**Table 5.** 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 6.** 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 7.** 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 8.** 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 9.** 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).

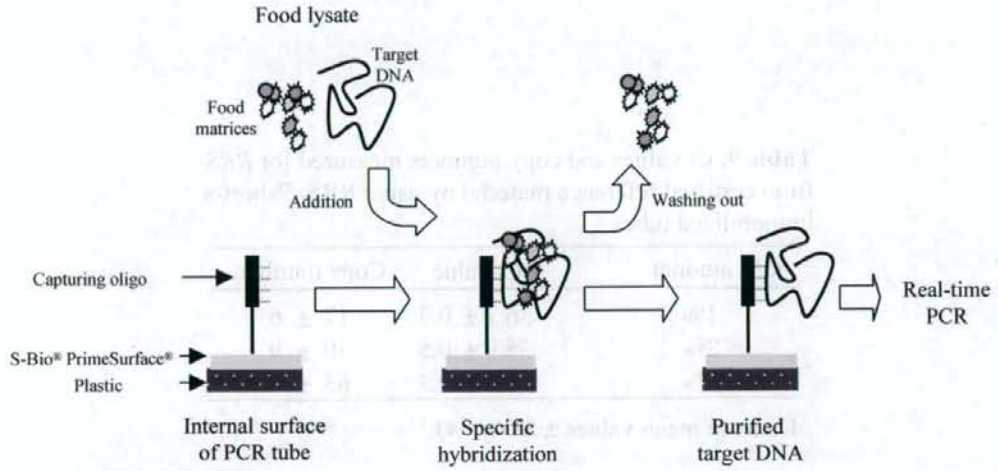


**Figure 1.** Process for a real-time PCR method using capture oligo-immobilized PCR tube. A capture oligo is immobilized on the S-Bio® PrimeSurface®-treated PCR tube. Target DNA in food lysate hybridizes to the capture oligo on the surface of PCR tube. Food matrices are removed by washing. Purified target DNA in the PCR tube is directly used for real-time PCR.

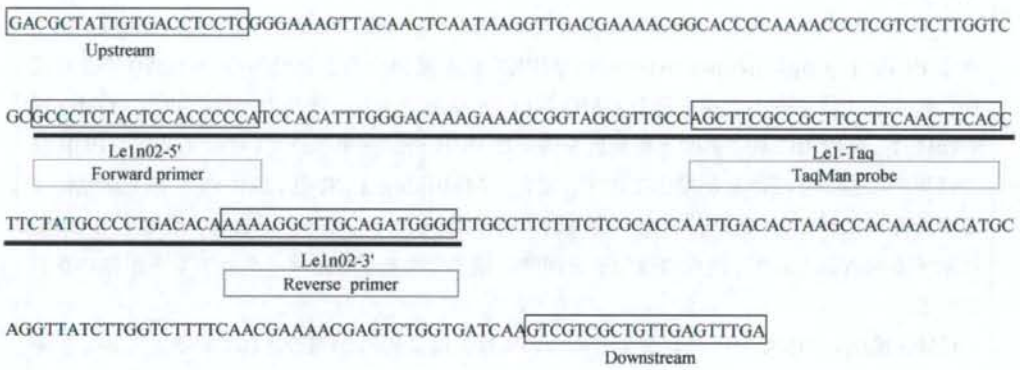


**Figure 2.** Position of capture oligos, PCR primers, and probe in *Le1*. DNA sequences of capture oligos for *Le1* are indicated by open boxes. We used DNA sequences of Le1n02-5' and Le1n02-3' and Le1-Taq as primers, and TaqMan probe for real-time PCR for *Le1*. Amplified region is shown with a bold line.

厚生労働科学研究費補助金（食品の安心・安全確保推進研究事業）

Ⅱ．分担研究報告書

# 2. PCR - multiple primer extension (MPEX) 法：遺伝子組換えトウモロコシと遺伝子組換え大豆に対するプラスチック基板上でのプライマー伸長反応を使用した複数遺伝子の同時検出法

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**研究要旨**

本研究の目的は、複数の遺伝子組換え食品(GMO)を可視にて簡便で短時間に同時検出できるPCR - multiple primer extension (MPEX)法を開発することである。平成20年2月現在、我が国では審査の手続を経たGMO数は88品種に上り、増加の一途を辿る。GMO検査法には、一般的に高い感度と特異性を備えたPCR法が使用されている。しかし、PCR法では単一の遺伝子だけしか検出できず、また、Multiplex PCR法においても電気泳動による分離能の限界から同時検出できる遺伝子の数は限られてしまう。そこで、増加するGMOに対する検査法として、複数の遺伝子を同時に検出できるDNAマイクロアレーが期待されている。

GMO検出のDNAマイクロアレーについては、既に幾つか報告されている。しかし、それらの中には、蛍光標識を使うため高価なレーザースキャナを必要とするものもある。また、すべての報告において、検出工程で1-18時間を要するハイブリダイゼーションが必要である。一方、検出工程で伸長反応を使用した方法は、MPEX法やarrayed primer extension法と呼ばれ、一塩基多型や変異を簡便で短時間に検出できる方法として使用されている。また、伸長反応時に標識核酸を取り込ませることで発色反応による検出も可能である。

本研究では、Multiplex PCR法による対象遺伝子の増幅とMPEX法を組み合わせたPCR-MPEX法をGMO検出に応用した。まず、トウモロコシと大豆の種特異的遺伝子の配列とGMOの構造特異的配列(Event176、Mon810、Bt11、GA21、T25トウモロコシ、ラウンドアップレディー大豆)に対応する8つのオリゴをプラスチックプレートに固定した。食品から抽出したDNAをMultiplex PCRしてこの8種類の遺伝子を増幅し、そのPCR産物をプレートに添加した。DNAポリメラーゼにてプレート上で伸長反応を行い、その際にビオチン11-dUTPを取り込ませた。そのビオチンをアビジン-ビオチン複合体とアルカリフォスファターゼの反応を利用して発色させた。この方法によって、遺伝子組換え(GM)トウモロコシ5品目とGM大豆1品目に対して各々1%のGMO含有サンプルまで可視で判定することができた。

今回の PCR-MPEX 法では、従来の DNA マイクロアレーのハイブリダイゼーションに相当する伸長反応の工程は 3 分であり、短時間での複数 GMO の可視での同時検出が可能となった。

## A. 研究目的

遺伝子組換え食品(GMO)は、食品の生産を量的及び質的に向上させ、食糧の安定供給に貢献しているが、一方で、食の安全、環境リスク、倫理上の問題が挙げられている。GMO が増加の一途をたどる中、多くの GMO を同時に検査できる方法が求められている。しかし、GMO 検査の代表的な方法である PCR 法では単一の遺伝子しか検出できない。また、Multiplex PCR 法にしても電気泳動での分離能の限界から、同時検出できる遺伝子の数に限りがある。

DNA マイクロアレーは、複数の遺伝子を同時検出するのに優れた方法である。実際に、GMO 検出に応用した例も報告されている[1-6]。しかし、それらのマイクロアレー法では、ラベル化した対象遺伝子をハイブリダイゼーションして検出するため、1-18 時間の長時間を要している。また、幾つかの方法では、高価なレーザースキャナが必要である。一方、ラベル化を含めた検出工程を伸長反応で行う multiple primer extension (MPEX)法や arrayed primer extension 法は、主に遺伝子多型や変異の検出に使われており、比較的短時間での検出が可能である。また、この方法は、発色反応での検出が可能で、近年では菌の検出にも応用されている[7]。

そこで、Multiplex PCR で対象遺伝子を増幅し、MPEX で検出する PCR-MPEX 法を GMO 検出に応用した(Fig. 1)。本研究では、遺伝子組換え(GM)トウモロコシ 5 品目と GM 大豆 1 品目を検査するための 8 配列の可視による検出を検討した。

## B. 研究方法

### B-1. サンプル

非組換え大豆とトウモロコシは兵庫県内のスーパーマーケットで購入し、AM-3 ホモジナイザー(Nihon Seiki Seisakusho)で粉末にした。GM 大豆標品(1、5%w/w ラウンドアップレディー大豆)と GM トウモロコシ標品(1、5%w/w Event176、Mon810、Bt11 と 1、4.3%w/w GA21)は、Fluka から購入した。一方、T25 の DNA 溶液は Generon から購入した。その DNA 溶液と非組換えトウモロコシ DNA 溶液を混ぜ合わせて、T25 の DNA の割合が 1 と 5%になるように調整した。

### B-2. DNA 抽出

粉末サンプルからの DNA 抽出は、DNeasy Plant Mini kit (Qiagen)を使用した。DNA 濃度は OD 260 nm あたり 50 μg/mL として算出した。

### B-3. Multiplex PCR

Multiplex PCR 反応液 25 μL は、QuantiTect Multiplex PCR Master Mix

(Qiagen) 12.5 μL、0.05 μM SSII、Event176、Bt11 プライマー、 0.1 μM Le1、RRS、Mon810、T25 プライマー、0.5 μM GA21 プライマー、DNA サンプル 100 ng、Nuclease Free 水で調整した。各プライマーは Table 1 に示したものを使用した。GeneAmp PCR System 9700 (Applied Biosystems)を使用して、50℃2 分と 95℃15 分のインキュベーション後、94℃60 秒と 61℃60 秒の 35 サイクルで反応した。

B-4. プラスチックプレートへのオリゴ固定

プレートに固定したオリゴは Table 1 に示したものを使用した。これらの配列は主に定量 PCR のプローブ配列を利用したものであった。また、SSII は報告の配列から微調整したものを使用し、その配列の特異性は BLAST 検索で確認した。各オリゴの 5'末端にアミノリンカー修飾を施したものを Nippon EGT で合成した。

オリゴ固定プレートの作製では、S-Bio® PrimeSurface®処理プラスチックプレート (Sumitomo Bakelite) に、オリゴ固定溶液で調整した 10 μM 5'アミノリンカー修飾オリゴ溶液を 1 μL スポットして、室温で 3 時間放置した。プレートを 0.1 N NaOH 溶液に浸して反応を停止した。更に、プレートを沸騰した水に浸して余分なオリゴを除去し、Nuclease Free 水で再度洗浄した後、プレートを乾かした。

B-5. 伸長反応

伸長反応液 50 μL は、5U TERMIPol DNA polymerase (Solis Biodyne)、1 × 緩衝液 C、2 mM $MgCl_2$、50 μg/mL salmon sperm DNA、0.05% triton-X 100、100 μM dATP、100 μM dGTP、100 μM dCTP、65 μM dTTP、35 μM biotin-11-dUTP、PCR 産物 5 μL、Nuclease Free 水で調整した。95℃で 5 分間加熱後、72℃に温めておいたオリゴ固定プレートに添加し、その溶液の上にカバーを乗せて 72℃3 分間反応した。

B-6. 発色反応

伸長反応後のプレートを洗浄液 1 (0.1% triton X-100 - 50 mM tris buffer (pH 7.5)) と洗浄液 2 (50 mM tris buffer (pH 7.5))で洗浄した。VectaStain ABC-AP kit (Vector Laboratories)の A 液と B 液を 2% BSA - 0.01% salmon sperm DNA を含む 50 mM tris buffer (pH 7.5)で 200 倍希釈して 20 分間室温で反応したものをプレートに添加し、37℃20 分間反応した。プレートを洗浄液 1 と洗浄液 2 で洗浄し、NBT/BCIP 溶液 (Roche)を添加して、37°C10 分間反応した。プレートを精製水で洗浄後、乾かして発色したスポットを確認した。

C. 結果・考察

PCR-MPEX 法は、Multiplex PCR による増幅と MPEX による検出の 2 つの工程に分かれる。Multiplex PCR では、各プライマー濃度や PCR 反応のサイク数を調整することで、各遺伝子の増幅効率の偏りや非特異的増幅を減らした。また、MPEX では、伸長反応に使用するオリゴの配列や反応温度を調整することで、交差反応や自己伸張反応による非特異的な発色を抑制した。

最適化された条件で検討したところ、非

組換えトウモロコシと非組換え大豆のDNAでは、それぞれ種特異的遺伝子のSSIIbとLe1のスポットのみに発色が認められた(Fig. 2)。これらは、このマイクロアレーにおけるポジティブコントロールになると考えられた。更に、1%と5%GMを含むGMトウモロコシ及びGM大豆標品から抽出したDNA(GA21は1、4.3%w/w。T25はDNA比率として1%と5%。)では、各対応するスポットで発色が認められた。また、5種類のGMトウモロコシのDNAを各1%含むサンプルでも、対応する5カ所のスポットで発色が認められた(data not shown)。これらのことから、伸長反応を利用したDNAマイクロアレーによって、簡便に短時間で複数のGMO遺伝子を可視で検出できることが示唆された。

## D. 結論

本研究では、トウモロコシと大豆の種特異的遺伝子の配列、GMOの構造特異的配列(Event176、Mon810、Bt11、GA21、T25トウモロコシ、ラウンドアップレディー大豆)の合計8配列を可視で検出するPCR-MPEX法を開発した。この方法によって、GMトウモロコシ5品目とGM大豆1品目を1%まで検出できた。また、従来のDNAマイクロアレーではハイブリダイゼーション工程に1-18時間を要するが[1-6]、この方法ではそれに相当する伸張反応工程は3分であった。このように、PCR-MPEX法は短時間でGMOの可視による同時検出を可能にした。今後、感度や定量性に関して更なる改良が必要と考えられる。

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F. 研究発表

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G. 知的財産権の出願・登録状況（予定を含む）

特になし

**Table 1.** 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 | 8 |
| | Reverse | GCCCATCTGCAAGCCTTTTT | 8 |
| | Extension[a] | AGCTTCGCCGCTTCCTTCAACTTCAC | 8 |
| RRS | Forward | CCTTTAGGATTTCAGCATCAGTGG | 8 |
| | Reverse | GACTTGTCGCCGGAAATG | 8 |
| | Extension[a] | CGCAACCGCCCGCAAATCC | 8 |
| SSIIb | Forward | CTCCCAATCCTTTGACATCTGC | 8 |
| | Reverse | TCGATTTCTCTCTTGGTGACAGG | 8 |
| | Extension[a] | GCAATGCAAAACGCAACGAGTGGGGG | This study |
| Event176 | Forward | TGTTCACCAGCAGCAACCAG | 8 |
| | Reverse | ACTCCACTTTGTGCAGAACAGATCT | 8 |
| | Extension[a] | TCGATGTGGTAGTCGGTCACGTCGG | 8[b] |
| Mon810 | Forward | GATGCCTTCTCCCTAGTGTTGA | 8 |
| | Reverse | GGATGCACTCGTTGATGTTTG | 8 |
| | Extension[a] | TTGTTGTCCATGGCCGCTTGGTATCT | 8[b] |
| Bt11 | Forward | ACATTTAATACGCGATAGAAAAC | 4 |
| | Reverse | ACACCTACAGATTTTAGACCAAG | 4 |
| | Extension[a] | TATGTTACTAGATCTGGGCCTCGTG | 4 |
| GA21 | Forward | GAAGCCTCGGCAACGTCA | 8 |
| | Reverse | ATCCGGTTGGAAAGCGACTT | 8 |
| | Extension[a] | CGGCCATGCACCGGATCCTT | 8[b] |
| T25 | Forward | GCCAGTTAGGCCAGTTACCCA | 8 |
| | Reverse | TGAGCGAAACCCTATAAGAACCCT | 8 |
| | Extension[a] | TGCAGGCATGCCCGCTGAAATC | 8 |

[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.

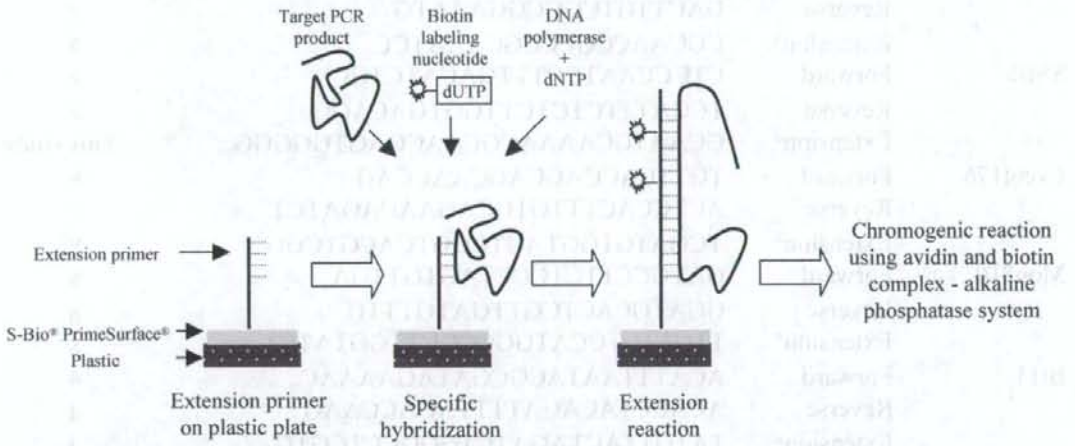


**Figure 1.** Process for primer extension on a plastic plate. An extension primer is immobilized on the S-Bio® PrimeSurface®-treated plastic plate. Target PCR product amplified by multiplex PCR hybridizes to the primer. Extension and incorporation of biotin-dUTP are carried out by DNA polymerase. The extension reaction is optically detected by an avidin and biotin complex - alkaline phosphatase system.

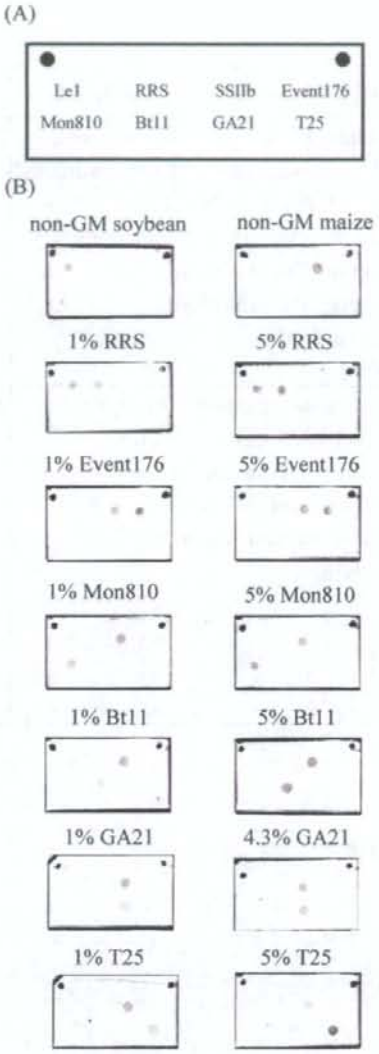


**Figure 2.** Detection of GMO genes using multiplex (8-plex) PCR coupled with primer extension. (A) Layout of the extension primer-immobilized plastic plate. The sequences of the primer are given in Table 1. (B) Image of the primer extension reaction of non-GM soybean, GM soybean (Roundup Ready soybean), 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.

## Ⅲ. 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 |
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## Ⅳ. 研究成果の刊行物・印刷物

次のページ以降に添付

Biosci. Biotechnol. Biochem., 72 (11), 2953–2958, 2008

# 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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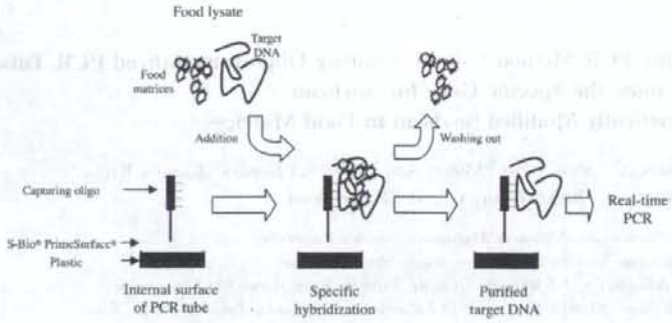


**Fig. 1.** Process for a Real-Time PCR Method Using a Capturing Oligo-Immobilized PCR Tube.
A capturing oligo is immobilized on the S-Bio® PrimeSurface®-treated PCR tube. Target DNA in a food lysate is hybridized to the capturing oligo on the surface of the PCR tube. Food matrices are removed by washing.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

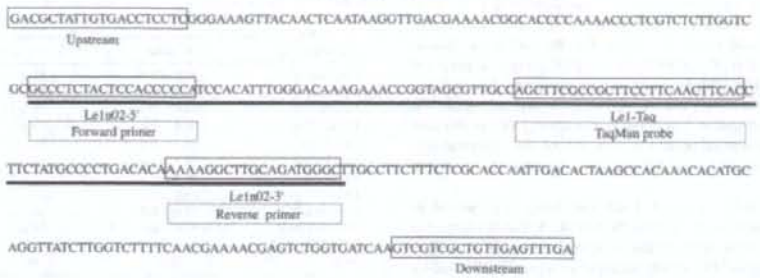


**Fig. 2.** Positions of the Capturing Oligos, PCR Primers, and Probe in *Le1*.
DNA sequences of the capturing oligos for *Le1* are indicated by open boxes. We used the DNA sequences of Le1n02-5' and Le1n02-3' as primers and Le1-Taq as the TaqMan probe for real-time PCR for *Le1*. The amplified region 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-a[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*, $110 \pm 16$ 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 $9068 \pm 497$ 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 | $34.2 \pm 0.5$ | $71 \pm 23$ |
| Upstream-a | $36.4 \pm 0.5$ | $15 \pm 4$ |
| Le1n02-5′-s | $33.9 \pm 0.3$ | $83 \pm 14$ |
| Le1n02-5′-a | $39.0 \pm 0.4$ | $3 \pm 1$ |
| Le1-Taq-s | $34.2 \pm 0.2$ | $69 \pm 11$ |
| Le1-Taq-a | $36.7 \pm 1.0$ | $13 \pm 6$ |
| Le1n02-3′-s | $36.8 \pm 0.4$ | $12 \pm 3$ |
| Le1n02-3′-a | $33.5 \pm 0.2$ | $110 \pm 16$ |
| Downstream-s | $36.6 \pm 0.4$ | $13 \pm 4$ |
| Downstream-a | $34.0 \pm 0.2$ | $76 \pm 10$ |
| 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], $14 \pm 4$ copies; Le1n02-3′-a [19 mer in length], $157 \pm 12$ copies; Le1n02-3′-a (+8 mer) [27 mer in length], $32 \pm 10$ 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, $2 \pm 1$ copies; 1 μM, $12 \pm 4$ copies; 10 μM, $101 \pm 28$ 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, $40 \pm 5$ copies;