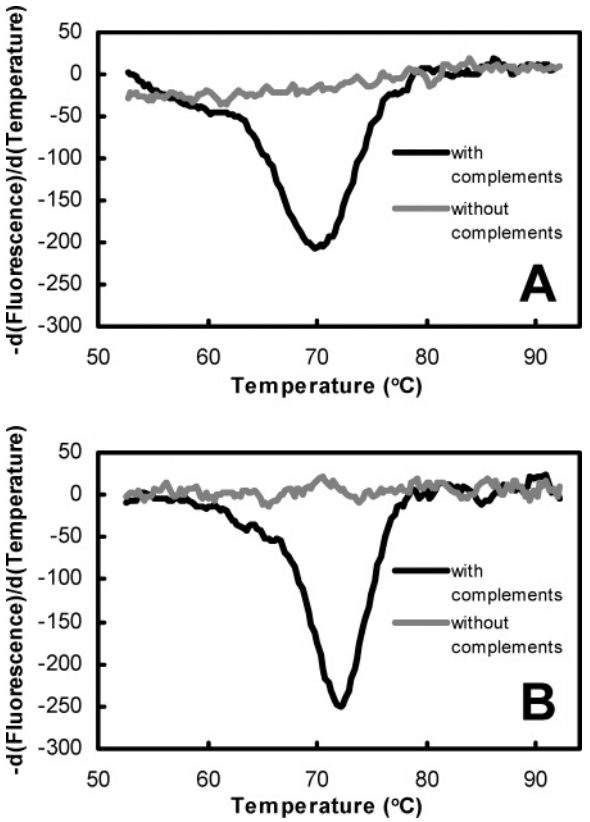

Figure 2. Derivative melting curve plots for Le1-QP (**A**) and RRS-QP (**B**) with complementary oligonucleotide (black line), and without complementary oligonucleotide (gray line). The peak indicates the melting point.

TaqMan Assay. The TaqMan assay was carried out using an ABI PRISM 7900 HT according to a report by the Ministry of Health, Labour and Welfare, Japan (*26*) with slight modification. The reaction mixture (25 μL) contained 100 ng of sample DNA or 5 μL of GM soybean (RRS) Detection Plasmid Set -ColE1/TE- (Nippon Gene), 0.2 μM TaqMan probe (Nippon Gene), 0.5 μM each primer, and 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems). PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 30 s and 59 °C for 60 s. The TaqMan assay was performed in triplicate for each template DNA. Results were analyzed using ABI PRISM 7900HT Sequence Detection System software 2.1 (Applied Biosystems). According to the report (*26*), GMO content was calculated using 1.04 as the C_V.

RESULTS AND DISCUSSION

Melting Curve Analysis To Measure T_m and Quench Rate of QProbes. The event-specific QProbes, Le1-QP and RRS-QP, were designed for *Le1* and RRS, respectively. Melting curve analyses of the QProbes with and without complementary oligonucleotide were performed. As shown in **Figure 2**, the T_m of Le1-QP and RRS-QP was 69.8 and 72.1 °C, respectively. The fluorescence quench rates of Le1-QP and RRS-QP (**Figure 3**) were 61.7 and 59.3%, respectively, at 59 °C which is the annealing temperature in the PCR for GMO quantification.

Standard Curves. Standard curves were constructed from reaction mixtures containing 0, 40, 250, 3000, 40 000, and 500 000 copies of the plasmid possessing *Le1* and RRS sequences as described in Materials and Methods. Since about 80 000 copies of *Le1* are contained in 100 ng of soybean DNA (*18*), 40−500 000 copies of RRS in the 100 ng of soybean DNA correspond to a GMO content of about 0.05−625%. This range meets the labeling requirement of regulations in the EU (0.9%) and Japan (5%). **Figure 4** shows amplification plots, standard curves, and melting curves for *Le1* and RRS obtained by QProbe-PCR. The calculated R^2 values of the standard curves for *Le1* and RRS were 0.9959 and 0.9987, respectively. Thus, the standard curves in QProbe-PCR were linear. The melting curve analysis for *Le1* and RRS showed that both PCR products contained the expected sequence. Then, the reproducibility of QProbe-PCR was evaluated using the Ct values obtained for constructing the standard curves. Copy numbers of *Le1* and RRS were calculated from the Ct values using the standard curves. The calculated mean copy number and relative standard deviation (RSD) of triplicate samples of *Le1* and RRS at each concentration are shown in **Table 2**. In *Le1* measurements, the RSDs of 3000−500 000 copies were less than 11% and, so, in a satisfactory range, but the RSDs of 40 and 250 copies were high (29.1 and 37.9%, respectively). Considering that 100 ng of soybean DNA contains about 80 000 copies of *Le1*, high RSDs for 40 and 250 copies would not affect the quantification of GM soybean in practical terms. In RRS measurements, the RSDs of 250−500 000 copies (corresponding to about 0.3−625% GMO) were less than 12%, but the RSD of 40 copies

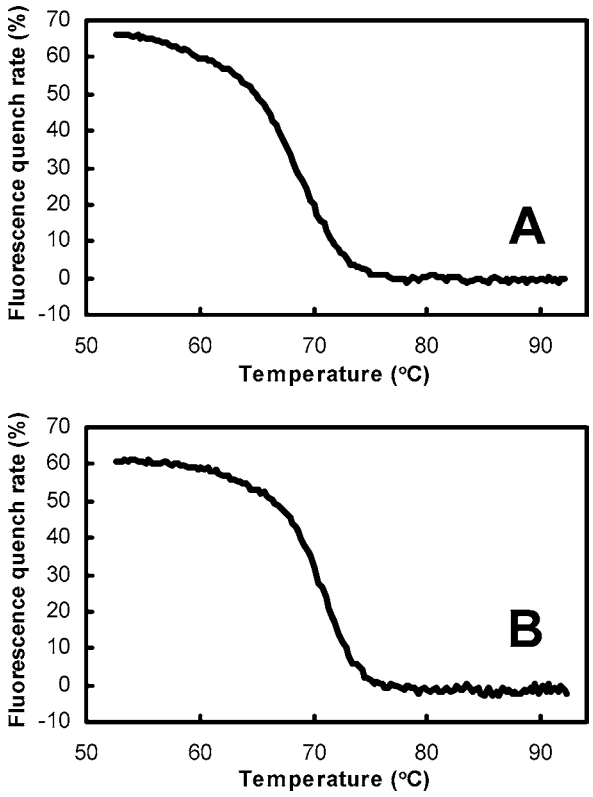

Figure 3. Fluorescence quench rates for Le1-QP (**A**) and RRS-QP (**B**).

Table 2. Reproducibility of Copy Numbers from 40 to 500 000 Copies Per Reaction for *Le1* and RRS

target	R^2	true value (copy)	mean (copy)	RSD[a] (%)
Le1	0.9959	40	38	29.1
		250	303	37.9
		3 000	3 235	5.5
		40 000	46 725	10.6
		500 000	435 680	6.4
RRS	0.9987	40	39	28.4
		250	261	11.5
		3 000	3 015	10.1
		40 000	40 406	6.0
		500 000	481 964	7.2

[a] RSD, relative standard deviation of triplicate reactions in a single experiment.

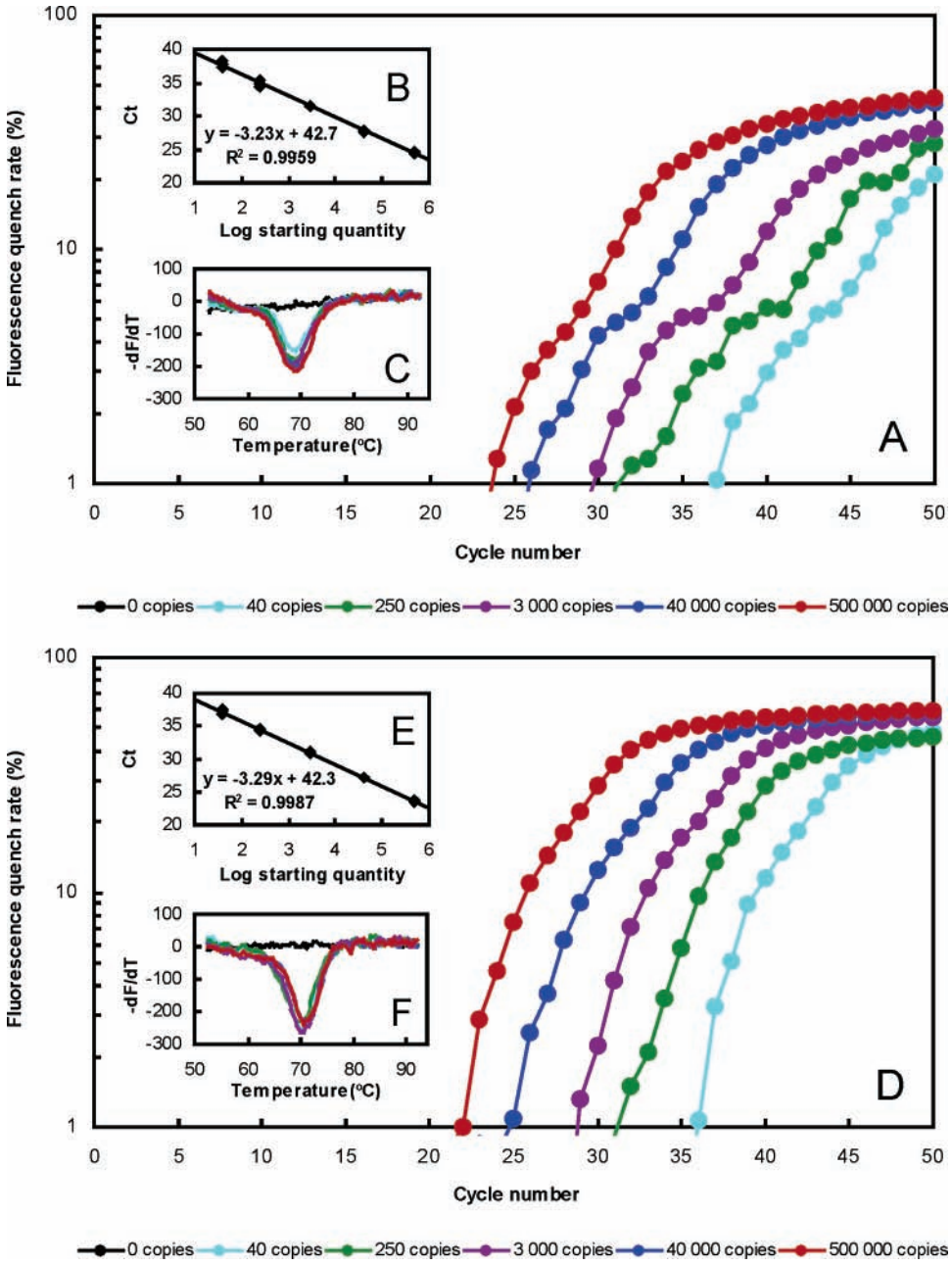

Figure 4. Soybean quantification in QProbe-PCR: (**A**) amplification plots of *Le1* in the plasmid; (**B**) standard curve for *Le1* (equation and fit of the line are shown); (**C**) melting curve analysis for *Le1*; (**D**) amplification plots of RRS in the plasmid; (**E**) standard curve for RRS (equation and fit of the line are shown); (**F**) melting curve analysis for RRS.

(corresponding to about 0.05%) was 28.4%. Considering the labeling requirements of regulations in the EU (0.9%) and Japan (5%), a high RSD for 40 copies would not affect the measurement of GM soybean content in practical terms. Therefore, the reproducibility of QProbe-PCR was satisfactory for practical use.

Accuracy and Precision of Quantification. To compare the performance of QProbe-PCR and the TaqMan assay for GM soybean quantification, we determined GMO contents of five levels of RRS reference materials, 0.1, 0.5, 1, 2, and 5%, using both methods. The experiments were repeated three times, and the mean GMO content, the difference between the experimental mean value and theoretical value (bias), SD, and RSD at each level of GMO content were calculated (**Table 3**). The bias of QProbe-PCR and that of the TaqMan assay were similar, but the RSDs of QProbe-PCR were slightly greater. This means that QProbe-PCR has a accuracy similar to, but a slightly lower precision than, the TaqMan assay. According to previous reports (*19*, *25*), the threshold of RSD for GMO measurement is 20 or 25%. As shown in **Table 3**, the RSDs in QProbe-PCR and the TaqMan assay for the samples containing more than 0.5% GMO were less than 20%, and the RSDs for the 0.1% sample were more than 20%. Since the labeling requirements of regulations in the EU and Japan are 0.9 and 5%, respectively, the performance of QProbe-PCR, as well as the TaqMan assay, is satisfactory to estimate the unintentional mixing of GM soybean in practical terms. In addition, in QProbe-PCR, a melting curve analysis was easily performed, and it was shown that each PCR product contained the expected sequence (data not shown). In this regard, QProbe-PCR can present more reliable data than the TaqMan assay.

In this study, we used QProbe-PCR to determine GMO contents and showed that QProbe-PCR has acceptable levels of accuracy and precision like the TaqMan assay. QProbe-PCR

Table 3. Accuracy and Precision Statistics for Quantitative Methods

		accuracy		precision	
method	true value (%)	mean (%)	bias[a] (%)	SD[b]	RSD[c] (%)
QProbe-PCR	0.10 ± 0.05	0.078	–21.8	0.03	33.0
	0.50 ± 0.10	0.48	–3.7	0.09	18.0
	1.0 ± 0.2	1.1	13.1	0.15	12.9
	2.0 ± 0.3	1.8	–8.6	0.20	11.0
	5.0 ± 0.6	4.2	–15.8	0.15	3.5
TaqMan assay	0.10 ± 0.05	0.10	0.0	0.03	26.5
	0.50 ± 0.10	0.44	–12.0	0.07	15.7
	1.0 ± 0.2	0.91	–9.0	0.05	5.0
	2.0 ± 0.3	1.7	–13.8	0.03	1.5
	5.0 ± 0.6	4.5	–10.5	0.24	5.4

[a] Bias = (mean value – true value)/true value × 100. [b] SD, standard deviation. [c] RSD, relative standard deviation. All experiments were performed three times.

has several advantages over the TaqMan assay, as follows: (i) The design and synthesis of probes are simpler and more cost-effective. (ii) A melting curve analysis can be performed after the PCR, so one can easily confirm whether the PCR products include expected sequences or not, and consequently, eliminate pseudopositive results. QProbe-PCR is sensitive enough to monitor labeling systems and useful for the quantification of GM soybeans. This method is also applicable to examination of other GMOs.

ABBREVIATIONS USED

QProbe, quenching probe; GMO, genetically modified organism; PCR, polymerase chain reaction; Ct, cycle of threshold; C_V, coefficient value

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