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Real-Time PCR Method Using Capturing Oligo-Immobilized PCR Tubes to Determine the Specific Gene for Soybean and Genetically Modified Soybean in Food Matrices

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A new real-time PCR method using capturing oligo-immobilized PCR tubes is described. This method was used to detect specific genes for soybean and genetically modified (GM) soybean in food matrices. In a standard reaction using soybean genomic DNA and a capturing oligo for the lectin gene (*Le1*) immobilized on the tube, we examined the effects of such hybridization conditions as the location, length, and amount of the capturing oligo, and the incubation time and temperature. Under optimized conditions, the copy number of *Le1* was determined in a concentration-dependent manner from soybean genomic DNA and soybean lysate (DNA 10–1000 ng, $r = 0.99$; lysate 1–100%, $r = 0.99$). The copy number of a Roundup Ready soybean (*RRS*) gene was also successfully detected in a concentration-dependent manner (1–100%, $r = 0.99$) from GM soybean lysate, using PCR tubes with an immobilized capturing oligo for the transgene. Our data indicate that this is a rapid and simple method to determine specific genes for soybean and GM soybean in food matrices.

Key words: DNA extraction; genetically modified organism; hybridization; polymerase chain reaction; single-tube method

Molecular genetic methods are widely used to detect microbial pathogens, genetically modified organisms (GMOs), and food allergens. Among these methods, the polymerase chain reaction (PCR) technique is the most commonly used. Real-time PCR is a useful tool for obtaining precise and quantitative information. However, DNA extraction methods are time-consuming, often including column-based steps and precipitation and centrifugation steps with toxic organic solvents. In addition, the DNA extraction step is thought to be difficult to automate and downscale to a small sample volume.

It is possible to avoid these problems by using the hybridization-bead method. The bead method can reduce the total detection time, remove inhibitors of the PCR amplification reaction, and remove excess non-target DNA.[1,2)] However, the hybridization bead method is not used directly in real-time PCR as the beads block the optical path. Ideally, the number of tube-to-tube transfers should be kept to a minimum to avoid the loss of template and decrease the risk of contamination.

A "single-tube method" has recently been developed, in which nucleic acid extraction, amplification, and detection are carried out in a single tube.[3,4)] This method is rapid and requires few manipulations. Single-tube real-time PCR methods, using an aluminum oxide filter and a heated guanidine solution, have also been attempted.[3,4)] However, these extraction processes required special handling, such as pressure filtration and dry evaporation of samples. In addition, these methods could extract total DNA from a sample but could not specifically isolate target DNA.

We describe in this report a novel single-tube method using hybridization as shown in Fig. 1. We focused on a unique plastic surface treatment which provides a unique biocompatible phospholipid polymer and a functional ester moiety to covalently bind the attachment site for amino-oligonucleotides.[5)] A capturing oligonucleotide can be immobilized on the surface-treated PCR tube.

We developed in the present study a novel, simple, and specific real-time PCR method using capturing oligo-immobilized PCR tubes. The method detected target DNA in food matrices in a concentration-dependent

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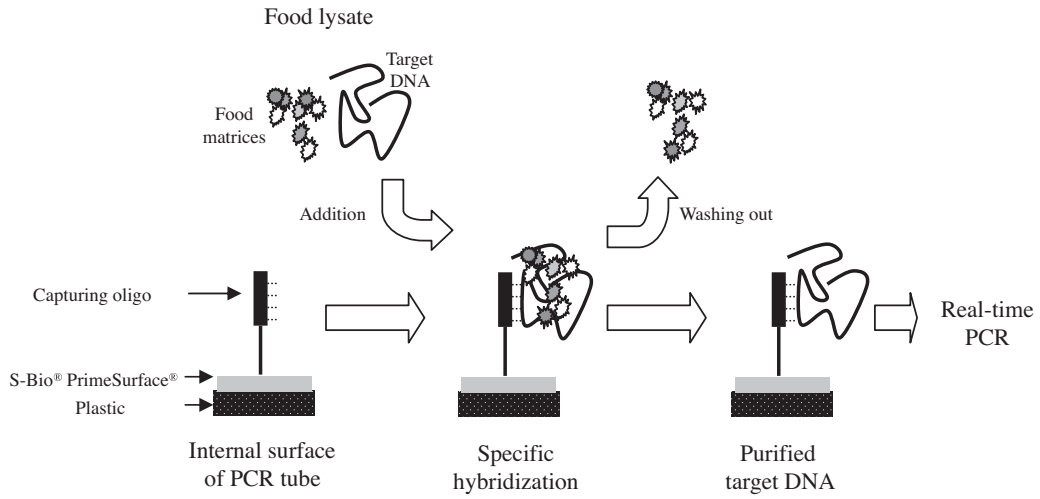

Fig. 1. Process for a Real-Time PCR Method Using a Capturing Oligo-Immobilized PCR Tube.
A capturing oligo is immobilized on the S-Bio® PrimeSurface®-treated PCR tube. Target DNA in a food lysate is hybridized to the capturing oligo on the surface of the PCR tube. Food matrices are removed by washing. Purified target DNA in the PCR tube is directly used for real-time PCR.

manner. This study focused on the detection of soybean, which is an allergenic food, and is often also a GMO. Initially, the hybridization conditions for soybean genomic DNA and soybean lysate were examined by using the species-specific lectin gene, *Le1*, as the capturing oligo immobilized on the PCR tube. Finally, concentration-dependent detection of the transgene in Roundup Ready soybean (*RRS*) from genetically modified (GM) soybean lysate was examined by using an *RRS* capturing oligo immobilized on the PCR tube.

Materials and Methods

Materials. Soybean, wheat flour, and corn flour were purchased from a local market in Hyogo, Japan. Roundup Ready soybean seeds were kindly provided by Monsanto (MO, USA). Soybeans were ground to a powder (AM-3, Nihon Seiki Seisakusho Company, Tokyo, Japan). Certified reference materials containing 1, 2 and 5% Roundup Ready soybean were purchased from Fluka (Buchs, Switzerland).

Heat treatment of the samples. Soybean powder (1 g) was suspended in 7.5 ml of distilled water and autoclaved (KS-323, Tomy Seiko Co., Tokyo, Japan) at 110 °C for 30 and 60 min (not including the temperature increase/decrease time), as described elsewhere.[6)]

DNA samples. Genomic DNA was extracted from the soybean powder by using a silica membrane-type kit (DNeasy Plant Mini Kit, Qiagen, Hilden, Germany). The soybean DNA [4 ng/µl in a lysis buffer (100 mM Tris HCl (pH 8.0), 10 mM $CaCl_2$, 0.4 M NaCl, and 0.5% SDS) with 10 mM EDTA] was added to the capturing oligo-immobilized PCR tube in the experiments optimizing the hybridization conditions. After hybridization and washing, the purified DNA in the PCR tube was analyzed by real-time PCR. In the concentration-dependent DNA experiment, 0.04 and 0.4 ng/µl of soybean DNA was prepared with a lysis buffer with 10 mM EDTA. In the experiment investigating the effects of food matrices, 4 ng/µl of the soybean DNA solution in 50 mg/ml of the wheat or corn lysate was added to the capturing oligo-immobilized PCR tube.

Lysate samples. Powdered soybean (50 mg) was added to a 1.5-ml tube. After adding 980 µl of 400 µg/ml of proteinase K (Sigma, MO, USA), 200 µg/ml of RNase A (Nacalai Tesque, Kyoto, Japan), and 14 U/µl of α-amylase (Nippon Gene, Tokyo, Japan) in a lysis buffer, the sample was incubated at 60 °C for 60 min with occasional vortexing. After incubating at 95 °C for 5 min, 20 µl of 0.5 M EDTA was added. The sample was centrifuged at 13000 *g* for 10 min. The resulting supernatant was used as the 50-mg/ml soybean lysate sample for the PCR method, using capturing oligo-immobilized PCR tubes. The lysate sample is defined as 100% and was diluted to 1% and 10% with the lysis buffer in the experiment to analyze the concentration effects. Lysates of wheat and corn (50 mg/ml) were prepared in the same way.

Capturing oligo design. The capturing oligo sequences for *Le1* were selected from both inside and outside the PCR amplification region for *Le1* (Fig. 2). The primer and probe sequences for *Le1* were selected from inside the region, and the 64-bp upstream and 89-bp downstream sequences of the PCR amplification region were selected from outside the region (Table 1). The sequence of the soybean lectin gene was obtained from GenBank (accession no. GI 170005) and was reconfirmed by a DNA sequence analysis. The capturing

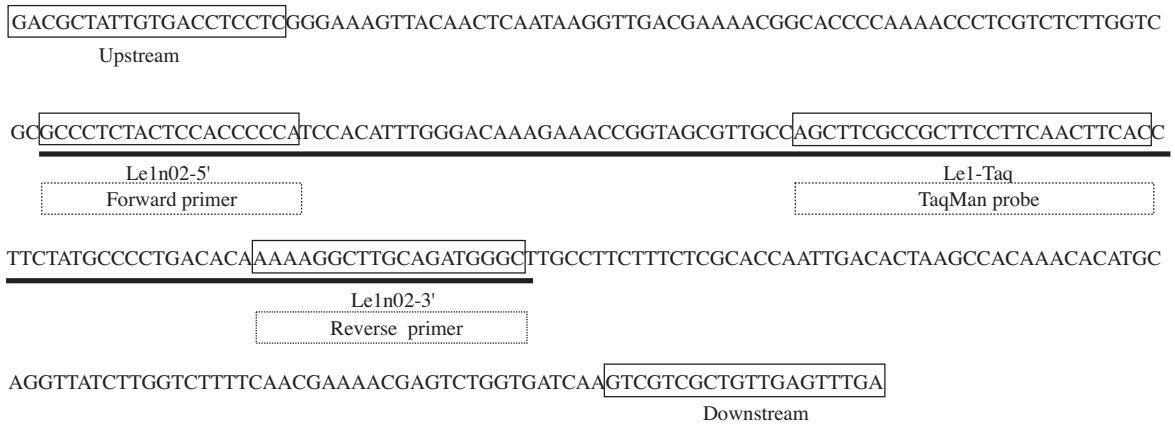

Fig. 2. Positions of the Capturing Oligos, PCR Primers, and Probe in *Le1*.

DNA sequences of the capturing oligos for *Le1* are indicated by open boxes. We used the DNA sequences of Le1n02-5′ and Le1n02-3′ as primers and Le1-Taq as the TaqMan probe for real-time PCR for *Le1*. The amplified region is shown with a bold line.

Table 1. List of Capturing Oligos

Capturing oligo	Orientation	Sequence
Upstream-s[a]	sense	5′-NH_2-GACGCTATTGTGACCTCCTC-3′
Upstream-a[a]	antisense	5′-NH_2-GAGGAGGTCACAATAGCGTC-3′
Le1n02-5′-s[a]	sense	5′-NH_2-GCCCTCTACTCCACCCCCA-3′
Le1n02-5′-a[a]	antisense	5′-NH_2-TGGGGGTGGAGTAGAGGGC-3′
Le1-Taq-s[a]	sense	5′-NH_2-AGCTTCGCCGCTTCCTTCAACTTCAC-3′
Le1-Taq-a[a]	antisense	5′-NH_2-GTGAAGTTGAAGGAAGCGGCGAAGCT-3′
Le1n02-3′-s[a]	sense	5′-NH_2-AAAAGGCTTGCAGATGGGC-3′
Le1n02-3′-a[a,b]	antisense	5′-NH_2-GCCCATCTGCAAGCCTTTT-3′
Downstream-s[a]	sense	5′-NH_2-GTCGTCGCTGTTGAGTTTGA-3′
Downstream-a[a]	antisense	5′-NH_2-TCAAACTCAACAGCGACGAC-3′
Le1n02-3′-a(−8 mer)[b]	antisense	5′-NH_2-GCAAGCCTTTT-3′
Le1n02-3′-a(+8 mer)[b]	antisense	5′-NH_2-GAAGGCAAGCCCATCTGCAAGCCTTTT-3′
SSIIb3-3′[c]	antisense	5′-NH_2-GATCAGCTTTGGGTCCGGA-3′
RRS-5′short-s[d]	sense	5′-NH_2-CCTTTAGGATTTCAGCATCA-3′

[a]Capturing oligos for *Le1* used to investigate the optimal location to bind target DNA.
[b]Capturing oligos for *Le1* used to investigate the optimal length of the capturing oligo.
[c]Capturing oligo for the starch synthase IIb gene in maize (negative control oligo).
[d]Capturing oligo for *RRS*.

oligo sequence for *RRS* was selected from a PCR primer. The PCR primer sequence for the starch synthase IIb gene (SSIIb) described in the previous report was used as the negative control oligo.[6)]

Preparation of capturing oligo-immobilized PCR tubes. PCR tubes were treated with S-Bio® PrimeSurface® (Sumitomo Bakelite, Hyogo, Japan), providing a unique biocompatible phospholipid polymer and a highly active functional ester moiety to covalently bind the attachment site for amino-modified oligonucleotides under alkaline conditions.[5)] The 5′-amino-modified oligo solution (Nippon Gene) was prepared at 10 μM with an alkaline solution, and was added to the PCR tube. After incubating for 90 min, the oligo-immobilized PCR tube was treated with a 0.1 M sodium hydroxide solution to block the remaining functional ester moieties, and then washed with water. To investigate the effects of different amounts of the capturing oligo, 0.1 and 1 μM oligo solutions were also used.

Single-tube real-time PCR. Each sample (25 μl) was added to the oligo-immobilized PCR tube. After preheating at 95 °C for 5 min, the tube was incubated at 50 °C for 30 min. To investigate the effects of incubation temperature and time, incubation was also carried out at 25, 40, and 60 °C for 15, 60, and 120 min. The tube was washed three times with 200 μl of a washing buffer (10 mM Tris HCl (pH 8.0), 1 mM EDTA, and 0.2 M NaCl). A 25 μl volume of the reaction solution containing 11 μl of nuclease-free water, 12.5 μl of the FastStart Universal Probe Master (Roche, IN, USA), 0.5 μl of a 10 μM probe solution, and 0.5 μl each of a 25 μM primer solution was added to the washed tube. The PCR system for *Le1* detection is shown in Fig. 2. The probe and primer sets had previously been used for the quantification of GM soybean based on real-time PCR.[7)] The probe and primer set for *RRS* were purchased from Fasmac (Kanagawa, Japan). The real-time PCR assay was performed by using a 7300 Real-time PCR system (Applied Biosystems, CA, USA). The

thermal cycle program for all primers was as follows: 2 min at 50 °C, 10 min at 95 °C, and subsequent amplification of the DNA for 40 cycles of 30 s at 95 °C and 60 s at 59 °C. A standard curve was prepared from the GM Soybean (RRS) Detection Plasmid Set (Nippon Gene), and was run on each plate to validate the method and to determine the efficiency of the reaction which was taken into account in the final calculations. The standard curve for each gene was linear ($r > 0.99$).

Statistical analysis. Each data value is presented as the mean with standard deviation. A linear regression analysis of data for the correlation between Ct values and log of DNA or soybean lysate (%) was performed by the least-squares method. The real-time PCR efficiency was calculated by using the slope of the linear regression plot according to the equation $E = 10^{(-1/\text{slope})}$.[8] A PCR efficiency (E) of 2.00 corresponds to a doubling of the copy number per PCR cycle. Analyses were performed by using Sequence Detection Software version 1.4 (Applied Biosystems) and Excel 2000 (Microsoft, WA, USA).

Results and Discussion

DNA is generally isolated by using organic extraction and silica-membrane column extraction steps. However, these extraction steps are time-consuming and are difficult to automate and downscale to a small sample volume. The hybridization-bead method can resolve these problems. However, the hybridization-bead method is not directly used in real-time PCR, and the number of tube-to-tube transfers still needs to be minimized. We developed a novel real-time PCR method using hybridization as shown in Fig. 1. In this study, we first analyzed the reaction conditions, such as the capturing oligo design and hybridization conditions, by using soybean genomic DNA and lysate. We then used the method to detect GM soybean.

Capturing oligo

The design of capturing oligos is an essential factor in the specific recovery of target DNA in a hybridization system. We investigated the optimal location for target DNA, and the effects of its orientation, length and concentration when immobilized on PCR tubes. We chose capturing oligo sequences from the region between approximately 100-bp upstream and downstream of the PCR amplification region (Fig. 2). The capturing oligos were designed by using sequences from several regions and from both orientations (Table 1). Among all the capturing oligos, the highest copy number of *Le1*, 110 ± 16 copies, was detected by using Le1n02-3′-a (Table 2). Therefore, Le1n02-3′-a was used for all subsequent soybean DNA detection in this study. For comparison, DNA was extracted from soybean powder by using a silica membrane-type kit, and was directly assayed by a general real-time PCR method which was carried out with the PCR reagents and thermal cycle program described in the Materials and Methods section. The copy number of *Le1* in 100 ng of soybean DNA was 9068 ± 497 copies ($n = 4$). Accordingly, the recovery of soybean DNA by using an Le1n02-3′-a-immobilized tube was estimated to be 1.2%. *Le1* could not be detected by using an SSIIb3-3′-immobilized tube which was designed to capture maize DNA. These results suggest that the oligo orientation and secondary structure of target DNA are important factors for the selection of capturing oligos. Furthermore, this method could isolate target DNA by specific hybridization to the capturing oligo.

Table 2. Ct Values and Copy Numbers Measured for *Le1* from 100 ng of Soybean DNA Using Different Capturing Oligos

Capturing oligo	Ct value	Copy number
Upstream-s	34.2 ± 0.5	71 ± 23
Upstream-a	36.4 ± 0.5	15 ± 4
Le1n02-5′-s	33.9 ± 0.3	83 ± 14
Le1n02-5′-a	39.0 ± 0.4	3 ± 1
Le1-Taq-s	34.2 ± 0.2	69 ± 11
Le1-Taq-a	36.7 ± 1.0	13 ± 6
Le1n02-3′-s	36.8 ± 0.4	12 ± 3
Le1n02-3′-a	33.5 ± 0.2	110 ± 16
Downstream-s	36.6 ± 0.4	13 ± 4
Downstream-a	34.0 ± 0.2	76 ± 10
SSIIb3-3′	ND	ND
None	ND	ND

None, no oligo was immobilized on the tube.
Data are mean values ± SD ($n = 4$).
ND, not detected.

For a DNA microarray probe, the optimal oligonucleotide probe length in a hybridization system is typically 35- to 70-mer.[9] We examined the effect of oligonucleotide length for our method. Among the examined oligonucleotides, the 19-mer oligonucleotide gave the highest copy number of *Le1* (Le1n02-3′-a (−8 mer) [11 mer in length], 14 ± 4 copies; Le1n02-3′-a [19 mer in length], 157 ± 12 copies; Le1n02-3′-a (+8 mer) [27 mer in length], 32 ± 10 copies; $n = 4$). Consequently, this length was considered to be the most suitable. We also investigated the effect of different concentrations of the capturing oligo immobilized on the PCR tube. The copy number of *Le1* was correlated with Le1n02-3′-a concentrations from 0.1 to 10 μM (0.1 μM, 2 ± 1 copies; 1 μM, 12 ± 4 copies; 10 μM, 101 ± 28 copies; $n = 4$). Thus, 10 μM was considered to be the optimal oligo concentration for immobilizing on the PCR tube.

Incubation temperature and time

Temperature is an important factor for hybridization, and various temperatures from 42 to 65 °C are used with the hybridization bead methods.[1,10–12] We tested an incubation temperature from 25 to 60 °C. The copy number of *Le1* in an Le1n02-3′-a-immobilized tube was successfully detected at 40 to 65 °C, especially at 50 °C, but detection at 25 °C was poor (25 °C, 40 ± 5 copies;

40 °C, 115 ± 35 copies; 50 °C, 169 ± 43 copies; 60 °C, 104 ± 22 copies; $n = 4$). This result suggested 50 °C to be the optimum incubation temperature with this method.

We also investigated the effect of incubation time on the detection of *Le1*. The copy number of *Le1* in an Le1n02-3′-a-immobilized tube appeared to be correlated with the incubation time; however, there was little difference in the detected copy number among incubations ranging from 15 to 120 min (15 min, 111 ± 35 copies; 30 min, 119 ± 16 copies; 60 min, 151 ± 15 copies; 120 min, 185 ± 33 copies; $n = 4$). In previous reports, the hybridization times were 2 h for a one-step system and 4 h for a two-step system.[1,10] Our data show that this method can be carried out with a relatively short hybridization time. We subsequently used 30-min hybridization in this study.

Effect of food matrix on the determination of Le1

To investigate the food matrix effects, we detected *Le1* from 100 ng of soybean DNA in 50 mg/ml of wheat and corn lysates. The copy number of *Le1* in an Le1n02-3′-a-immobilized tube was not significantly affected by the presence of wheat and corn lysates (lysis buffer only, 74 ± 18 copies; corn lysate, 85 ± 35 copies; wheat lysate, 106 ± 25 copies; $n = 4$). This result indicates that the method can detect soybean DNA from a food lysate containing other food product; in this case, a wheat or corn extract.

Concentration-dependent detection

We investigated whether the copy number of *Le1* was detected from soybean DNA and lysate in a concentration-dependent manner. The copy number of *Le1* in the Le1n02-3′-a-immobilized tube was linearly correlated with the soybean DNA and lysate amounts (Table 3). A strong correlation was appeared between the log values for the soybean DNA and lysate amounts and the Ct values for *Le1* [DNA, $Ct = -3.41 \times \log(\text{soybean DNA ng}) + 39.19$, $r = 0.99$; lysate, $Ct = -3.14 \times \log(\text{soybean lysate (\%)}) + 35.19$, $r = 0.99$]. The detection limits were 10 ng of soybean DNA and 1% soybean lysate (data not shown). The PCR efficiency (E), where 2.00 corresponds to a doubling of the copy number per PCR cycle, was 1.96 in soybean DNA and 2.08 in the lysate sample. The PCR efficiency (E) in soybean DNA samples has reportedly ranged from 1.80 to 2.10 for various DNA extraction methods.[13] These results indicate that the proposed method allows concentration-dependent detection of soybean genes in both DNA and crude samples without inhibiting the PCR efficiency.

Effect of heat treating a sample on the determination of Le1

The DNA extracted from heat-treated soybean by using the silica membrane kit was degraded into small fragments, and a lower copy number of *Le1* was detected in the degraded DNA than that from the intact DNA.[6] To test the effect of heat treating a sample on the determination of *Le1*, we heated the soybean sample for various times. The copy number of *Le1* in the Le1n02-3′-a-immobilized tube was detected in all samples (0 min, 3578 ± 527 copies; 30 min, 15967 ± 800 copies; 60 min, 8011 ± 783 copies; $n = 4$). DNA microarray analyses are affected by the secondary structures and long nucleic acid molecules which reduce the hybridization efficiency.[14] The difference in copy number of *Le1* in the Le1n02-3′-a-immobilized tubes between the heat-treated samples is likely to have been due to differences in the secondary structures and lengths of degraded DNA. Our data suggest that this method can easily detect the copy number of *Le1* in a heat-treated soybean sample.

Table 3. Soybean DNA and Lysate Concentration Effects on Ct Values and Copy Numbers Measured for *Le1* by Using Le1n02-3′-a-Immobilized Tubes

Sample amount	Ct value	Copy number
Soybean DNA		
10 ng	35.8 ± 0.4	17 ± 5
100 ng	32.3 ± 0.2	174 ± 28
1000 ng	29.0 ± 0.4	1677 ± 493
Soybean lysate		
1%	35.7 ± 0.2	22 ± 3
10%	32.1 ± 0.2	285 ± 51
100%	28.7 ± 0.1	3089 ± 206

Soybean lysate (50 mg/ml) is defined as 100%.
Data are mean values ± SD ($n = 4$).

Table 4. GM Soybean Lysate Concentration Effects on Ct Values and Copy Numbers Measured for *RRS* by Using RRS-5′Short-s Immobilized Tubes

Sample amount	Ct value	Copy number
1%	36.5 ± 0.6	17 ± 7
10%	33.4 ± 0.3	143 ± 33
100%	29.5 ± 0.2	2111 ± 229

GM soybean lysate (50 mg/ml) is defined as 100%.
Data are mean values ± SD ($n = 4$).

GM soybean detection

To apply this method to detect GM soybean, an RRS-5′short-s oligo based on the *RRS* PCR primer was immobilized on the PCR tube. The copy number of *RRS* in the RRS-5′short-s immobilized tube was correlated with the GM soybean lysate amount in a concentration-dependent manner (Table 4). The log value for the GM soybean lysate amount was correlated with the Ct value for *RRS* [$Ct = -3.54 \times \log(\%\text{ GM soybean lysate}) + 36.66$, $r = 0.99$]. The PCR efficiency (E) was 1.92. Certified reference materials are frequently used as calibrators for GMO quantification by real-time PCR. The copy number of *RRS* in the RRS-5′short-s immobilized tube was also detected in the 50 mg/ml lysates of certified reference materials containing 1%, 2% and 5%

Roundup Ready soybean (1%, 17 ± 6 copies; 2%, 30 ± 9 copies; 5%, 63 ± 16 copies; $n = 4$). These results indicate that the method can detect specific genes in Roundup Ready soybean by using a corresponding capture oligo.

Conclusion

The real-time PCR method using capturing oligo-immobilized PCR tubes described in this study could detect soybean and GM soybean genomic DNA in samples comprising 1–100% soybean and GM soybean. In addition, this method could also detect GM soybean genomic DNA in certified reference materials containing 1, 2 and 5% Roundup Ready soybean. Many countries and areas have specified GMO labeling regulations to protect consumers' rights; labeling thresholds for GMOs are 3% in Korea and 5% in Japan.[15,16] The method described in this study can detect these threshold levels of GM soybean.

We have described in this study the development and validation of a novel real-time PCR method using capturing oligo-immobilized PCR tubes. This method is simple and specific, and can detect the copy number of target DNA from crude food matrices in a concentration-dependent manner. This method would be useful in many types of food analysis.

Acknowledgments

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