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Real-time PCR Detection Methods for Food Allergens (Wheat, Buckwheat, and Peanuts) Using Reference Plasmids

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Supporting Information

ABSTRACT: Specific and sensitive real-time qualitative polymerase chain reaction (PCR) methods for the detection of food allergens including wheat, buckwheat, and peanuts were developed that could cancel between instrument effects and avoid risks of false-positives and false-negatives. In these real-time PCR analysis, the cutoff for determination of positive samples was set in every PCR run by using reference plasmids containing known copies of the target sequences. The copy numbers of the plasmids were used to detect the allergenic ingredients corresponding to 10 ppm (w/w) protein in highly processed foods (cooked for more than 30 min at 122 °C). Reference plasmid analysis for each real-time PCR run helped to minimize variability between runs and instruments (7900HT Real-Time PCR systems and Light Cycler Nano). It also helped to avoid false positives due to trace levels of contaminants from the laboratory environment or agricultural products. The specificity of the real-time PCR method was verified using 79 commonly used food materials and some of their relatives. The method was sensitive enough to detect those allergenic ingredients corresponding to 10 ppm (w/w) in seven types of incurred samples. The current official Japanese method was not able to detect the allergens in some of the incurred samples. The developed method can avoid false negatives due to lack of sensitivity and is useful to confirm positive ELISA screening tests.

KEYWORDS: *food allergen, internal transcribed spacer (ITS), polymerase chain reaction (PCR), positive/negative threshold, DNA fragmentation*

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INTRODUCTION

Food allergies have recently become a serious food safety issue in many countries, including Japan.[1,2] The ingestion of very small amounts of a food allergen can cause severe anaphylactic shock in sensitive patients. Therefore, an effective preventative measure for patients is to avoid taking in foods that include amounts of the allergen sufficient to cause an allergic reaction. In 1999, the Joint FAO/WHO Codex Alimentary Commission agreed to recommend the labeling of these ingredients (milk, eggs, peanuts, soybeans, tree nuts, fish, crustaceans, sulfites, and cereals containing gluten) known as major food allergens.[3] The Japanese government requires mandatory labeling of seven ingredients, eggs, milk, wheat, buckwheat, peanuts (from 2002), shrimp/prawns, and crab (from 2008),[4] after periodic review. They have designated 10 μg of soluble protein from an allergic ingredient in 1 g of food as the labeling threshold.[5] To verify the validity of food labeling and to ensure the safety of food products, the Japanese Consumer Affairs Agency has announced official methods for detecting these seven ingredients.[5] When these three allergens, wheat, buckwheat, and peanuts, in food are analyzed, screening tests are carried out by validated enzyme-linked immunosorbent assay (ELISA) kits. If the ELISA shows the presence of more than 10 μg allergen protein/g food, a confirmation test is carried out by a polymerase chain reaction (PCR) method to exclude false positives. However, several reports indicate that the sensitivity of the wheat PCR might be insufficient to detect wheat levels of 10 μg protein/g of highly processed food or incurred samples.[6−8]

Our group has already developed several PCR methods for food allergens by using the internal transcribed spacer (ITS) region, which exists in high copy number.[9,10] This is among the most sensitive of PCR detection methods for food allergens that have been reported, which decrease the risk of false negatives.

Sensitive PCR methods, however, may increase the risk of false positive misjudgments caused by the carry-over of PCR products or trace contaminants from the laboratory environment. To avoid the false positive risk, we improved on real-time PCR when developing a detection method for buckwheat.[9] In this real-time PCR method, gel electrophoresis for the detection of the product is not used. Rather, the signals from the products at the exponential step in each reaction tube are monitored without analyzing the product in an open environment. However, while real-time PCR has its merits, it also has disadvantages in that the method recommends using a specific model of real-time PCR instrument, especially in food

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analysis. Moreover, it is still difficult to differentiate positive samples from a trace level of contamination from the laboratory environment.

There are many quantitative and qualitative real-time PCR methods to detect food contaminants, including allergenic ingredients.[11−24] In Japan, real-time PCR methods have been officially adopted to identify and quantify genetically modified crops,[25] food poisonings,[26,27] and parasitic worms in raw fish.[28] In the case of qualitative methods, a sample is considered positive if its amplification curve reaches a predetermined threshold line or if its C_t value is less than a predetermined value. However, threshold lines are calculated automatically, and C_t values are different among PCR runs or real-time PCR instruments. These variations can lead to different judgments, especially in the analysis of trace levels of target DNA. For this reason, some official Japanese methods for genetically modified crops recommend using a specific model of instrument.

We have developed real-time PCR methods for wheat, buckwheat, and peanuts that, in addition to sample DNA, analyze reference plasmids containing predetermined copy numbers of amplicons from target allergens. We use the C_t value as the cutoff for determining positive samples in each PCR run. This is useful not only to avoid risks of false-positives and false-negatives but also to minimize differences between real-time PCR runs or instruments.

In this study, we determined the specificity and sensitivity of our wheat, buckwheat, and peanut real-time PCR methods. The results of a two-laboratory validation study using incurred samples with three real-time PCR instruments are reported.

MATERIALS AND METHODS

Samples Used for DNA Isolation. A total of 79 food materials, *Fagopyrum esculentum* (common buckwheat), *Fagopyrum tataricum* (Tartarian buckwheat), *Oryza sativa* subsp. *Japonica* (rice), *Zea mays* (corn), *Triticum aestivum* (wheat), *Hordeum vulgare* (barley, Shunrai), *Hordeum vulgare* (barley, fiber-snow), *Secale cereale* (rye), *Coix lacryma-jobi* var. *ma-yuen* (adlay), *Avena sativa* (oat), *Setaria italica* (foxtail millet), *Panicum miliaceum* (proso millet), *Echinochloa esculenta* (Japanese barnyard millet), *Amaranthus* (pigweed), *Fallopia convolvulus* (wild buckwheat), *Glycine max* (soybean), *Vigna angulariz* (adzuki bean), *Phaseolus coccineus* (runner bean), *Cicer arietinum* (chickpea), *Vigna unguiculata* (black-eyed pea), *Phaseolus vulgaris* (common bean), *Phaseolus vulgaris* (pinto bean), *Arachis hypogaea* (peanut), *Helianthus annuus* (sunflower seed), *Piper nigrum* (pepper), *Capsicum annuum* (chili pepper), *Sinapis alba* L. (mustard), *Zingiber officinale* (ginger), *Ocimum basilicum* (basil), *Trigonella foenum-graecum* (fenugreek), *Malus domestica* (apple), *Actinidia deliciosa* (kiwifruit), *Prunus dulcis* (almond), *Juglans* spp. (walnut), *Macadamia integrifolia* (macadamia nut), *Anacardium occidentale* (cashew), *Corylus avellana* (hazelnut), *Carya illinoinensis* (pecan), *Pistacia vera* (pistachio), *Pinus koraiensis* (pine nut), *Sesamum indicum* (sesame), *Aloe vera* (aloe plants), *Cucumis melo* (melon), *Diospyros kaki* (persimmon), *Ficus carica* (fig tree), *Vitis* spp. (grape), *Rheum rhabarbarum* (rhubarb), *Porphyra* spp. (seaweed), *Oncorhynchus keta* (salmon), *Scomber* spp. (mackerel), *Gadus macrocephalus* (cod), *Sardinops* spp. (sardine), *Todarodes pacificus* (Japanese common squid), *Penaeus monodon* (black tiger prawn), *Portunidae trituberculatus* (swimming crabs), *Mizuhopecten yessoensis* (Japanese scallop), *Turbo cornutus* (horned turban), *Gallus gallus* (chicken), *Sus scrofa* (pork), *Bos taurus* (beef), *Ovis aries* (mutton), *Solanum lycopersicum* (tomato), *Dioscorea japonica* (Japanese yam), *Solanum tuberosum* (potato), *Allium cepa* (onion), *Spinacia oleracea* (spinach), *Brassica campestris* (Chinese cabbage), *Daucus carota* subsp. *sativus* (carrot), *Abelmoschus esculentus* (okra), *Cucumis sativus* (cucumber), *Lentinula edodes* (shiitake mushroom), *Agaricus bisporus* (common mushroom), *Arctium lappa* (edible burdock), *Persea americana* (avocado), *Citrus sinensis* (orange), *Musa* spp. (banana), *Mangifera indica* (mango), *Vaccinium corymbosum* (blueberry), and *Ananas comosus* (pineapple) were purchased at local food markets or online shops. Seeds of common buckwheat, Tartarian buckwheat, rice, Shunrai barley, fiber snow barley, rye, oat, foxtail millet, proso millet, pigweed, soybean, adzuki bean, runner bean, mustard, fenugreek, and rhubarb were sowed in pots in a greenhouse to obtain sprout and leaf samples. Commercial food products and ingredients for the incurred samples were purchased from local supermarkets.

Preparation of Incurred Samples. For the sensitivity study, we prepared seven varieties of incurred sample: rice gruel, mixed juice, instant miso soup, tomato pasta sauce, freeze-dried vegetable soup, retort packed chicken meatballs with vegetables, and retort packed Asari clams. The spiking materials were prepared according to the appendix[5] shown in the official Japanese method of analysis for food allergens. Equal amounts of the 14 kinds of wheat seeds were mixed and ground. The ground powder was passed through a 1.18 mm sieve and used as a spiking material for wheat. Equal amounts of buckwheat seeds from Ibaraki prefecture in Japan and northern China were mixed and ground. The ground powder was passed through a 1.18 mm sieve and used as a spiking material for buckwheat. Peanuts of the Virginia variety made in Chiba prefecture in Japan were ground with a mortar and pestle. To remove the fat, 1 g of ground peanuts was vigorously shaken with 10 mL of acetone, then centrifuged for 30 min at 10,000 × *g*, and the procedure was repeated three times. The defatted peanut powder was dried for 7 h at 45 °C on an aluminum block bath (Sansho Corp., Japan) and used as a spiking material for peanuts. The protein contents in the three kinds of allergen powder were determined using a 2-D Quant Protein Assay kit (GE Healthcare, Buckinghamshire, UK). The 10 ppm (w/w) incurred samples were prepared using the procedures described below. To obtain a final soluble wheat, buckwheat, and peanut protein content of 10 μg/g in the food, the amounts of spiking materials were calculated. We also prepared 0 ppm samples as blanks, which did not contain any spiking material.

Rice gruel was made with rice and water and cooked with the spiking materials of the three allergens in a rice cooker (SR-DG 10 H, Panasonic National, Japan). The mixed juice was made with orange juice and Mexican mangoes (KING OVLADA). The mangoes were peeled and pureed in a food processor (DLC-NXJPG, Cuisinart, Stamford, CT, USA). Orange juice and the mango puree were mixed at 1:1, and the spiking materials were added and heated with a hot stirrer (MRK MAGMIX HOT STIRRER, Mitamura Riken Industry, Japan) at 90 °C for 10 min. The pH of the mixed juice was 4.0.

The instant miso soup was made with rice miso paste, concentrated kelp stock, freeze-dried pumpkin, and freeze-dried green beans. Miso paste, concentrated kelp stock, and water were thoroughly mixed with the spiking materials. The mixture was packaged in an aluminum pouch, boiled for 7 min, and cooled in flowing water for 5 min, and then the freeze-dried pumpkin and freeze-dried green beans were added.

The freeze-dried vegetable soup was made with potato starch, salad oil, onion, sweet cooking rice wine, chicken stock, potato, pumpkin, broccoli, carrot, and bacon. Potato starch, salad oil, onion, sweet cooking rice wine, chicken stock, water, and the spiking materials were stirred over medium heat until thickened. Small pieces of bacon, boiled potato, pumpkin, broccoli, and carrot were added to the soup mixture, which was then cooled at −80 °C for overnight in a freezer and then freeze-dried for 2 days.

The tomato pasta sauce was made with tomato puree, olive oil, garlic, salt, basil, black pepper, and rosemary leaf. Chopped garlic sautéed in olive oil, tomato puree, chopped basil, and rosemary leaf were mixed and simmered over low heat for 15 min, and then salt, pepper, and the spiking materials were added. The mixture was packaged in an aluminum pouch, treated in a constant-temperature water bath at 85 °C for 30 min, and then cooled in water for 5 min.

The retort packed chicken meatballs with vegetables was made with minced chicken meat, carrots, Japanese radishes, potato starch, salt, sweet cooking rice wine, and concentrated soup stock. Chicken meatballs were made with minced chicken meat, potato starch, salt,

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and water, which was then thoroughly kneaded, and balls were made of about 7 g each and then boiled for 3 min. The chicken meatballs, carrots, Japanese radishes, sweet cooking rice wine, concentrated soup stock, water, and the spiking materials were packaged in a retort pouch, heated in a retort sterilizer at 122 °C for 15, 25, 30, 35, and 40 min at a pressure of 1.5 bar, and then cooled in water for 10 min.

The retort packed Asari clams was made with frozen Asari (Japanese littleneck) clams, sodium citrate, disodium hydrogenphosphate, salt, and citric acid. The frozen Asari clams were boiled for 3 min, then soaked for 30 min in an 80 mM citric acid-disodium and 30 mM hydrogen phosphate solution (pH 8.8). The seasoning liquid mixture was made with water, salt, sodium citrate, and citric acid, and its pH was adjusted from 4 to 6. The soaking solution was drained off the clams, and the clams and seasoning liquid mixture were packaged in a retort pouch. For calculating the *F*-value, the sample was heated in a retort sterilizer at 122 °C for 0−60 min at 1.5 bar of overpressure.

Each food was homogenized using a mixer mill (IFM-800DG; Iwatani Corp., Japan), a multisample mixer mill (MM300; Retsch, Haan, Germany), a multibeads shocker (Yasui-Kikai, Japan), or a food processor (DLC-XG, Cuisinart). All homogenized samples were stored in a freezer at −20 °C until use.

Determination of Sterile Conditions of Highly Processed Model Foods. We selected "retort packed chicken meatballs with vegetables" and "retort packed Asari clams" as highly processed foods to set the positive/negative threshold for the real-time PCR methods. In order to determine the sterilization condition of the retort packed chicken meatballs with vegetables, we compared the degree of plant DNA fragmentation in four packaged products containing chicken meatballs and vegetables (two kinds of baby food, soft food for the elderly, and general food) and the five incurred samples prepared under different conditions (122 °C for 15, 25, 30, 35, and 40 min). We analyzed the DNA fragmentation with short PCR and long PCR targeting the plant 18S rRNA gene.[29] The primers and the probe targeting the plant 18S rRNA sequences were as follows: the common forward primer (short and long PCR) (5′-GTT GGC CTT CGG GAT CGG AGT A-3′), the reverse short primer (short PCR) (5′-TTG TTC GTC TTT CAT AAA TCC AAG AAT TTC ACC-3′), the reverse long primer (long PCR) (5′-GCA TCG TTT ATG GTT GAG ACT AG-3′), and the TaqMan probe (5′-TCG GGG GCA TTC GTA TTT CAT AGT CAG A-3′) labeled with 6-carboxyfluorescein (FAM) at the 5′ end and with carboxytetramethylrhodamine (TAMRA) at the 3′ end. PCR was performed using 50 ng of total DNA as a template in a 25 μL reaction consisting of 2× QuantiTect PCR probe Master Mix (Qiagen), 0.2 μM F-Primer, 0.065 μM R-Primer for the "short PCR", 0.055 μM R-Primer for the "long PCR", and 0.2 μM TaqMan probe (FASMAC, Kanagawa, Japan). A thermal cycler 7900HT Real-Time PCR system (Thermo Fisher Scientific) was used for PCR under the cycling conditions of a hot start at 95 °C for 15 min, followed by 45 cycles of 95 °C for 30 s and 63 °C for 120 s. The threshold line was set at 0.256, and the baseline was set at 3−15 for the amplification plot. The C_t value of "short PCR" was subtracted from that of "long PCR" to calculate ΔC_t. The sterilization condition of the Asari clams was fixed at a *F*-value of 35 as described in the recipe for canned Asari clams.[30]

DNA Extraction and Purification. For the specificity study of the real-time PCR methods, DNA was extracted from 79 materials (0.1−2 g) with 7.5−20 mL of buffer G2 (Qiagen, Hilden, Germany) supplemented with 20 μL of 100 mg/mL RNase A (Qiagen) and 100 μL of proteinase K solution (Qiagen), and purified using Genomic-tip 20/G (Qiagen) according to the manufacturer's instructions with slight modification. The DNA concentration was determined by measuring the absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany). The DNA was diluted to 50 ng/2.5 μL with Tris-EDTA buffer (pH 8.0) (Promega Corporation, Madison, WI, USA). For the materials that did not give any expected PCR products from the Genomic-tip DNA in plant, animal, or fungus universal PCR, the DNA was extracted by using a DNeasy plant mini kit (Qiagen), DNeasy mericon Food kit (Qiagen), or GM quicker4 (NIPPON GENE Co., Ltd., Japan) according to the manufacturers' instructions with slight modification. To confirm the sensitivity of the methods, the DNA from wheat, common buckwheat, Tartarian buckwheat, and peanuts was serially diluted with 50 ng/2.5 μL salmon testes DNA solution (Sigma Chemical Co., St. Louis, MO) to obtain PCR templates containing 50 fg/2.5 μL and 500 fg/2.5 μL of the DNA.

The DNA was also extracted from 2.0 g of incurred samples and commercial food products by using Genomic-tip 20/G (Qiagen) according to the procedure described in the official Japanese method for food allergens.[5] In the DNA extraction experiment, we used an air-displacement pipet and a clean bench after UV irradiation for 1−2 h to decompose contaminant DNA from the laboratory environment.

Quality Check of the Extracted DNA. To avoid false-negative results due to the impurities in template DNA preparation that inhibit PCR amplification, or from template DNA degradation, universal primer pairs for plants, animals, and fungi were used for a quality check of the extracted DNA. According to the official Japanese method for food allergens, plant primer pairs (CP03-5′, CP03-3′) designed to target a partial region of plant chloroplast DNA were used for the plant samples, and animal primer pairs (AN1-5′/AN2-5′, AN-3′) designed to target a partial region of mitochondrial DNA were used for samples of animal ingredients.[5] Fungi (shiitake mushroom and common mushroom) PCR was carried out in a final volume of 25 μL containing 1× buffer (PCR buffer II), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.625 U of AmpliTaq Gold (Thermo Fisher Scientific), 0.2 μM of each primer pair (ITS1F, 5′-CTT GGT CAT TTA GAG GAA GTA A-3′; ITS4, 5′-TCC TCC GCT TAT TGA TAT GC-3′) for amplification of the ITS region in fungi, and 50 ng of template DNA.[31] The PCR conditions were as follows: preincubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 55 °C for 15 s, and extension at 72 °C for 0.5 min, followed by a final extension at 72 °C for 7 min. PCR was conducted on a GeneAmp PCR system 9700 or Veriti 200 (both from Thermo Fisher Scientific), and PCR products were analyzed by MultiNA microchip electrophoresis (Shimadzu, Japan). All of the DNAs used in the specificity and sensitivity studies gave the expected PCR products with one or more universal primers. All of these primers were purchased from Eurofins Genomics (Tokyo, Japan).

Quantification of Incurred Samples Using ELISA. The protein contents of three kinds of allergens in 10 and 0 ppm (w/w) samples were measured using the Wheat/Gluten (Gliadin) ELISA Kit, Buckwheat ELISA Kit, and Peanut ELISA Kit (Morinaga Institute of Biological Science, Inc., Japan). For the 10 ppm (w/w) incurred samples, duplicate protein extracts were prepared from 1 g of each six portions in a homogenized sample. For the 0 ppm (w/w) samples, duplicate protein extracts were prepared from 1 g of one portion in a homogenized sample.

Oligonucleotide Primers and Probes. The sequences of primers and TaqMan probes for wheat, buckwheat, and peanuts used in this study are listed in Table 1. In addition to the primer pairs and the probes reported in our previous reports,[9,10] we newly designed the wheat probe for the ITS-1 region to detect all *Triticum* sp., and the peanut primer pair and probe for the ITS-2 region to detect *Arachis hypogaea* (i.e., GenBank AY615267 and FJ212319). All primers and the probes were synthesized by Eurofins Genomics Inc. and Thermo Fisher Scientific Inc.

Preparation of Reference Plasmid DNA Solutions. The target sequences of wheat, buckwheat, and peanut PCR were tandemly synthesized as a molecule of 236 nucleotides by FASMAC. The synthesized double stranded DNA molecule was inserted into the *Sma*I site of the pUC19 plasmid. A deliberate base substitution was introduced in each target sequence to differentiate PCR amplicons from plasmid DNAs from those of genomic DNAs (wheat sequence: 5′-CAT GGT GGG CGT CCT CGC TTT ATC AAT GCA GTG CAT CCG GCG C**C**C AGC TGG CAT TAT GGC CTT T-3′; buckwheat sequence: 5′-CGC CAA GGA CCA CGA ACA GAA GCG CGT CCC GAG CCT CCC GGT CCC CGG GCG GCA CGG CGG CGT CGC GTC GTT TCT AC**C** AAA CAG AAC GAC TCT CGG CAA CG-3′; peanut sequence: 5′-TTG GTT CAA AGA GAC GGG CTC TT**C** GTG GGG AGC GGC ACC GCG GCA GAT GGT GGT CGA GAA CAA CCC TCG TG-3′; the underlined

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Table 1. Primers and Probes Used in This Study

detection	sequence	concentration (μM)	amplicon size (bp)
Wheat PCR			
forward primer	5′-CAT GGT GGG CGT CCT C-3′	0.4	64
reverse primer	5′-AAA GGC CAT AAT GCC AGC TG-3′	0.2	
	5′-TGA GGC CGT CAT GCC GGC TG-3′	0.1	
	5′-TGA GGC CAT AAT GTC GGC TG-3′	0.1	
TaqMan probe	5′-FAM-CGG ATG CAC TGC ITT GAT AAA G-MGB-3′	0.1	
Buckwheat PCR			
forward primer	5′-CGT TGC CGA GAG TCG TTC TGT TT-3′	0.2	101
reverse primer	5′-CGC CAA GGA CCA CGA ACA GAA G-3′	0.2	
TaqMan probe	5′-FAM-CGG GAC GCG CTT C-MGB-3′	0.1	
Peanut PCR			
forward primer	5′-TTG GTT CAA AGA GAC GGG CTC-3′	0.4	71
reverse primer	5′-CAC GAG GGT TGT TCT CGA CC-3′	0.4	
TaqMan probe	5′-FAM-ACC GCG GCA GAT GG-MGB-3′	0.1	

nucleotides are deliberate base substitutions from G to C). The plasmid DNA was linearized by *Nde* I digestion and used as the reference plasmid DNA. The concentration of 15 ng/μL of reference plasmid DNA solution was determined by measuring the absorbance at 260 nm with a Cary 60 spectrophotometer (Agilent Technologies, USA). The copy number per microliter of the reference plasmid DNA solution was determined based on the absorbance at 260 nm and was denoted as T_{abs}. The T_{abs} was calculated using the following equation, where A_{260} is the absorbance at 260 nm and 2686 + 236 bp is the length of pUC19 DNA plus the synthesized DNA.

$$T_{abs} = \frac{A_{260} \times 50[\text{ng}/\mu\text{L}] \times 6.02 \times 10^{23} \times 10^{9}[\text{copies}]}{660[\text{MW/bp}] \times (2686 + 236)[\text{bp}]} \quad \text{(I)}$$

The calculated 15 ng/μL reference plasmid DNA solution was serially diluted to T_{abs} = 3000 and 20 copies/μL with 20 ng/μL salmon testes DNA solution in 1× Tris-EDTA buffer (pH 8.0) using an analytical balance with 0.01 mg resolution gravimetrically. The T_{abs} = 1000 copies/μL reference plasmid DNA solution was similarly diluted from a 3000 copies/μL reference plasmid DNA solution using micropipettes.

Digital PCR Analysis of Reference Plasmid. Digital PCR amplifications of the peanut target sequence on the reference plasmid were performed using QuantStudio 3D (Thermo Fisher Scientific) in a total volume of 15 μL. The reaction mixtures contained a final concentration of 1× QuantStudio 3D Digital PCR Master Mix (Thermo Fisher Scientific), 0.4 μM each of the forward and reverse primers for peanuts, 0.1 μM of peanut TaqMan probe, 6 μL of the T_{abs} = 3000 copies/μL reference plasmid DNA solution, and water. The thermal cycling conditions for amplification were an initial denaturation at 96 °C for 10 min, followed by 34 cycles each consisting of a step at 58 °C for 2 min and at 98 °C for 30 s, and a final step at 58 °C for 2 min on digital chips. After PCR amplification, the chips were read on a QuantStudio 3D Digital PCR System. The copy number per microliter of the reference plasmid DNA solution was evaluated based on digital PCR analysis with QuantStudio 3D AnalysisSuite Software. Three samples of the T_{abs} = 3000 copies/μL reference plasmid DNA solutions were diluted independently from 15 ng/μL of reference plasmid DNA solution and were analyzed in duplicate.

Real-time PCR Analysis. The plasmid DNA was diluted to T_{abs} = 50 copies/2.5 μL (the reference plasmid, hereinafter called "rP") and T_{abs} = 2500 copies/2.5 μL (the high concentration plasmid, hereinafter called "hP"). The PCR reaction mixtures were placed in a 25 μL final volume containing 50 ng of DNA sample or 2.5 μL of "rP" or "hP" and 12.5 μL of the 2× QuantiTect probe PCR Master Mix (Qiagen); each concentration is shown in Table 1 of the primer pairs and TaqMan probes. The amplification plot of "hP" was used to determine the threshold line, and the C_t value of "rP" was used to determine positive samples. These plasmid DNA and genomic DNA samples were always analyzed in the same run in different tubes. Real-time PCR was performed using a 7900HT Real-Time PCR system (Thermo Fisher Scientific) and LightCycler Nano (Roche Diagnostics), and the PCR conditions were as follows: preincubation at 95 °C for 15 min, 38 cycles consisting of denaturation at 95 °C for 0.5 min, and annealing and extension at 68 °C for 1 min. In the 7900HT Real-Time PCR system, the C_t values of "rP" and the DNA samples were calculated by using the threshold line. The threshold line was preliminarily determined automatically only from the amplification data of "hP" (n = 2) to avoid an inappropriately low threshold line in a run. A sample is considered positive if its C_t value is smaller than that of "rP". In the LightCycler Nano (Roche Diagnostics), the C_t values (denoted as Cq in its manual) of the reference plasmids and DNA samples were calculated by autoanalysis. The average C_t value of "rP" was used as the cutoff for determination of a positive sample. To determine the optimal cutoff for positive samples in wheat, buckwheat, and peanuts in real-time PCR, we analyzed 16 replicates of 50 copies/2.5 μL reference plasmid DNA solution and 50 ng of DNA of the 10 ppm (w/w) incurred samples: the retort packed Asari clams or the chicken meatballs with vegetables. The false negative property was calculated from the C_t value of 50 ng DNA of the 10 ppm (w/w) incurred samples and the mean of the two C_t values of "rP" according to the following equation; $P\{N(X_2 - X_1, S_2^2/2 + S_1^2) \leq 0\}$, where X_1 and S_1 are mean and standard deviation of C_t values of 16 replicate incurred sample determined experimentally, respectively, and X_2 and S_2 are mean and standard deviation of C_t values of 16 replicate 50 copies/2.5 μL of the reference plasmid DNA solution, respectively (Figure 1).

Comparison with Reported Methods. To compare the sensitivity of our developed real-time PCR methods with that of reported PCR methods, we also tested the seven 10 ppm (w/w) incurred samples of wheat, buckwheat, and peanuts via PCR using official Japanese methods.[5] Each primer sequence was as follows: Wtr01-5′ (5′-CAT CAC AAT CAA CTT ATG GTG G-3′) and

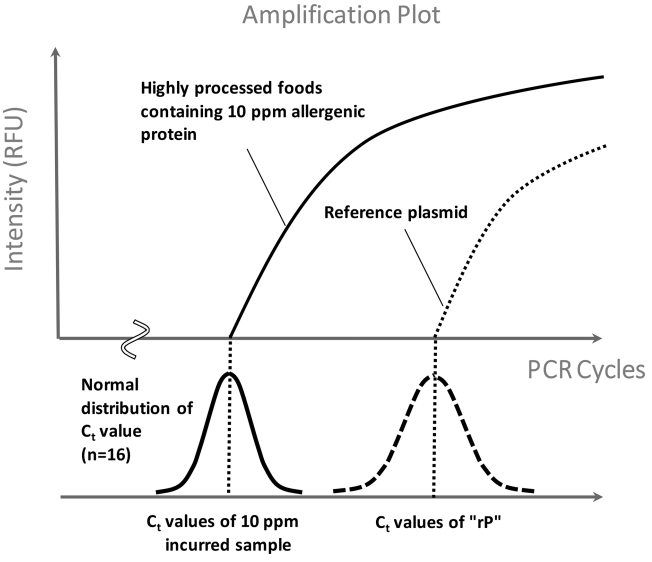

Figure 1. Determination of the optimal copy number of the reference plasmid.

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Table 2. Calculated False Negative Probabilities of the Samples Analyzed in the 7900HT Real-time PCR System[a]

	retort packed Asari clams			retort packed chicken meatballs and vegetables			rP[c] (50 copies)	
	probability (%)[b]	X_1[d]	S_1[e]	probability (%)[b]	X_1[d]	S_1[e]	X_2[f]	S_2[g]
wheat PCR	0.0012	33.30	0.23	0.0000	32.92	0.12	34.50	0.23
buckwheat PCR	0.0024	33.88	0.12	0.0000	33.15	0.11	34.63	0.20
peanut PCR	0.0000	33.54	0.13	0.0000	32.59	0.09	34.59	0.22

[a]All of the false negative probabilities, calculated from the results of LightCycler Nano using the same two 10 ppm (w/w) incurred samples were 0.0000%. [b]The probability that the C_t value of 50 ng of DNA of the 10 ppm (w/w) incurred sample is larger than the mean of the two C_t values of "rP" is calculated using $P\{N(X_2 - X_1, S_2^2/2 + S_1^2) \leq 0\}$. [c]Reference plasmid DNA. [d]Mean of C_t values of 16 replicate incurred samples. [e]Standard deviation of C_t values of 16 replicate incurred samples. [f]Mean of C_t values of 16 replicate 50 copies/2.5 μL of the reference plasmid DNA solution. [g]Standard deviation of C_t values of 16 replicate 50 copies/2.5 μL of the reference plasmid DNA solution.

Table 3. Verification with 10 ppm Incurred Foods

average C_t values (n = 2)	rice gruel	mixed juice	instant miso soup	tomato pasta sauce	freeze-dried vegetable soup	chicken meatballs with vegetables	retort packed Asari clams	rP[c] (50 copies)	hP[d] (2500 copies)
Wheat PCR									
7900HT[a]	24.70	25.02	24.91	26.00	29.04	33.95	33.90	35.23	29.43
7900HT[b]	23.68	24.11	24.03	25.16	28.03	33.13	33.25	34.48	28.87
LightCycler Nano	23.67	23.73	22.46	23.74	26.72	31.67	31.95	32.86	27.60
Buckwheat PCR									
7900HT[a]	25.36	25.71	25.55	27.95	29.70	33.91	33.75	35.47	29.48
7900HT[b]	24.38	24.97	24.73	26.76	28.70	33.13	33.67	35.08	29.04
LightCycler Nano	22.17	22.66	21.68	24.06	26.10	30.94	31.24	32.90	27.06
Peanut PCR									
7900HT[a]	26.39	24.06	26.03	28.57	30.12	32.68	34.11	34.76	28.90
7900HT[b]	25.43	23.33	25.70	28.38	29.43	31.91	33.49	33.80	27.94
LightCycler Nano	23.75	21.54	23.48	26.52	27.63	30.29	31.48	32.05	26.03

[a]Real-time PCR was performed by the National Institute of Health Sciences using a 7900HT Real-Time PCR system (Thermo Fisher Scientific). [b]Real-time PCR was performed by the House Foods Group Inc. using a 7900HT Real-Time PCR system (Thermo Fisher Scientific). [c]Reference plasmid DNA. [d]High concentration plasmid DNA.

Wtr10-3′ (5′-TTT GGG AGT TGA GAC GGG TTA-3′) for wheat detection, FAG19-5′ (5′-AAC GCC ATA ACC AGC CCG ATT-3′) and FAG22-3′ (5′-CCT CCT GCC TCC CAT TCT TC-3′) for buckwheat detection, and agg04-5′ (5′-CGA AGG AAA CCC CGC AAT AAA T-3′) and agg05-3′ (5′-CGA CGC TAT TTA CCT TGT TGA G-3′) for peanut detection. The PCR reaction mixtures were as follows: a reaction volume of 25 μL containing 50 ng of the DNA, 0.2 μM of each primer, 1× PCR buffer II, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.625 U of AmpliTaq Gold (Thermo Fisher Scientific). The PCR conditions were as follows: preincubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 0.5 min, followed by a final extension at 72 °C for 7 min. All of the PCR products were analyzed by MultiNA microchip electrophoresis (Shimadzu).

RESULTS AND DISCUSSION

Incurred Samples Used To Set Cutoffs for Positive Samples. Our real-time PCR methods for wheat, buckwheat, and peanuts are designed to avoid false negatives due to lack of sensitivity and not to determine trace contamination signals as positive. Based on this, we set the positive/negative threshold using highly processed 10 ppm (w/w) incurred samples. It is very difficult to detect target DNA from highly processed foods by PCR because their DNA is severely fragmented.[32] Breaks in DNA occur randomly during food processing, and the proportion of short DNA fragments in highly processed foods is higher than that in common processed foods. As ΔC_t is defined as the C_t value of "long PCR" minus the C_t value of "short PCR", it correlates well with the proportion of short DNA fragments in the sample DNA.[29] The ΔC_t of the five retort packed chicken meatballs and vegetables model samples that were sterilized at 122 °C for 15, 25, 30, 35, and 40 min were 2.5, 5.0, 5.2, 6.5, and 7.0, respectively. The ΔC_t of the two kinds of baby food, soft food for the elderly, and general food were 5.8, 6.3, 4.4, and 4.6, respectively. The average ΔC_t of these commercial food products was 5.2. So, we supposed that the DNA fragmentation level of the common retort packed products containing chicken meatballs and vegetables was at the same level as a sample sterilized at 122 °C for 30 min.

At the laboratory scale, the sterilization condition of retort packed Asari clams was at 122 °C for 33 min, which is equivalent with the F-value of 35 described in the recipe.[30] Therefore, these sterilization conditions were adopted to prepare 10 ppm incurred samples used for the cutoff determination of positive samples of wheat, buckwheat, and peanuts using real-time PCR.

Cutoff for the Determination of Positive Samples. Based on the C_t values obtained from 16 replicates of 50 copies/2.5 μL reference plasmid DNA solution, 50 ng of DNA incurred samples of chicken meatballs with vegetables (10 ppm, w/w) and retort packed Asari clams (10 ppm, w/w) were analyzed simultaneously with the wheat, buckwheat, and peanut real-time PCR, and we determined the optimal cutoff copy number of the reference plasmid DNA as T_{abs} = 50 copies/2.5 μL (rP).

The calculated highest false negative probability of the incurred samples analyzed in all real-time PCR methods was

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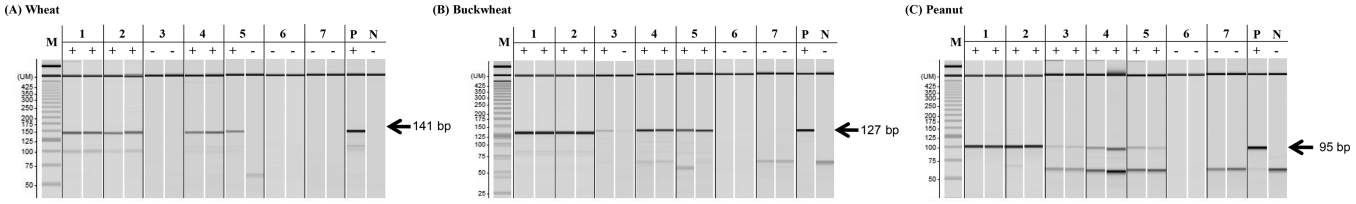

Figure 2. Ten ppm (w/w) incurred samples were tested with three kinds of official PCR methods. (A) Wheat, Japanese PCR method for wheat; (B) Buckwheat, Japanese PCR method for buckwheat; (C) Peanut, Japanese PCR method for peanut; M, DNA marker (25 bp ladder, Thermo Fisher Scientific); N, negative control (no template); P, positive control; + , positive; −, negative; (Samples 1−7) genomic DNA of rice gruel (1), mixed juice (2), instant miso soup (3), tomato pasta sauce (4), freeze-dried vegetable soup (5), retort packed chicken meatballs with vegetables (6), retort packed Asari clams (7). All the DNA samples were analyzed twice (lanes 1−2).

small enough to 0.0024% (Table 2, Asari clams/peanut PCR in 7900HT). In addition, all the calculated false negative probabilities analyzed in the LightCycler Nano were 0.0000% (data not shown).

Specificity and Sensitivity of the Real-time PCR Methods. DNA from 79 food materials was used for this experiment. No amplification signal was observed from 50 ng DNA in any food materials except the target materials (Table S1). For buckwheat and peanut real-time PCR, the amplification signal was observed from 500 fg of DNA of each target plant material (buckwheat or peanut). For wheat real-time PCR, the amplification signal was observed from 50 fg of DNA of wheat. This data suggested that the detection methods for wheat, buckwheat, and peanuts were specific enough for the detection of each target as designed.

In the experiment of the specificity, wheat amplification signals were sometimes observed from DNAs in food materials other than wheat extracted in the clean bench without preliminary treatment of UV irradiation. We realized that a treatment of UV irradiation was useful for reducing trace allergen contamination from laboratory environment.

Analysis of Incurred Samples. The sensitivities of the wheat, buckwheat, and peanut real-time PCR methods were confirmed using seven kinds of 10 ppm (w/w) incurred samples: rice gruel, mixed juice, instant miso soup, tomato pasta sauce, freeze-dried vegetable soup, retort packed chicken meatballs with vegetables, and retort packed Asari clams. As shown in Table 3, the amplification signals were observed from all the incurred samples with the three PCR instruments. These incurred samples (10 ppm, w/w) were determined as positive because their C_t values were smaller than the cutoff C_t values determined with "rP". However, no amplification signal was observed from any of the samples with 0 ppm (w/w).

The results of ELISA analysis showed that all 10 ppm (w/w) incurred samples contained 9.2−14.1 ppm (w/w) wheat protein, 7.2−14.8 ppm (w/w) buckwheat protein, and 4.9−9.2 ppm (w/w) peanut protein as designed. We also confirmed that all of the 0 ppm (w/w) foods were below the LOD of ELISA (1 ppm, w/w). Under Japanese food allergy labeling regulations, specified allergenic ingredients must be declared on the food label when 10 ppm (μg/g or μg/mL) or more of its total protein is present in the food. Therefore, we concluded that our real-time PCR methods for wheat, buckwheat, and peanuts would be sensitive enough as a confirmation method for positive ELISA screening tests. In real-time PCR analysis, threshold lines or C_t values vary among PCR runs or instruments. The cutoff C_t values for positive samples determined with "rP" worked well in different real-time PCR instruments. This makes these methods easy to use in various laboratories without specifying a real-time PCR instrument.

To compare the sensitivity of our real-time PCR methods with that of reported PCR methods, we also analyzed the 10 ppm (w/w) incurred samples with the official Japanese PCR methods for wheat, buckwheat, and peanuts. As shown in Figure 2, 50 ng of DNA of the 10 ppm (w/w) incurred samples from "retort packed chicken meatballs with vegetables" (n = 2/2) and "retort packed Asari clams" (n = 2/2) did not give any wheat, buckwheat, or peanut PCR products of target size and were judged as negative. The 10 ppm (w/w) incurred samples from "miso soup" (n = 2/2) and "freeze dried vegetable soup" (n = 1/2) were judged as wheat negative, and "miso soup" (n = 1/2) was judged as buckwheat negative. These results show that our methods are more sensitive than the official Japanese methods and are thus useful to decrease false negative results.

In summary, we developed specific and highly sensitive real-time PCR methods for detecting wheat, buckwheat, and peanuts. The cutoff C_t values for determining positive samples efficiently nullify positive signals due to trace levels of contaminants from the environment or agricultural products. The cutoff C_t values set in each PCR run using a predetermined copy number of a reference plasmid can also effectively minimize the differences between real-time PCR runs or instruments. Compared to reported methods, our method accurately detects trace amounts of allergenic ingredients corresponding to 10 ppm (w/w) wheat, buckwheat, or peanut protein, especially in highly processed foods. These methods can avoid false negatives due to their sensitivity and are potentially useful for confirming positive ELISA screening tests.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b01234.

Quality check of DNA samples and specific detection using real-time PCR methods (PDF)

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Notes

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■ ABBREVIATIONS USED

PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; C_t, cycle threshold; ITS, internal transcribed spacer; SD, standard deviation

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SUPPORTING INFORMATION

Real-Time PCR Detection Methods for Food Allergens (Wheat, Buckwheat, and Peanuts) Using Reference Plasmids

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Table S1. A: Quality check of DNA samples. B: Specific detection using real-time PCR methods.

Samples		A: Universal PCR			B: Real-time PCR (C_t)		
No,	species (common name)	Plants	Animals	Fungi	Wheat	Buckwheat	Peanut
1	*Fagopyrum esculentum* (common buckwheat)	+	NT	NT	nd	14.79	nd
2	*Fagopyrum tataricum* (Tartarian buckwheat)	+	NT	NT	nd	18.29	nd
3	*Oryza sativa* subsp. *Japonica* (rice)	+	NT	NT	nd	nd	nd
4	*Zea mays* (corn)	+	NT	NT	nd	nd	nd
5	*Triticum aestivum* (wheat)	+	NT	NT	15.21	nd	nd
6	*Hordeum vulgare* (barley, Shunrai)	+	NT	NT	nd	nd	nd
7	*Hordeum vulgare* (barley, fiber-snow)	+	NT	NT	nd	nd	nd
8	*Secale cereale* (rye)	+	NT	NT	nd	nd	nd
9	*Coix lacryma-jobi* var. *ma-yuen* (adlay)	+	NT	NT	nd	nd	nd
10	*Avena sativa* (oat)	+	NT	NT	nd	nd	nd
11	*Setaria italica* (foxtail millet)	+	NT	NT	nd	nd	nd
12	*Panicum miliaceum* (proso millet)	+	NT	NT	nd	nd	nd

13	*Echinochloa esculenta* (Japanese barnyard millet)	+	NT	NT	nd	nd	nd
14	*Amaranthus* (pigweed)	+	NT	NT	nd	nd	nd
15	*Fallopia convolvulus* (wild buckwheat)	+	NT	NT	nd	nd	nd
16	*Glycine max* (soybean)	+	NT	NT	nd	nd	nd
17	*Vigna angularis* (adzuki bean)	+	NT	NT	nd	nd	nd
18	*Phaseolus coccineus* (runner bean)	+	NT	NT	nd	nd	nd
19	*Cicer arietinum* (chickpea)	+	NT	NT	nd	nd	nd
20	*Vigna unguiculata* (black-eyed pea)	+	NT	NT	nd	nd	nd
21	*Phaseolus vulgaris* (common bean)	+	NT	NT	nd	nd	nd
22	*Phaseolus vulgaris* (pinto bean)	+	NT	NT	nd	nd	nd
23	*Arachis hypogaea* (peanut)	+	NT	NT	nd	nd	17.19
24	*Helianthus annuus* (sunflower seed)	+	NT	NT	nd	nd	nd
25	*Piper nigrum* (pepper)	+	NT	NT	nd	nd	nd
26	*Capsicum annuum* (chili pepper)	+	NT	NT	nd	nd	nd
27	*Sinapis alba* L. (mustard)	+	NT	NT	nd	nd	nd

28	*Zingiber officinale* (ginger)	+	NT	NT	nd	nd	nd
29	*Ocimum basilicum* (basil)	+	NT	NT	nd	nd	nd
30	*Trigonella foenum-graecum* (fenugreek)	+	NT	NT	nd	nd	nd
31	*Malus domestica* (apple)	+	NT	NT	nd	nd	nd
32	*Actinidia deliciosa* (kiwifruit)	+	NT	NT	nd	nd	nd
33	*Amygdalus dulcis* (almond)	+	NT	NT	nd	nd	nd
34	*Juglans* (walnut)	+	NT	NT	nd	nd	nd
35	*Macadamia integrifolia* (macadamia nut)	+	NT	NT	nd	nd	nd
36	*Anacardium occidentale* (cashew)	+	NT	NT	nd	nd	nd
37	*Corylus avellana* (hazelnut)	+	NT	NT	nd	nd	nd
38	*Carya illinoinensis* (pecan)	+	NT	NT	nd	nd	nd
39	*Pistacia vera* (pistachio)	+	NT	NT	nd	nd	nd
40	*Pinus koraiensis* (pine nut)	+	NT	NT	nd	nd	nd
41	*Sesamum indicum* (sesame)	+	NT	NT	nd	nd	nd
42	*Aloe vera* (aloe plants)	+	NT	NT	nd	nd	nd

43	*Cucumis melo* (melon)	+	NT	NT	nd	nd	nd
44	*Diospyros kaki* (persimmon)	+	NT	NT	nd	nd	nd
45	*Ficus carica* (fig tree)	+	NT	NT	nd	nd	nd
46	*Vitis* spp. (grape)	+	NT	NT	nd	nd	nd
47	*Rheum rhabarbarum* (rhubarb)	+	NT	NT	nd	nd	nd
48	*Porphyra* (seaweed)	+	NT	NT	nd	nd	nd
49	*Oncorhynchus keta* (salmon)	NT	+	NT	nd	nd	nd
50	*Scomber* (mackerel)	NT	+	NT	nd	nd	nd
51	*Gadus macrocephalus* (cod)	NT	+	NT	nd	nd	nd
52	*Sardinops* (sardine)	NT	+	NT	nd	nd	nd
53	*Todarodes pacificus* (Japanese common squid)	NT	+	NT	nd	nd	nd
54	*Penaeus monodon* (black tiger prawn)	NT	+	NT	nd	nd	nd
55	*Portunidae* (swimming crabs)	NT	+	NT	nd	nd	nd
56	*Mizuhopecten yessoensis* (Japanese scallop)	NT	+	NT	nd	nd	nd
57	*Turbo cornutus* (horned turban)	NT	+	NT	nd	nd	nd

58	*Gallus gallus* (chicken)	NT	+	NT	nd	nd	nd
59	*Sus scrofa* (pork)	NT	+	NT	nd	nd	nd
60	*Bos taurus* (beef)	NT	+	NT	nd	nd	nd
61	*Ovis aries* (mutton)	NT	+	NT	nd	nd	nd
62	*Solanum lycopersicum* (tomato)	+	NT	NT	nd	nd	nd
63	*Dioscorea japonica* (Japanese yam)	+	NT	NT	nd	nd	nd
64	*Solanum tuberosum* (potato)	+	NT	NT	nd	nd	nd
65	*Allium cepa* (onion)	+	NT	NT	nd	nd	nd
66	*Spinacia oleracea* (spinach)	+	NT	NT	nd	nd	nd
67	*Brassica campestris* (Chinese cabbage)	+	NT	NT	nd	nd	nd
68	*Daucus carota* subsp. *sativus* (carrot)	+	NT	NT	nd	nd	nd
69	*Abelmoschus esculentus* (okra)	+	NT	NT	nd	nd	nd
70	*Cucumis sativus* (cucumber)	+	NT	NT	nd	nd	nd
71	*Lentinula edodes* (shiitake mushroom)	NT	NT	+	nd	nd	nd
72	*Agaricus bisporus* (common mushroom)	NT	NT	+	nd	nd	nd

73	*Arctium lappa* (edible burdock)	+	NT	NT	nd	nd	nd
74	*Persea americana* (avocado)	+	NT	NT	nd	nd	nd
75	*Citrus sinensis* (orange)	+	NT	NT	nd	nd	nd
76	*Musa* spp. (banana)	+	NT	NT	nd	nd	nd
77	*Mangifera indica* (mango)	+	NT	NT	nd	nd	nd
78	*Vaccinium corymbosum* (blueberry)	+	NT	NT	nd	nd	nd
79	*Ananas comosus* (pineapple)	+	NT	NT	nd	nd	nd

C_t, Cycle threshold; +, positive detection; NT, not tested; nd, not detected