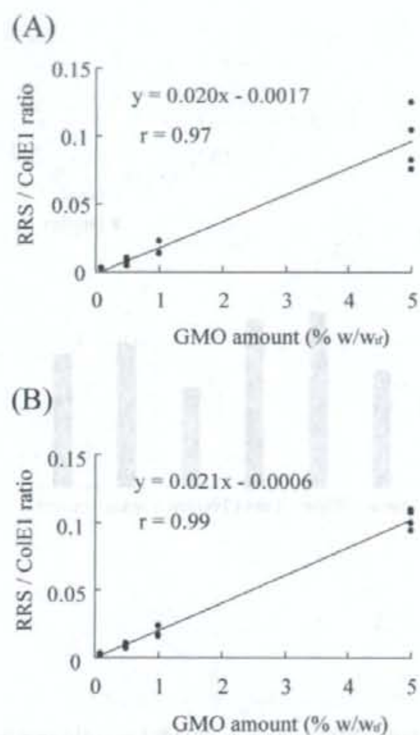

Figure 7. Correlations between GMO amounts (GMO weight per weight of total food (w/w_{tf})) and RRS/ColE1 ratio in simplex (A) and duplex (B) real-time PCR assays. Lines represent the least squares regression lines.

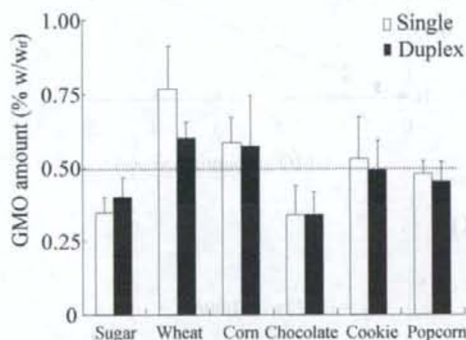

Figure 8. Comparison of calculated GMO amounts (GMO weight per weight of total food (w/w_{tf})) between simplex and duplex real-time PCR assays. Food samples were spiked with 5% w/w GM soybean powder consisting of GMO and non-GMO (0.5% w/w_{tf} GMO). Horizontal line represents expected GMO amounts (w/w_{tf}) in the samples. Data are means ± SD ($n = 4$).

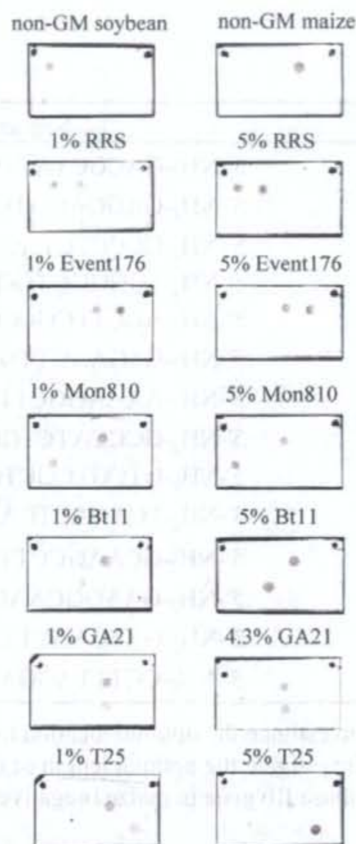

Figure 9. Detection of GMO genes using multiplex (8-plex) PCR coupled with primer extension. (A) Layout of the extension primer-immobilized plastic plate. The sequences of the primer are given in Table 1. (B) Image of the primer extension reaction of non-GM soybean, GM soybean (Roundup Ready soybean), non-GM maize and GM maize (Event176, Mon810, Bt11, GA21 and T25). The GMO contents of the samples are 1 and 5%, except for 4.3% GA21. In the case of T25, the percentages indicate the percentages of GM DNA relative to non-GM DNA. The positive signals appear as gray spots. The dots in upper corners denote the position of the plate.

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*.

Table 2. List of forward and reverse primers used for multiplex (8-plex) PCR and extension primers used for an extension reaction on a plastic plate

Target	Orientation	Sequence (5'-3')	Reference
Le1	Forward	GCCCTCTACTCCACCCCCA	9
	Reverse	GCCCATCTGCAAGCCTTTTT	9
	Extension[a]	AGCTTCGCCGCTTCCTTCAACTTCAC	9
RRS	Forward	CCTTTAGGATTTCAGCATCAGTGG	9
	Reverse	GACTTGTCGCCGGAAATG	9
	Extension[a]	CGCAACCGCCCGCAAATCC	9
SSIIb	Forward	CTCCCAATCCTTTGACATCTGC	9
	Reverse	TCGATTTCTCTCTTGGTGACAGG	9
	Extension[a]	GCAATGCAAAACGCAACGAGTGGGGG	This study
Event176	Forward	TGTTCACCAGCAGCAACCAG	9
	Reverse	ACTCCACTTTGTGCAGAACAGATCT	9
	Extension[a]	TCGATGTGGTAGTCGGTCACGTCGG	9[b]
Mon810	Forward	GATGCCTTCTCCCTAGTGTTGA	9
	Reverse	GGATGCACTCGTTGATGTTTG	9
	Extension[a]	TTGTTGTCCATGGCCGCTTGGTATCT	9[b]
Bt11	Forward	ACATTTAATACGCGATAGAAAAC	5
	Reverse	ACACCTACAGATTTTAGACCAAG	5
	Extension[a]	TATGTTACTAGATCTGGGCCTCGTG	5
GA21	Forward	GAAGCCTCGGCAACGTCA	9
	Reverse	ATCCGGTTGGAAAGCGACTT	9
	Extension[a]	CGGCCATGCACCGGATCCTT	9[b]
T25	Forward	GCCAGTTAGGCCAGTTACCCA	9
	Reverse	TGAGCGAAACCCTATAAGAACCCT	9
	Extension[a]	TGCAGGCATGCCCGCTGAAATC	9

[a] Modification with amino linkers at the 5' end due to immobilization of the extension primer on a plastic plate.

[b] Use of complement sequence of the reference.

Table 3. DNA yield from certified reference materials (CRMs)

GMO amount (%)	DNA quantity (ng)	
	Extraction from Sample	Usage for PCR
0	907 ± 81	11.3 ± 1.0
0.1	1063 ± 112	13.3 ± 1.4
0.5	1093 ± 101	13.7 ± 1.3
1	1202 ± 92	15.0 ± 1.2
5	1246 ± 233	15.6 ± 2.9

Values are means ± SD (n = 4).
Weight per weight of GMO in mixtures of GMO and non-GMO is expressed as w/w.
GMO weight per weight of total food is expressed as w/w_{tf}.

Table 4. Calculations of copy number ratios of RRS to Le1 or ColE1 from real-time PCR assay results. DNA was extracted from certified reference materials (CRMs)

GMO amount (%)	Copy number			Copy number ratio	
	RRS	Le1	ColE1	RRS/Le1	RRS/ColE1
0		22010 ±2090	12533 ± 940		
0.1	26 ± 6	25999 ±2725	12623 ± 724	0.0010 ± 0.0001	0.0020 ± 0.0006
0.5	107 ± 24	27480 ±3107	14013 ± 635	0.0039 ± 0.0005	0.0076 ± 0.0015
1	227 ± 42	29043 ±2932	14682 ± 805	0.0078 ± 0.0008	0.0156 ± 0.0033
5	1102 ± 264	28530 ±7546	13894 ± 710	0.0392 ± 0.0073	0.0792 ± 0.0180

Values are means ± SD (n = 4).
Weight per weight of GMO in mixtures of GMO and non-GMO is expressed as w/w.
GMO weight per weight of total food is expressed as w/w_{tf}.

Table 5. DNA yield from food samples spiked with 5% w/w GM soybean powder (0.5% w/w_{tf} GMO)

Sample	DNA quantity (ng)	
	Extraction from Sample	Usage for PCR
Sugar	57 ± 19	0.7 ± 0.2
Wheat	862 ± 28	10.8 ± 0.4
Corn	140 ± 11	1.8 ± 0.1
Chocolate	68 ± 11	0.8 ± 0.13
Cookie	144 ± 21	1.8 ± 0.26
Popcorn	135 ± 18	1.7 ± 0.23

Values are means ± SD (n = 4).
Weight per weight of GMO in mixtures of GMO and non-GMO is expressed as w/w.
GMO weight per weight of total food is expressed as w/w_{tf}.

Table 6. Calculations of copy number ratios of RRS to Le1 or ColE1 from real-time PCR assay results. DNA was extracted from food samples spiked with 5% w/w GM soybean powder (0.5% w/w_{tf} GMO)

Sample	Copy number			Copy number ratio	
	RRS	Le1	ColE1	RRS/Le1	RRS/ColE1
Sugar	49 ± 14	1344 ± 225	6593 ± 1938	0.0364 ± 0.0062	0.0076 ± 0.0012
Wheat	97 ± 17	2875 ± 156	11020 ± 1121	0.0336 ± 0.0049	0.0088 ± 0.0015
Corn	117 ± 9	2976 ± 260	11665 ± 261	0.0394 ± 0.0037	0.0100 ± 0.0008
Chocolate	68 ± 14	2011 ± 290	12487 ± 556	0.0337 ± 0.0027	0.0055 ± 0.0012
Cookie	87 ± 6	3139 ± 660	11951 ± 1166	0.0284 ± 0.0061	0.0073 ± 0.0010
Popcorn	89 ± 22	3454 ± 323	12749 ± 502	0.0257 ± 0.0058	0.0069 ± 0.0016

Values are means ± SD (n = 4).
Weight per weight of GMO in mixtures of GMO and non-GMO is expressed as w/w.
GMO weight per weight of total food is expressed as w/w_{tf}.

Table 7. 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

Table 8. Ct values and copy numbers measured for *Le1* from 100 ng of soybean DNA using capture oligos with different lengths and concentrations

Capture oligo	Ct value	Copy number
Oligo length		
Le1n02-3'-a (-8 mer)	36.8 ± 0.5	14 ± 4
Le1n02-3'-a	33.1 ± 0.1	157 ± 12
Le1n02-3'-a (+8 mer)	35.6 ± 0.5	32 ± 10
Oligo concentration		
0.1 uM Le1n02-3'-a	39.1 ± 1.0	2 ± 1
1.0 uM Le1n02-3'-a	35.9 ± 0.5	12 ± 4
10 uM Le1n02-3'-a	32.8 ± 0.4	101 ± 28

Data are mean values ± SD (n=4).

Table 9. Effects of different incubation temperatures and times on Ct values and copy numbers measured for *Le1* from 100 ng soybean DNA using Le1n02-3'-a-immobilized tubes

Hybridization conditions	Ct value	Copy number
Temperature (°C)		
25	35.1 ± 0.2	40 ± 5
40	33.5 ± 0.4	115 ± 35
50	32.9 ± 0.4	169 ± 43
60	33.6 ± 0.3	104 ± 22
Time (min)		
15	33.7 ± 0.5	111 ± 35
30	33.6 ± 0.2	119 ± 16
60	33.2 ± 0.2	151 ± 15
120	32.9 ± 0.3	185 ± 33

Data are mean values ± SD (n=4).

Table 10. 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 11. Effect of sample matrices on Ct values and copy numbers measured for *Le1* from 100 ng soybean DNA using Le1n02-3'-a-immobilized tubes

Sample matrix	Ct value	Copy number
Binding buffer	33.4 ± 0.3	74 ± 18
Corn extract	33.3 ± 0.7	85 ± 35
Wheat extract	32.9 ± 0.4	106 ± 25

Data are mean values ± SD (n=4).

Table 12. Effect of heat treatment on Ct values and copy numbers measured for *Le1* from soybean samples using Le1n02-3'-a-immobilized tubes

Heating time	Ct value	Copy number
0 min	28.3 ± 0.2	3578 ± 527
30 min	26.0 ± 0.1	15967 ± 800
60 min	27.0 ± 0.1	8011 ± 783

Data are mean values ± SD (n=4).

Table 13. 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).

Table 14. Ct values and copy numbers measured for *RRS* from certified reference material by using RRS-5'short-s immobilized tubes

GM amount	Ct value	Copy number
1%	36.7 ± 0.7	17 ± 6
2%	35.9 ± 0.5	30 ± 9
5%	34.7 ± 0.3	63 ± 16

Data are mean values ± SD (n=4).

Ⅱ．研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Harikai N, Saito S, Abe M, Kondo K, Kitta K, Akiyama H, Teshima R, Kinoshita K.	A real-time PCR method using capture oligo-immobilized PCR tubes to determine the specificgene for soybean and genetically modified soybean in food matrices.	Bioscience, Biotechnology, and Biochemistry	72(11)	2953-2958	2008
Tanaka A, Harikai N, Saito S, Yakabe T, Funaoka S, Yokoyama K, Fujiwara K, Iwao-Koizumi K, Murata S, Kinoshita K.	All-in-one tube method for quantitative gene expression analysis in oligo-dT30 immobilized PCR tube coated with MPC polymer.	Analytical Sciences	25(1)	109-114	2009

Ⅲ．研究成果の刊行物・印刷物

次のページ以降に添付

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JSBA

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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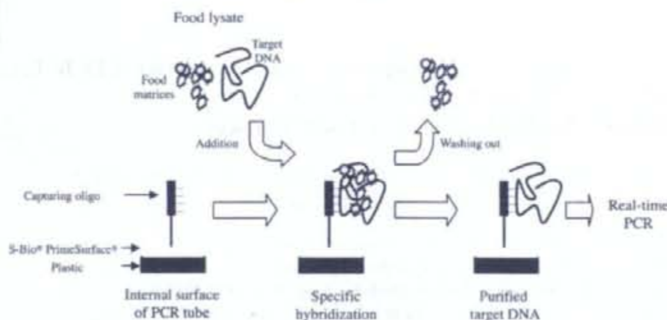

Fig. 1. Process for a Real-Time PCR Method Using a Capturing Oligo-Immobilized PCR Tube.
A capturing oligo is immobilized on the S-Bio® PrimeSurface®-treated PCR tube. Target DNA in a food lysate is hybridized to the capturing oligo on the surface of the PCR tube. Food matrices are removed by washing.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