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Detection of SNPs in Fish DNA: Application of the Fluorogenic Ribonuclease Protection (FRIP) Assay for the Authentication of Food Contents

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The fluorogenic ribonuclease protection (FRIP) assay was used to detect single nucleotide polymorphisms (SNPs) in commercially produced fish products. By using fluorescence resonance energy transfer (FRET) between fluorophore and quencher labeled probes, the species-specific cleavage of sample RNA was detected by measuring the fluorescence intensity during the FRIP assay. We were able to discriminate raw and thermally processed eel and tuna species using the FRIP-based SNP detection method. Furthermore, the intensity of fluorescence was correlated with the mutant/wild-type ratio. These results suggest that the FRIP assay is a useful method for the in situ confirmation of labels of fishery foods during food production.

KEYWORDS: Fluorogenic ribonuclease protection (FRIP) assay; single nucleotide polymorphism (SNP); fluorescence resonance energy transfer (FRET); authentication

INTRODUCTION

The attitude of consumers toward the health and safety of food products has led to strict regulations regarding the labeling and traceability of foods in some countries (*1, 2*). For the labeling, information regarding the species, the presence of genetically modified organisms, and the expiration date is required, and contamination of more than 5% of other species should be labeled in Japan (*3*). It is vital that food is correctly labeled to ensure that the consumer is correctly informed about the quality and quantity of the ingredients. However, closely related food species often appear very similar, which can result in misidentification. In addition, species discrimination in processed foods such as in filleted, broiled, or canned products is not possible because the morphological characteristics are often destroyed. To address these issues, a series of studies has focused on the molecular based discrimination of food species and validation of analytical methods (*4, 5*).

DNA based analytical methods, such as restriction fragment length polymorphism (RFLP), single strand conformational polymorphism (SSCP), and random amplified polymorphic DNA (RAPD) (*6–9*) are practically employed techniques for the routine discrimination of the species that are present in a variety of food products, including heat-processed products. However, these methods have drawback of requiring the use of hazardous chemicals. Application of such procedures to quantitative determination is also highly technical, which limits their use in routine and high-throughput analysis. Although PCR based techniques, including real-time PCR and PCR-ELISA (*10–13*), have recently been applied for quantitative and semiquantitative determination of species presence, requirement for expensive realtime PCR equipments or time-consuming procedures limit their use in in situ (in the markets and factories) food inspection.

To evaluate labels simply, we developed the fluorogenic ribonuclease protection (FRIP) assay. The FRIP assay uses fluorescent probes and ribonucleases for the detection of single-base mutations (**Scheme 1**). The assay also allows quantification of the mutant ratio in a heterogeneous population of mutant/wild-type species by the end point measurement of the fluorescence intensity (*14*) using fluorometers, which have prices that are from one-fifth to one-tenth of realtime PCR equipments. The lower initial cost is the advantage for the quantitative discrimination of food species from mixed food products at the downstream of the distribution process. We describe the application of the FRIP assay for the detection of species types in raw and thermally processed eels and tunas.

MATERIALS AND METHODS

Sample Preparations. Broiled European eel (*Anguilla anguilla*) and Japanese eel (*A. japonica*) and raw muscles of Atlantic bluefin (*Thunnus*

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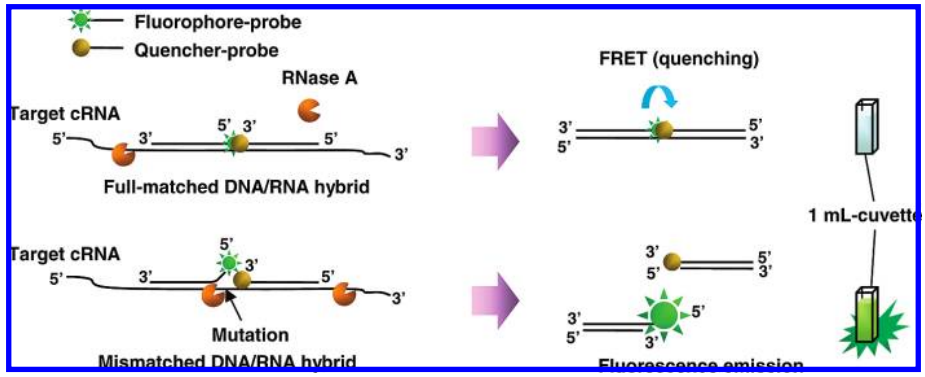
Scheme 1. Illustration of the FRIP Assay[a]



[a] The mismatched RNA base is digested by ribonuclease, generating a fluorescence signal. The intensity of the fluorescence detected by the fluorophotometer is linearly correlated with the wild type/mutant ratio in the solution, allowing the quantitative discrimination of food products.

Table 1. PCR Primers

name	sequence (5′ → 3′)	species
T7-eel	ATGATCACTAATACGACTCACTATAGGGACTTTACTACGGCTCATA	European eel and Japanese eel
rev-eel	TCCTCATGGAAGTACATATCCTACGAATG	
T7-bft	ATGATCACTAATACGACTCACTATAGGGCCCATCAAGCACACGCATACC	Pacific bluefin and Atlantic bluefin
rev-bft	GACAGGAGGGGTGTGGTGTCCTTG	
T7-sbf	ATGATCACTAATACGACTCACTATAGGGACTTGCATTCCCTCTCTG	Southern bluefin
rev-sbf	CTGTTAACCGCACTCCAAGTGCTAAGGG	
T7-byt	ATGATCACTAATACGACTCACTATAGGGCCACAAATGAGCCGTTCTA	Bigeye tuna
rev-byt	TTGAGAGATAGTTGAGTAG	
T7-yft	ATGATCACTAATACGACTCACTATAGGGCTACAGCAGCAACTGTC	Yellowfin tuna and Albacore
rev-yft	ATGTGGTATGCGTGTGCTTGA	

Table 2. Discrimination Probes

	5′ FITC probe		3′ BHQ1 probe	
species	name	sequence (5′ → 3′)[a]	name	sequence (5′ → 3′)
European eel	F-AA	TTTGTAAAGGTATGAGCCGT	Q-AA	ACTCCGATGTTTCATGTTTC
Japanese eel	F-AJ	CTGTTATTATTACTAATAGG	Q-AJ	AGTAAATATCCTACGAATG
Pacific bluefin	F-PBF	CAACTGCACCTGTTAATGGT	Q-PBF	TGACGTTATTAGTAGGGCAG
Atlantic bluefin	F-ABF	ACATTGTAAGGAGCAGGAGG	Q-ABF	ATGTCTCGTCATCATTGGT
Southern bluefin	F-SBF	AATAACAGTTGCTAATCAG	Q-SBF	GGTTGGTTTCGCATGCCAAT
Bigeye tuna	F-BYT	AAATTAGGAATAACATTAGG	Q-BYT	GAGTCCTAGTATGTTTAGGG
Yellowfin tuna	F-YFT	GCAGGGTTGCTGTTAGGAT	Q-YFT	TAGAAGTGTTAGAAGGAAAA
Albacore	F-ALB	AAAGGCTTAGGAGTAGGACA	Q-ALB	ATAGACGTTTTCTTGTAGGT

[a] Underlined DNA bases hybridize to target SNPs.

thynnus thynnus), Pacific bluefin (*T. thynnus orientalis*), Southern bluefin (*T. maccoyii*), bigeye tuna (*T. obesus*), yellowfin tuna (*T. albacares*), and albacore (*T. alalunga*) were commercially obtained. Three samples of muscle tissue were removed from each specimen. Total DNA was extracted using a DNeasy Tissue kit (Cat. No. 69504; QIAGEN, Tokyo, Japan). Each extracted DNA (approximately 200 μg/μL) was diluted with 0.1× TE buffer to obtain the concentration of 0.05, 0.5, 5.0, 50, and 200 μg/μL. The amount of template DNA for PCR was examined three parallels and three replications for the limit of determination (LOD) and optimal amount.

Canned yellowfin tuna (n = 8), albacore (n = 7), and an unknown species (n = 3) were also purchased. The canned products were uniformly mixed in a food mixer prior to the FRIP analysis. Aliquots (N = 3) of each sample were suspended in hot water then centrifuged. The supernatant was decanted, and DNA was extracted with a DNeasy Tissue kit (QIAGEN, Japan). Extracted DNA (approximately 20 μg/μL) was diluted with 0.1× TE buffer to the concentration of 0.5 and 5.0 μg/μL, then subjected to the determination of LOD and optimal amount. The raw muscle of yellowfin tuna and albacore were mixed

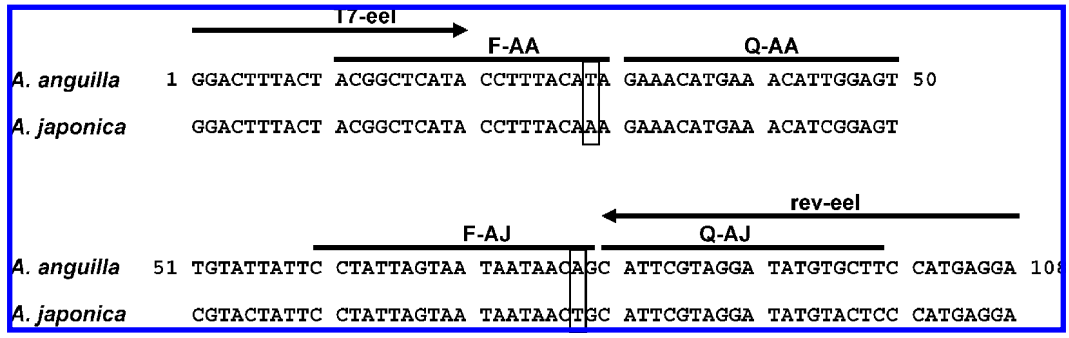


Figure 1. DNA sequences and SNP positions used for the Targeted of eel species (*15*). Targeted SNPs are shown in squares.

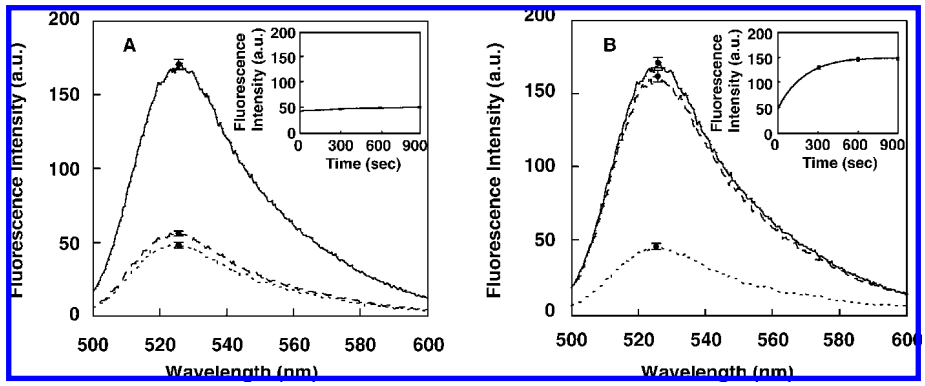


Figure 2. Changes in fluorescence intensity after hybridization of RNA samples with F-AA/Q-AA (dotted line) and following RNase A digestion (broken line). Probes were hybridized to (**A**) Japanese eel sample (perfectly match) and (**B**) European eel sample (mismatch). Insets show the time course after RNase A addition. Error bars represent standard deviations.

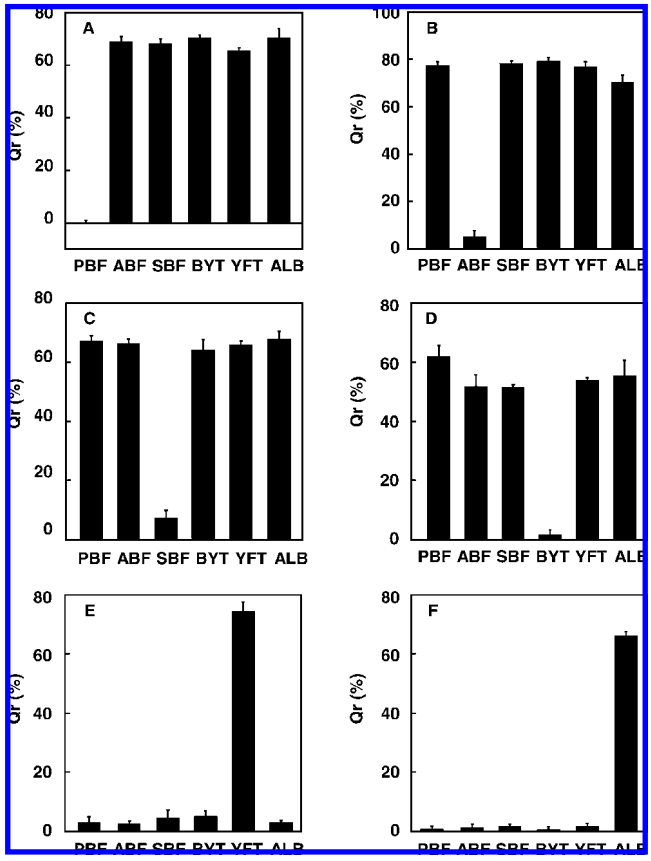


Figure 3. Quenching rates of fluorophore-probes following RNase A digestion. Probes are: (**A**) F-PBF/Q-PBF; (**B**) F-ABF/Q-ABF; (**C**) F-SBF/Q-SBF; (**D**) F-BYT/Q-BYT; (**E**) F-YFT/Q-YFT; (**F**) F-ALB/Q-ALB. The samples are: PBF, Pacific bluefin; ABF, Atlantic bluefin; SBF, Southern bluefin; BYT, bigeye tuna; YFT, yellowfin tuna; ALB, albacore. Error bars show the standard error.

in different ratios, steam-heated at 115 °C for 90 min in salad oil, and subjected to a semiquantitative assay.

Oligonucleotides. PCR primers and dye-labeled oligonucleotide probes (listed in **Tables 1** and **2**) were custom-synthesized by Hokkaido System Science Co. Ltd. (Sapporo, Japan). The labeled oligonucleotides were stored at 4 °C in the dark in a 1× TE buffer (pH 7.4).

Preamplification of Gene Targets by PCR. Target DNA fragments were preamplified by PCR using *Pyrobest* DNA polymerase (Cat. No. R005A; Takara Bio, Shiga, Japan) with the primer combinations listed in **Table 1**. The amplified regions in the mitochondrial cytochrome *b* of eels and in the mitochondrial region between ATPase 6 and cytochrome *c* oxidase subunit 3 (an ATCO region) of the tuna were determined based on reported DNA sequences (*15, 16*). To generate the T7 promoter sequence in the PCR products, 5′-ATGATCAC-TAATACGACTCACTATAGGG-3′ tailed primers were used for PCR amplification of target genes. The PCR mixture (20 μL) contained 10 ng of extracted DNA, 20 pmol of primers, and 0.5 U of *Pyrobest* DNA polymerase. The PCR procedure was as follows: (1) for eels, 94 °C for 15 s, 54 °C for 15s, and 72 °C for 15s for 35 cycles; and (2) for tuna, 94 °C for 15 s, 50 °C for 15s, and 72 °C for 15s for 35 cycles. The amplified fragments were cloned in pUC18 plasmids, and the sequences were confirmed using the fluorescent dideoxy termination reaction method in a CEQ 8000 (Beckman Coulter, Tokyo, Japan). The plasmids were used as standards without any further modification.

RNA Amplification by in Vitro Transcription. RNA was synthesized by in vitro transcription using a T7 RiboMAX Express large scale RNA production Ssystem (Cat. No. P1320; Promega, Tokyo, Japan). Two microliters (approximately 0.08 μg) of PCR amplicon was added to the reaction mixture (20 μL volume), which was incubated at 37 °C for 30 min, then stored at room temperature until the FRIP analysis.

Discrimination and Quantification of Mutations Using the FRIP Assay. Tris-EDTA buffer solution (100×, Cat. No. T9285) and Triton X-100 (Cat. No. T8787) were purchased from Sigma-Aldrich (Tokyo, Japan). Magnesium chloride hexahydrate (Cat. No. 135−15055) was purchased from Wako Pure Chemical Industries (Osaka, Japan). The RNA transcription product (4 μL) was added directly to 1 mL of hybridization buffer (10 mM Tris-HCl, 1 mM EDTA, 50 mM $MgCl_2$, 0.001% Triton X-100, pH 7.4) containing 20 pmol of a fluorescent probe and 100 pmol of a quencher probe in a 1.5 mL polypropylene tube. The reaction mixture was incubated at 25 °C for 15 min, then treated with 10 μg of bovine pancreatic ribonuclease A (Cat. No. 313−01461; Nippon Gene, Tokyo, Japan) at 25 °C for 15 min. Fluorescene emission of FITC at 532 nm, with excitation at 490 nm, was measured with a luminescence spectrophotometer, LS 55 (Perkin-Elmer, Yokohama, Japan), equipped with a cutoff filter at 500 nm. The quenching rate (Qr) of fluorescence was calculated as

$$\mathrm{Qr}\,(\%) = (F_p - F) \times 100/F_p \quad (1)$$

where F_p is the fluorescence intensity of a donor probe before hybridization and F is the fluorescence intensity after RNase A digestion.

The blending ratio of mixed ingredients was estimated by the following equation:

$$\mathrm{Br}\,(\%) = (F_{sample} - F_{fullmatch}) \times 100/(F_{mismatch} - F_{fullmatch}) \quad (2)$$

where F_{sample}, $F_{fullmatch}$, and $F_{mismatch}$ are the fluorescence intensity of the sample, full-match, and mismatch target after RNase A digestion.

RESULTS AND DISCUSSION

Discrimination of Eel Species. Previously, the Japanese and European eels were distinguished from six other eel species based on SNPs found in the flanking region of mitochondrial cytochrome *b* (*15*). To discriminate between eel species using

Table 3. Repeatability of the FRIP Assay Affected by the Amount of DNA Extracted from Canned Tunas

amount of DNA (ng) in 20 μL PCR	positive signals	mean fluorescence intensity (perfectly matched)	SD[a]	RSD[a] (%)	mean fluorescence intensity (mismatched)	SD[a]	RSD[a] (%)
yellowfin tuna							
0.5	6/9						
5.0	9/9	37.65	0.89	2.36	167.8	2.56	1.53
20	9/9	38.49	1.29	3.35	169.0	3.14	1.86
200	9/9	38.63	1.57	4.06	171.0	2.75	1.60
albacore							
0.5	6/9						
5.0	9/9	41.35	1.03	2.49	185.65	2.61	1.40
20	9/9	43.23	0.75	1.73	186.09	2.72	1.46
200	9/9	42.19	1.06	2.51	185.19	3.16	1.71

[a] SD and RSD show the standard deviation and relative standard deviation, respectively.

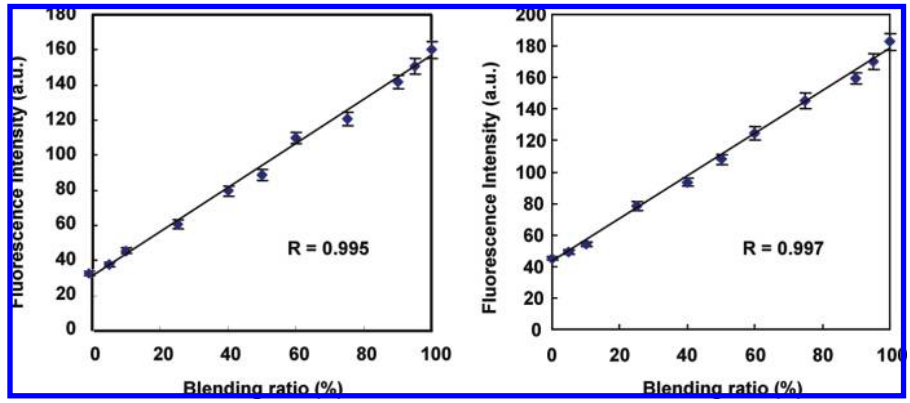


Figure 4. Correlation between the fluorescence quenching rate and the blending ratio of yellowfin tuna (**A**) and albacore (**B**). Error bars show the standard deviations, and *R* represents the correlation coefficient.

Table 4. Quantitative Discrimination of Commercially Available Canned Tuna

sample no.	calculated content of yellowfin tuna (%) ± SD[a]	calculated content of albacore (%) ± SD[a]	ingredient on label
1	95.2 ± 2.0	6.07 ± 0.3	tuna
2	99.4 ± 1.5	0.95 ± 0.8	tuna
3	101.3 ± 0.1	0.784 ± 0.8	tuna
4	99.9 ± 6.3	0.95 ± 0.8	yellowfin tuna
5	99.9 ± 5.0	−0.01 ± 0.1	yellowfin tuna
6	98.2 ± 4.8	−0.04 ± 1.5	yellowfin tuna
7	101 ± 1.2	1.77 ± 0.7	yellowfin tuna
8	102 ± 1.9	2.06 ± 1.5	yellowfin tuna
9	102 ± 3.1	−2.40 ± 3.4	yellowfin tuna
10	99.6 ± 2.4	1.35 ± 0.2	yellowfin tuna
11	98.8 ± 2.2	−1.74 ± 0.8	yellowfin tuna
12	−0.10 ± 2.2	100 ± 1.7	albacore
13	2.64 ± 1.2	103 ± 0.5	albacore
14	2.06 ± 3.4	98.0 ± 1.3	albacore
15	2.21 ± 1.9	100 ± 3.6	albacore
16	−2.42 ± 2.4	103 ± 0.7	albacore
17	−1.91 ± 2.9	97.6 ± 2.0	albacore
18	−2.59 ± 3.8	98.5 ± 4.1	albacore

[a] SD shows the standard deviation.

the FRIP assay, two sets of donor−accepter probes were designed (**Table 2**, **Figure 1**, and **Figure S1** of the Supporting Information). For the designing of probe sequences that gives the largest difference between the cleavage speed of perfectly matched hybrid and mismatched hybrid by RNase A, the binding/cleavage mechanism of RNase A was exploited. RNase A is known to have three subsites (B1, B2, and B3) to recognize RNA bases, and the B1 subsite binds to only pyrimidine bases, which 3′ end phosphate groups are cleaved (*17, 18*). Because of this, the largest difference in the cleavage speeds is obtained when perfectly matched RNA bases are purine bases and mismatched RNA bases are pyrimidine bases. Moreover, our experiments revealed that the unpaired RNA was cleaved at the fastest rate when pyrimidine RNA bases hybridize to the penultimate DNA bases to the 5′ end of fluorophore-probes.

DNA fragments of 108 bp were amplified by PCR from the broiled-eel DNA extracts, transcribed to cRNAs, then analyzed with the FRIP assay. The fluorescence intensity of the probe declined after hybridization with sample RNAs, resulting in high quenching rates (∼75%), as shown in **Figure 2**. Along with following digestion of the RNA/probe duplexes with RNase A, the fluorescent recovery was observed in response to the complementarity of the duplexes (**Figure 2**). The quenching rates after RNase A digestion were 68.11 ± 1.13 (Japanese eels) and 3.48 ± 2.49 (European eels) with F-AA/Q-AA probes. The quenching rates were 70.02 ± 3.83 (European eels) and 1.24 ± 1.17 (Japanese eels) with F-AJ/Q-AJ probes. These differences between the quenching rates of two eel species suggested that they can be distinguished simply based upon fluorescence recovery. Although at a slow rate compared to ultimate- and penultimate-mismatch hybrids, full-match hybrids and internal-mismatch hybrids are also digested by RNase A (insets in **Figure 2**). Therefore, regulation of the incubation time with RNase A is necessary for the accuracy of the determination.

LOD of the FRIP assay depends on amplification efficiency by the PCR reaction. When 0.05 ng of extracted eel DNA was subjected to the PCR (20 μL volume), the target region was not amplified efficiently in some reaction tubes under our condition. As a result, several hybridization mixtures showed the quenching rate of only ∼10% after addition of cRNA. Obtained positive signals were 6/9 and 5/9 for European eels and Japanese eels, respectively. Sufficient hybridization occurred when 0.5−200 ng of extracted DNAs were applied (**Table S1** of the Supporting Information). However, the amplification

efficiency declined again when 2.0 μg of extracted DNA was subjected, and extinction did not occur after addition of transcription products. We concluded that 2.0 μg of template DNA is not suitable for the FRIP assay. The absolute LOD for eel is assumed to be about 0.5 ng. For the discrimination of a lower concentration of DNA, an increment of the PCR cycle should be examined.

Discrimination of *Thunnus* Tuna Species. Previous studies suggested that SNPs in the mitochondrial flanking ATCO region were divided into distinctive genotypes among the six main commercial tuna species in Japan (*16, 19*). By using primer sets of T7-bft/rev-bft (141 bp), T7-sbf/rev-sbf (179 bp), T7-byt/rev-byt (110 bp), and T7-yft/rev-yft (151 bp) in **Table 1**, four different fragments were amplified and analyzed by the FRIP assay. Since RNase A does not recognize/cleave dG/rU or dT/rG mismatches, we designed complementary probes for yellowfin tuna and albacore and noncomplementary probes for the remaining species (**Table 2** and **Figure S2** of the Supporting Information). After hybridization, the fluorescence intensity of the probes declined to 20−30%. The addition of RNase A altered the fluorescence intensity, depending on the complementarity of the DNA/RNA hybrids (**Figure 3**), suggesting that the FRIP assay may be a useful tool for discriminating between tuna species using raw tissue. Because of the three-dimensional recognition/cleavage of RNA base by RNase A (*14, 17*), internal mismatches observed in the tuna probe/cRNA hybrids had little effect on RNase A mediated point mutation detection in the FRIP analysis. The LOD and optimal amount of extracted DNAs for the accurate discrimination were determined to be 0.5 ng and 0.5−200 ng, respectively.

Application of the FRIP Assay for Determination of Species in Cans of Tuna. Electrophoresis profiles of DNA isolated from highly processed foods, such as canned fish, are often obscured because heat sterilization causes nonspecific fragmentation of DNAs (*20, 21*). The FRIP assay is potentially able to detect SNPs in these products as it requires only a short DNA fragment, typically less than 150 bp (*14*). As shown in **Table 3**, a large amount of DNA was required for the accurate determination compared to the raw samples, presumably because of the degradation of DNA. However, similar results were obtained using DNA from both canned fish and raw fillets, suggesting that the FRIP assay is able to discriminate species in heat-processed cans of tuna.

Fluorescence intensity is intrinsically correlated with the mutant/native ratio; therefore, we examined whether the FRIP assay could be used to quantitatively determine the ratio of species in a sample. As shown in **Figure 4**, the quenching rate was proportional to the ratio of DNA from each tuna species by the following equations:

$$y_{\text{yellowfin tuna}} = 1.25x + 32.00 \quad (3)$$

$$y_{\text{albacore}} = 1.35x + 41.71 \quad (4)$$

We were also able to calculate the ratio of the target species in commercially obtained canned tunas, using eq 2 (**Table 4**), indicating that this method is an appropriate screening test.

The FRIP assay takes 2.5 h (1.5 h for PCR, 0.5 h for in vitro transcription, and 0.5 h for hybridization and RNase A treatment) to estimate the contamination of more than 5% of other species that should be labeled in Japan. It also has a wide range of applications for the determination of raw or processed food. The assay consists of four steps: (1) amplification of dsDNA, (2) in vitro transcription of ssRNA, (3) hybridization with fluorophore and quencher probes, and (4) digestion with ribonucleases. Premix solution for all these steps can be prepared and stored for at least three months under −20 °C, and users are only required to add a specific amount of extracted DNA depending on the condition of the samples. In the future, using various fluorescent dyes, it will be possible to operate a multiplex analytical procedure. This simple method can be used for the in situ screening test for the confirmation of the labeling along with the traceability of food products.

Supporting Information Available: Partial alignments of the sequences of eels and *Thunnus* tunas hybridized to fluorophore and quencher probes and table of the repeatability of the FRIP assay for eel. This material is available free of charge via the Internet at http://pubs.acs.org.

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