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Two Quantitative Real-Time PCR Assays for the Detection of Penaeid Shrimp and Blue Crab, Crustacean Shellfish Allergens

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Supporting Information

ABSTRACT: Food allergen detection methods must be able to specifically detect minute quantities of an allergenic food in a complex food matrix. One technique that can be used is real-time PCR. For the work described here, real-time PCR assays were developed to detect penaeid shrimp and blue crab, crustacean shellfish allergens. The method was tested using shrimp meat and crab meat spiked into several types of foods, including canned soups, deli foods, meat, seafood, and prepared seafood products. Foods were spiked with either shrimp or crab at levels ranging from 0.1 to 10^6 parts per million (ppm) and analyzed either raw or cooked by a variety of methods. Real-time PCR data were used to generate linear standard curves, and assays were evaluated with respect to linear range and reaction efficiency. Results indicate that both assays performed well in a variety of food types. High reaction efficiencies were achieved across a linear range of 6−8 orders of magnitude. Limits of detection were generally between 0.1 and 1 ppm. Cooking methods used to simulate thermal processing of foods had little effect on assay performance. This work demonstrates that real-time PCR can be a valuable tool in the detection of crustacean shellfish.

KEYWORDS: *reaction efficiency, internal amplification control, thermal cycling, multiplex*

INTRODUCTION

Food allergy has negative impacts on both health and quality of life. Approximately 4−6% of Americans suffer from some form of food allergy. Allergy to crustacean shellfish affects up to 2% of adults and is the leading cause of food allergy in American adults.[1] Sensitive individuals can have a very strong reaction to even trace amounts of an allergen, so patients rely on prevention of allergic reactions through avoidance of offending foods and information provided on food labels. This necessitates analytical methods that can detect minute quantities of the allergenic food against a high background of nonallergenic material in a complex food matrix.

Detection methods for allergenic foods can be protein-based or DNA-based. Protein-based methods—such as ELISA—detect the allergenic protein itself. DNA-based methods—such as PCR—detect the genome of the allergenic food and therefore serve as a surrogate for the allergen. Whether or not the DNA is a good surrogate depends primarily on two factors: one is whether the allergenic food is inherently high in DNA content; the second is how the allergenic food is used in food products. Of the eight major allergenic foods and food groups identitfied in the Food Allergen Labeling and Consumer Protection Act (FALCPA), four are amenable to DNA-based detection: tree nuts, peanuts, fish, and crustacean shellfish. These four are inherently high in DNA and are likely to be present in foods as the whole plant or animal tissue, which contains both proteins and DNA. In such cases, DNA is a good surrogate for allergenic protein. DNA-based detection is less appropriate for the remaining four allergenic foods: soy and wheat contain DNA, but are often present in food as protein fractions; eggs and milk contain inherently low levels of DNA. The relative advantages and disadvantages of protein- and DNA-based detection methods have been discussed in detail elsewhere.[2,3] Some advantages of PCR-based detection are that DNA can be efficiently extracted from difficult food matrices, and it is less prone to damage under harsh laboratory extraction conditions than proteins. Because DNA is generally more stable than proteins with respect to food-processing techniques, it has the potential to overestimate the allergenicity of a food product. However, this may not always be the case: protein allergenicity can be increased, decreased, or unaffected by food processing. DNA-based assays can help determine whether a given allergenic food has been introduced into a food product at any point, regardless of processing. Finally, PCR-based assays are not limited to the genes that code for allergenic proteins. Gene targets for PCR can be chosen on the basis of desirable sensitivity and specificity characteristics. Specificity can be controlled by the researcher, as PCR-based assays can be designed to differentiate particular species or to detect broad groups of organisms.

Both ELISA and PCR assays have been used for crustaceans. Crustacean ELISA assays do not distinguish one crustacean from another because of similarities in the primary allergenic protein, tropomyosin, among crustaceans.[2,4,5] Brzezinski[6] and Taguchi et al.[5] have developed methods to differentiate crustaceans using end-point PCR and RFLP analysis. Compared to end-point PCR, real-time PCR assays are generally more sensitive and rapid. Monitoring of PCR reactions in real time using an oligonucleotide probe enables

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Table 1. Assay Design Information

description	sequence information
shrimp 12S target	
accession numbers	AF217843, NC_009626, DQ518969, EF584003, EU497054, EU517503, AP006346, DQ534543
forward primer	5′-TTCTAGGTACACTTTCCAGTACACC-3′
reverse primer	5′-TACACATCGCCCGTCGCTCTC-3′
probe	5′-Cy5-ACTATGTTACGACTTATCTCGCTT-Iowa Black RQ-3′
amplicon	TTCTAGGTACACTTTCCAGTACACCTACTATGTTACGACT
	TATCTCGCTTTAATTAACGAGAGCGACGGGCGATGTGTA
shrimp 16S target	
accession numbers	AF217843, NC_009626, DQ518969, EF584003, EU497054, EU517503, AP006346, DQ534543, DQ656600
forward primer	5′-ATAGAAACCGACCTGGCTCACG-3′
reverse primer	5′-TTTAGGGATAACAGCGTAATCTTCTTTG-3′
probe	5′-6-FAM-ATCATGTAA/ZEN/GGATTTAAAGGTCGAACAGACC-Iowa Black FQ-3′
amplicon	AGATAGAAACCGACCTGGCTCACGCCGGTCTGAACTCAAATCATGTAAGGATTTAAAGGTCGAAC
	AGACCCTCCTTTATAACTGCTGCATTATAAGGAAACCTTAATTCAACATCGAGGTCGCAACCCTTC
	CTGTCGATATGGACTCTCAAAGAAGATTACGCTGTTATCCCTAAA
crab CO-1 target	
accession number	NC_006281
forward primer	5′-AGCTCCTGATATAGCCTTCCC-3′
reverse primer	5′-GACCATACCTCTTATTAGTAATAGAGTTAG-3′
probe	5′-Cy5-AACATAAGATTCTGACTCCTACCTCCATCA-Iowa Black RQ-3′
amplicon	AGCTCCTGATATAGCCTTCCCACGAATAAATAACATAAGATTCTGA
	CTCCTACCTCCATCACTAACTCTATTACTAATAAGAGGTATGGTC
crab cyt-b target	
accession number	NC_006281
forward primer	5′-TTTCTCAAGAGTAGCACACATTTGTC-3′
reverse primer	5′-CCAATATGAATGTAGATGCAAATAAAGAAG-3′
probe	5′-6-FAM-AAACTATGG/ZEN/CTGACTATTACGAACTATACATGC-Iowa Black FQ-3′
amplicon	TTTCTCAAGAGTAGCACACATTTGTCGTGACGTAAACTATGGCTGACTATTACGA
	ACTATACATGCTAATGGAGCCTCATTCTTCTTTATTTGCATCTACATTCATATTGG

quantitation, increases specificity, and eliminates the need for post-PCR confirmation of results.[2] Herrero et al.[7] adapted the work of Brzezinski[6] to real-time PCR, although the method of Herrero et al.[7] does not distinguish among different types of crustaceans.

This paper is a report on the development of two probe-based, quantitative real-time PCR assays for the detection of penaeid shrimp and blue crab. Both assays target mitochondrial genes because they are high copy number and can therefore result in a more sensitive assay. In each assay, two different genes were targeted to provide internal confirmation of results and additional data for cross-reactivity analysis. An internal amplification control[8] was included to account for the potential presence of PCR inhibitors from food matrices. Assay performance was determined in various food types including processed and cooked foods. The method was evaluated with respect to reaction efficiency, linear range, and limit of detection.

MATERIALS AND METHODS

Primer and Probe Design. Primers and probes for shrimp and crab were designed from Genbank sequences using Allele ID software (Premier Biosoft, Palo Alto, CA, USA) and obtained from Integrated DNA Technologies (Coralville, IA, USA). For the shrimp assay, 12S and 16S rRNA gene sequences from several species of penaeid shrimp were aligned; primers and probes were designed from conserved regions of the alignment. For the crab assay, primers and probes were designed from cytochrome *b* (cyt-b) and cytochrome oxidase I (CO-I) sequences for blue crab. PCR products ranged from 79 to 176 bp in size. Genbank accession numbers used in assay design as well as primer, probe, and amplicon sequences are reported in Table 1. For shrimp, species used in primer and probe design included *Penaeus monodon* (AF217843), *Litopenaeus vannamei* (NC_009626, EF584003, and DQ534543), *Fenneropenaeus chinensis* (DQ518969), *Farfantepenaeus californiensis* (EU497054), *Litopenaeus stylirostris* (EU5176503), and *Marsupenaeus japonicus* (AP006346). Primers and probes for crab were designed using a single sequence for *Callinectes sapidus* (NC_006281). These Genbank accession numbers refer to sequences for complete mitochondrial genomes; sequences for the genes of interest were manually extracted from the complete mitochondrial genome sequences prior to primer and probe design. The genes used for primer and probe design are not allergen genes. Early work on the shrimp assay was carried out using primers and probes targeting both the mitochondrial 16S gene and the nuclear gene that codes for the major crustacean allergen, tropomyosin. This early work showed that C_T values obtained using the tropomyosin gene were delayed by approximately four compared to C_T values obtained using the 16S gene (Figure S1 in the Supporting Information). This corresponds to a >10-fold difference in assay sensitivity. For the work described in this paper, mitochondrial genes were targeted because they are high copy number and therefore result in a more sensitive assay. The internal amplification control (IAC) used for this work is described in Deer et al.[8] Primers, probe (5′-HEX-AGCTAGTCGATGCACTCCAGT-CCTCCT-Iowa Black FQ-3′), and ultramer template DNA for the internal control were obtained from Integrated DNA Technologies.

Sample Preparation and DNA Extraction. For assay validation in foods, several different types of foods were tested, including seafood, meat, soups, deli foods, and prepared seafood products. For 10-fold serial dilution experiments, foods were spiked with crustacean meat at levels of 0.1, 1, 10, 100, 1000, 10^4, and 10^5 ppm prior to cooking and homogenization; for 2-fold serial dilution experiments, foods were spiked with crustacean meat at levels of 0.09, 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 ppm. One sample consisting entirely of crustacean meat (10^6 ppm) was also included in most experiments as a control not subject to the effects of the food matrix. Cooking

Table 2. Thermal Profile Data

	16S (FAM)			12S (Cy5)			IAC (HEX)	
thermal profile	range	efficiency (%)	R^2	range	efficiency (%)	R^2	range	$C_T \pm$ SD
shrimp DNA in buffer								
standard	0.005−50000 pg	90.0	0.97	0.005−50000 pg	102.4	0.99	0.005−500 pg	25.29 ± 0.80
modified	0.005−50000 pg	105.5	0.99	0.005−50000 pg	93.0	0.97	0.005−500 pg	24.81 ± 1.46
two-step	0.005−50000 pg	107.7	0.98	0.005−50000 pg	111.9	1.00	0.005−500 pg	25.72 ± 2.55
fast 1	50−5000 pg	47.1	0.58	5−50000 pg	61.3	0.79	0.005−500 pg	25.18 ± 3.44
fast 2	no amplification	n/a	n/a	misc amplification	n/a	n/a	0.005−500 pg	29.43 ± 2.42
shrimp spiked into salmon								
standard	$0.1-10^6$ ppm	97.9	0.97	$0.1-10^6$ ppm	101.8	0.95	$0.1-10^5$ ppm	24.41 ± 0.56
modified	$10-10^6$ ppm	79.4	0.98	$10-10^6$ ppm	76.4	0.95	$0.1-10^5$ ppm	24.75 ± 1.22
two-step	$10-10^6$ ppm	91.4	0.97	$1-10^6$ ppm	96.2	0.97	$0.1-10^5$ ppm	23.21 ± 1.47
fast 1	$100-10^6$ ppm	115.6	0.87	$10-10^6$ ppm	83.2	0.56	$0.1-10^5$ ppm	24.35 ± 1.40
fast 2	no amplification	n/a	n/a	$100-10^6$ ppm	97.0	0.37	$0.1-10^5$ ppm	25.98 ± 1.78

methods were used to help evaluate the effects of thermal processing; these included baking, boiling, microwaving, and autoclaving. Autoclaving was used to simulate the high pressure and temperature used in the canning process. Samples were mixed with 4 mL of buffer per gram of food matrix, homogenized in a commercial laboratory blender on high speed for 2−3 min, and stored at −80 °C until use. Meat and seafood samples were homogenized directly in 2% SDS. Soups and deli foods were homogenized in water to prevent excess foaming, and SDS was added to a final concentration of 1% for the last 15−30 s of homogenization.

DNA extraction was carried out using a hybrid of the method described by Aljanabi and Martinez[9] and the DNeasy kit (Qiagen, Valencia, CA, USA). First, 200 μL of homogenate was mixed with 50 μL of 5× salt homogenizing buffer and 10 μL of proteinase K (Thermo Scientific Fermentas, Glen Burnie, MD, USA), incubated at 55 °C for 4 h−overnight, and subjected to a salt precipitation step.[9] DNA was purified from the supernatant using spin columns in the DNeasy kit (Qiagen) according to the manufacturer's instructions. DNeasy columns were subject to two additional washes with 80% ethanol prior to elution of DNA. DNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer (NanoDrop/Thermo Scientific, Wilmington, DE, USA). Negative control (blank) samples were included for both homogenization and DNA extraction.

PCR. Real-time PCR was carried out using an Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). For evaluation of PCR performance, standard curves were generated using serial dilutions of only target (shrimp or crab) DNA in TE buffer. These ranged from 0.005 to 50000 pg (50 ng) of genomic DNA per reaction. For assay validation in foods, 50 ng total DNA was used in all reactions. A no-template control was included in all PCR experiments. PCR conditions were optimized for this work. Each reaction contained 1× PCR buffer, 4 mM Mg, 4% glycerol, 3% DMSO, 30 nM ROX reference dye, and 1.2 mM dNTPs. Platinum Taq polymerase (Invitrogen/Life Technologies, Grand Island, NY, USA) was added to a final concentration of 0.05 unit/μL, or 0.625 unit in a 12.5 μL reaction. Primers and probes for both target genes and the internal amplification control were included at 200 nM. The internal amplification control (IAC) template was held constant at 5×10^5 copies per reaction in all PCR reactions for the shrimp assay; it was held constant at 1.7×10^5 copies per reaction for the crab assay.

Thermal cycling consisted of an initial cycle at 95 °C for 10 min followed by 40−45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 60 or 65 °C for 2 min. Fluorescent signal was read at the end of the annealing step. Whether extension was carried out at 60 or 65 °C did not have a significant effect on data in brief side-by-side tests (data not shown); 65 °C was used for most PCR experiments reported here. In all, five different thermal profiles were tested with the shrimp assay using both standard curves of shrimp DNA in buffer and DNA from spike experiments (Table 2). Thermal profiles were as follows: standard, 95 °C/15 s + 55 °C/30 s + 65 °C/120 s; modified, 95 °C/15 s + 55 °C/30 s + 72 °C/60 s; two-step, 95 °C/15 s + 60 °C/120 s (read fluorescence after 30 s at 60 °C); fast 1, 95 °C/10 s + 55 °C/20 s + 72 °C/20 s; and fast 2, 95 °C/1 s + 60 °C/20 s. Fluorescent signal was read at the end of the annealing (second) step in all cases, except as noted for the two-step profile. All thermal profiles included an initial step at 95 °C for 10 min and 45 cycles. Combined data for two to three independent PCR runs are reported for each condition.

Data Analysis. PCR data were analyzed using MxPro software (Agilent Technologies). C_T values were determined from amplification curves and plotted as a function of log DNA quantity (for PCR performance) or ppm spiking level (for assay validation in foods). This generated linear standard curves for both crustacean target genes in each assay. Statistical R^2 values were calculated for the best-fit line. Reaction efficiencies were calculated using the equation $E = 10^{(-1/m)} - 1$, where E = reaction efficiency and m = slope of the linear standard curve.[11] An ideal real-time PCR reaction has an efficiency of 100%; the goal for assay development is to achieve reaction efficiencies between 90 and 110%. The IAC was expected to amplify with a constant C_T value regardless of crustacean spiking level. For the IAC, statistical average (avg) and standard deviation (SD) were calculated for the optimal range.

RESULTS AND DISCUSSION

PCR Performance. To test primers and probes and to find optimal PCR conditions, PCR performance was initially determined using serial dilution of target crustacean DNA in TE buffer. C_T values were determined from amplification plots (Figures 1a and 2a) and used to generate linear standard curves (Figures 1b and 2b). Data were analyzed as described above. The standard curve data (Figures 1b and 2b) show that for both gene targets in both the shrimp and crab assays, the PCR was linear over 7 orders of magnitude, with R^2 values >0.99 and reaction efficiencies between 96.8 and 104.5%. In each assay, C_T values for both gene targets were similar as both targeted mitochondrial genes.

The internal control was added to PCR reactions at a constant copy number within each assay (shrimp or crab); it was therefore expected to amplify with the same C_T value regardless of the amount of crustacean DNA. Data for the internal control (Figures 1b and 2b) indicate that it amplified consistently in most samples with C_T values of approximately 24−25. Amplification of the internal control was inhibited in samples containing high amounts of target crustacean DNA. Further optimization of PCR reaction conditions did not relieve this inhibition. The internal control was included to help evaluate samples in which amplification of target crustacean

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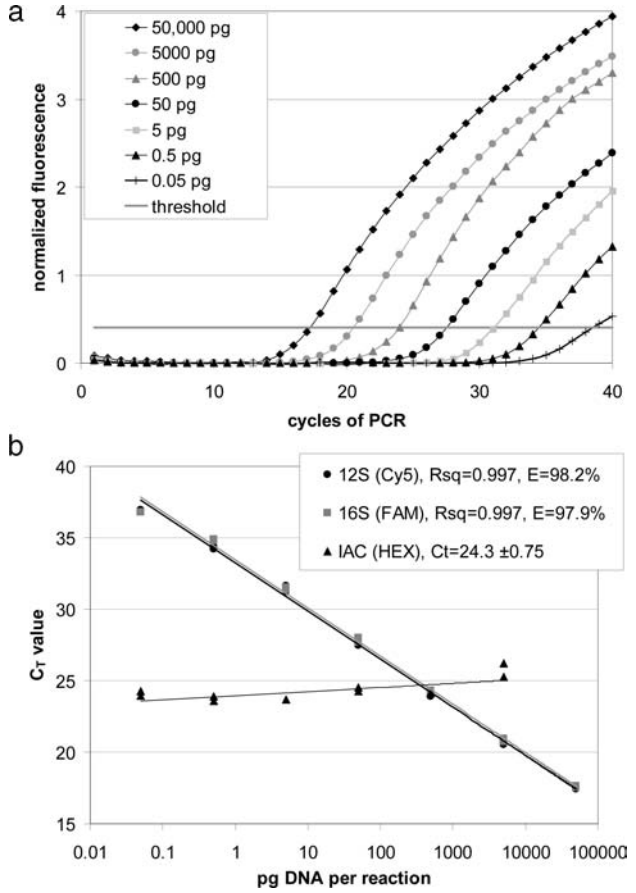

Figure 1. Shrimp assay PCR performance: (a) amplification plots generated using serial dilution of shrimp DNA in buffer (amplification plots are shown only for the 16S gene target for clarity; all PCR reactions were run with two crustacean targets (Table 1) as well as the internal control);[8] (b) standard curves for shrimp assay (data are shown for both gene targets as well as the internal control).

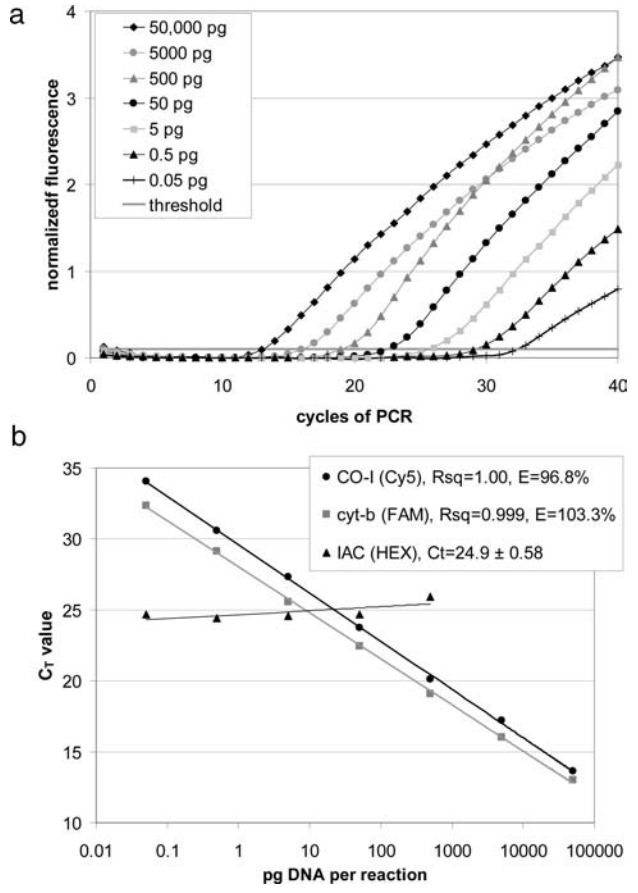

Figure 2. Crab assay PCR performance: (a) amplification plots generated using serial dilution of crab DNA in buffer (amplification plots are shown only for the cyt-b gene target for clarity; all PCR reactions were run with two crustacean targets (Table 1) as well as the internal control);[8] (b) standard curves for crab assay (data are shown for both gene targets as well as the internal control).

DNA was weak or absent. Therefore, its inhibition by strong target amplification was not considered problematic. Furthermore, in three independent PCR tests using only the internal control, amplification was linear over 8−9 orders of magnitude, R^2 values were 0.98−0.99, and reaction efficiencies were 93−106% (for an example, see Figure S2 in the Supporting Information). This shows that the internal control amplified with high efficiency under the PCR conditions used for this work. Copy numbers of IAC template added to PCR reactions were well within its linear range.

The effect of different thermal profiles on PCR performance was determined using the shrimp assay; thermal profiles were tested using both shrimp DNA diluted in TE buffer and DNA from spike experiments (Table 2). Details of thermal cycling are given under Materials and Methods. Amplification of the internal control (IAC) was consistent in all conditions with one exception (DNA in buffer, fast 2 profile). For the shrimp targets, linear range, reaction efficiency, and statistical R^2 values for the best-fit line were adversely affected to some extent using the modified and two-step profiles; data were adversely affected to a great extent using the fast 1 and fast 2 profiles. Using the fast 2 thermal profile, the 12S target was amplified in some cases, whereas the 16S target was not amplified at all. This is likely because of differences in amplicon size (Table 1): the 16S target (176 bp) is longer than the 12S target (79 bp) and therefore less likely to amplify well with shorter cycling. The thermal profile data indicate that, as reported by Hilsher et al.,[10] faster, shorter cycling times compromised assay performance. The standard thermal profile was used for assay validation in foods.

Assay Validation in Foods. *10-fold Serial Dilution Experiments.* Assay performance was determined in a variety of raw and cooked food matrices for both shrimp (Table 3) and crab (Table 4). Linear standard curves were generated by plotting C_T as a function of parts per million spiking level and analyzed as described above. The optimal linear range for each target as well as the R^2 and reaction efficiency values for that linear range are reported. Both shrimp and crab assays performed well in all food matrices and cooking conditions tested. Linear ranges spanned 6−8 orders of magnitude. Reaction efficiencies >90% and R^2 values >0.95 were observed in nearly all cases. In general, cooking methods used to simulate thermal processing had little effect on assay performance. Autoclaving, however, did result in slightly higher limits of detection and lower reaction efficiencies. It is important to note that for assay validation in foods, the *x*-axis values on standard curves (ppm) came from initial spiking of samples, which was done prior to cooking, homogenization, and DNA extraction. Therefore, results for assay validation in foods reflect the performance of not only the PCR but also the DNA extraction

Table 3. Assay Validation in Foods/Shrimp, 10-fold Serial Dilution Experiments

		16S (FAM)			12S (Cy5)			IAC (HEX)	
food matrix	cooking	range (ppm)	efficiency (%)	R^2	range (ppm)	efficiency (%)	R^2	range (ppm)	$C_T \pm SD$
canned soups									
Manhattan clam chowder	uncooked	$0.1-10^6$	102	0.97	$0.1-10^6$	104	0.99	$0.1-10^4$	23.43 ± 0.73
Manhattan clam chowder	autoclave 121 °C, 10 min	$10-10^5$	86	0.99	$0.1-10^5$	85	0.99	$0.1-10^5$	22.10 ± 0.57
New England clam chowder	uncooked	$0.1-10^6$	97	0.99	$0.1-10^6$	104	0.99	$0.1-10^5$	20.93 ± 0.54
New England clam chowder	boil 15 min	$1-10^6$	89	0.98	$1-10^6$	87	0.97	$0.1-10^6$	22.28 ± 0.58
New England clam chowder	autoclave 121 °C, 10 min	$1-10^5$	86	0.98	$1-10^5$	89	0.98	$1-10^5$	22.92 ± 0.20
deli foods									
macaroni salad	uncooked	$1-10^6$	98	0.97	$0.1-10^6$	119	0.95	$1-10^5$	22.32 ± 0.20
coleslaw	uncooked	$0.1-10^6$	91	0.96	$0.1-10^6$	101	0.99	$0.1-10^6$	21.37 ± 0.89
potato salad	uncooked	$0.1-10^6$	94	0.94	$0.1-10^6$	97	0.96	$0.1-10^4$	23.64 ± 1.06
meat and fish									
ground turkey	uncooked	$1-10^6$	91	0.99	$1-10^6$	89	0.99	$0.1-10^5$	23.82 ± 0.19
salmon	uncooked	$0.1-10^6$	91	0.97	$0.1-10^6$	100	0.99	$0.1-10^5$	22.79 ± 0.23
salmon	baked 400 °F, 25 min	$0.1-10^6$	97	0.99	$0.1-10^6$	117	0.99	$0.1-10^5$	21.85 ± 0.39
prepared seafood products									
fish sticks	uncooked	$1-10^6$	110	0.98	$0.1-10^6$	95	0.99	$0.1-10^5$	23.16 ± 0.77
fish sticks	microwave 700 W, 5 min	$0.1-10^6$	89	0.96	$0.1-10^6$	99	0.97	$0.1-10^5$	25.43 ± 0.60

Table 4. Assay Validation in Foods/Crab, 10-fold Serial Dilution Experiments

		cyt-b (FAM)			CO-I (Cy5)			IAC (HEX)	
food matrix	cooking	range (ppm)	efficiency (%)	R^2	range (ppm)	efficiency (%)	R^2	range (ppm)	$C_T \pm SD$
canned soups									
Manhattan clam chowder	uncooked	$0.1-10^6$	91	0.99	$1-10^6$	97	0.94	$0.1-10^4$	25.93 ± 1.51
Manhattan clam chowder	boil 10 min	$1-10^5$	98	0.99	$1-10^5$	105	0.99	$1-10^5$	23.67 ± 0.22
Manhattan clam chowder	microwave 1500 W, 2 min	$1-10^5$	100	0.98	$1-10^5$	101	0.98	$1-10^5$	21.92 ± 0.14
New England clam chowder	uncooked	$0.1-10^6$	95	0.99	$0.1-10^6$	96	0.99	$0.1-10^6$	21.82 ± 0.34
New England clam chowder	autoclave 121 °C, 10 min	$0.1-10^6$	100	0.99	$0.1-10^6$	103	0.99	$0.1-10^6$	20.34 ± 0.54
deli foods									
coleslaw	uncooked	$0.1-10^6$	90	0.98	$0.1-10^6$	90	0.97	$0.1-10^4$	24.0 ± 0.33
macaroni salad	uncooked	$0.1-10^6$	89	0.99	$1-10^6$	81	0.99	$0.1-10^4$	25.4 ± 0.30
potato salad	uncooked	$0.1-10^6$	108	0.98	$0.1-10^6$	94	0.95	$0.1-10^4$	24.5 ± 0.72
meat and fish									
ground pork	uncooked	$0.1-10^6$	89	0.98	$0.1-10^6$	102	0.97	$0.1-10^4$	23.4 ± 0.43
pork sausage	uncooked	$0.1-10^6$	108	0.98	$0.1-10^6$	99	0.99	$0.1-10^4$	23.6 ± 0.33
salmon	uncooked	$1-10^5$	92	0.95	$0.1-10^5$	94	0.98	$0.1-10^5$	21.95 ± 0.29
salmon	baked 400 °F, 25 min	$1-10^5$	103	0.97	$1-10^5$	102	0.96	$1-10^5$	26.10 ± 0.22
prepared seafood products									
fish sticks	uncooked	$1-10^5$	94	1.00	$10-10^5$	86	1.00	$0.1-10^5$	26.20 ± 0.22
fish sticks	baked 425 °F, 30 min	$1-10^5$	100	0.99	$1-10^5$	100	0.99	$1-10^4$	25.6 ± 0.18
salmon cakes	uncooked	$0.1-10^5$	96	0.96	$0.01-10^5$	103	0.92	$0.1-10^4$	25.4 ± 0.63
salmon cakes	baked 400 °F, 20 min	$0.1-10^5$	110	0.98	$0.1-10^5$	99	0.98	$0.1-10^4$	26.2 ± 0.36

and homogenization procedures. Optimal range and average C_T ± SD are reported for the internal control. As discussed above, amplification of the internal control was inhibited only in samples containing high levels of crustacean DNA, and this was not considered problematic. Overall, this internal control performed well in both the shrimp and crab assays.

2-fold Serial Dilution Experiments. Data reported above (Tables 3 and 4) were generated using 10-fold serial dilutions of crustacean meat in foods with a range that covered 8 orders of magnitude. The method was initially validated in this manner because it is the standard for real-time PCR assay validation: a well-developed real-time PCR assay should be linear over 6−8 orders of magnitude. However, a narrower range in the lower parts per million levels is of more immediate interest to the allergen community. More recent work on this method has been carried out in our laboratory using 2-fold serial dilution of crustacean meat in foods with spiking levels from 0.09 to 100 ppm. These experiments have shown that high reaction efficiencies and statistical R^2 values are achieved in this narrower spiking range (Table 5; Figure S3 in the Supporting Information). Compared to the initial 10-fold serial dilution experiments—which had a 0.1 ppm limit of detection—a limit

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Table 5. Assay Validation in Foods/Shrimp and Crab, 2-fold Serial Dilution Experiments

		16S (FAM)			12S (Cy5)			IAC (HEX)	
food matrix	assay (crustacean detected)	range (ppm)	efficiency (%)	R^2	range (ppm)	efficiency (%)	R^2	range (ppm)	$C_T \pm SD$
canned soups									
Manhattan clam chowder	shrimp	0.39−100	103	0.97	0.39−100	102	0.98	0.39−100	23.35 ± 0.15
Manhattan clam chowder	crab	0.78−100	97	0.92	0.78−100	89	0.91	0.78−100	23.01 ± 0.52
deli foods									
macaroni salad	shrimp	0.78−100	101	0.94	1.56−100	93	0.95	0.78−100	23.73 ± 0.17
macaroni salad	crab	0.78−100	106	0.97	0.78−100	93	0.96	0.78−100	23.79 ± 0.19

of 0.39−0.78 ppm was needed in these experiments to maintain high reaction efficiencies and R^2 values. Further work on this method will include validation using this narrow spiking range with a wider variety of food matrices and cooking conditions.

Brief tests of cross-reactivity were carried out for each assay using 500 pg of DNA from shrimp, blue crab, lobster, and crayfish purchased at local markets (Table S1 in the Supporting Information) and show that both the shrimp and crab assays have low cross-reactivity with other crustaceans. For the shrimp assay, some amplification was observed in blue crab for the 12S but not the 16S target; this underscores the utility of including two targets in each assay. Future work on this project will include more extensive cross-reactivity testing using vouchered samples collected as part of an ongoing collaboration with colleagues at FDA and other institutions. Overall, these results demonstrate that the real-time PCR method described here works well under a variety of conditions, including complex, highly processed foods and foods subjected to stringent conditions such as autoclaving. They also underscore the versatility of an internal control developed by our colleagues at FDA.[8] Use of this internal control is straightforward and cost-effective, and it can be readily adapted for use with any real-time PCR assay. Overall, this work shows that real-time PCR can be a valuable tool in the detection of crustacean shellfish.

■ ASSOCIATED CONTENT

Supporting Information

Additional table and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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