

High-Throughput Identification of Allergens in a Food System via Hybridization Probe Cluster-Targeted Next-Generation Sequencing

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ABSTRACT: Food allergies (FAs) are a crucial public health problem and a severe food safety issue, resulting in an urgent need for an accurate method to detect all of the hidden allergens that exist in food systems. Current methods for detecting allergens typically utilize ELISA, PCR, or LC-MS, which are suitable for the confirmatory analysis of allergens from ingredients rather than unintended contaminants. In this study, we demonstrate a hybridization probe cluster-targeted next-generation sequencing (HPC-NGS) platform for high-throughput screening of potential allergens in food systems. The HPC-NGS successfully captured target DNA fragments and identified 19 allergenic ingredients in a complex food system. Additionally, the HPC-NGS provided expected allergenic species matching rates of 94.24–100% in single food materials and 99.87–99.98% in processed food products. Thus, HPC-NGS enables the accurate characterization of allergenic ingredients and unintended allergenic contaminants in foods. Our results provide new perspectives on the use of HPC-NGS in the accuracy of high-throughput detection technologies for allergens imposed by the complex matrix effect.

KEYWORDS: allergens, screening, genome, complex food system

INTRODUCTION

Food allergies (FAs) and anaphylaxis represent increasingly important public health problems in industrialized countries and globally. The prevalence of food allergies in infants is estimated to be 1–5% in European countries and the United States, 7% in China, and over 10% in Australia.¹ To date, more than 989 proteins have been registered as allergens by the World Health Organization and the International Union of Immunological Societies (WHO/IUIS, www.allergen.org). Affected individuals can experience immunoglobulin E (IgE)-mediated food hypersensitivity, which results in severe acute systemic anaphylaxis, potentially including asthma, urticaria, gastrointestinal spasms, hypotension, and even shock.² Concerns related to these reactions have led to calls for more protection from accidental exposures to food allergens for affected individuals. According to the FDA, advanced processing technologies and complex source materials with “hidden” allergens may result in 30 000 emergency room visits, 2000 hospitalizations, and 150 deaths each year from accidental exposure to food allergens in the United States.³ Precautionary allergen labeling, therefore, is increasingly valued by all countries. In accordance with several countries and organizations,^{4–8} mandatory labeling of allergenic ingredients is required to be highlighted on food packages, which are included in the food recipe knowingly. Besides, the “May Contain” precautionary statement is voluntarily applied to indicate the presence of unintended allergenic ingredients on the food labels, which might be present due to unintentional cross-contamination during production, transformation, or processing.⁹ To guarantee the accuracy of the information provided about food ingredients and composition, managing

allergen risks, therefore, requires appropriate analytical methods for full risk assessment of unintended allergenic ingredients throughout the food supply chain.

Food allergens generally result from three sources: food materials, cross-contamination during transportation and storage, and cross-contamination of other foods from the same production line.^{10,11} Multiple sources of ingredients and unintended allergens from cross-contamination can increase the chance of missed detection, and allergen extraction is difficult because the abundant components in a food matrix can mask allergens. Furthermore, food processing may alter proteins and degrade DNA, resulting in decreased reliability of these conventional detection techniques in processed foods. All in all, there is an urgent need for reliable analytical methods enabling screening of intended and unintended allergens in food products, followed by confirmatory analysis. Current methods, such as immune-based [e.g., enzyme-linked immunosorbent assay (ELISA)], protein-based [e.g., liquid chromatography-mass spectrometry (LC-MS)], and DNA-based [e.g., polymerase chain reaction (PCR)] methods, provide relatively sensitive and reliable detection for the identification and confirmatory analysis of allergens.^{12–14} ELISA and LC-MS are the standard protocols for directly quantitating allergens in food ingredients rather than processed foods since some

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proteins and peptides may be degraded during food processing steps implying heating.^{15,16} The incomplete proteoform database for allergens also limits the application of LC-MS in food allergen analysis.¹⁷ DNA-based methods are indirect detection approaches as screening tools to trace the presence of allergenic ingredients by capturing segments of the gene encoding allergenic proteins or any specific DNA of the allergenic foods.^{18,19} Direct-multiplex PCR,²⁰ TaqMan real-time PCR,²¹ and LAMP²² methods have been used for identifying the plant- and animal-derived allergenic foods. The primers and probes are mainly designed upon single-copy or multicopy genes coding for allergenic proteins. However, PCR amplification could not be obtained from food products because of the presence of PCR inhibitors (e.g., fat, sugar) and the removal and degradation of target DNA fragments during processing. While all of the previously mentioned methods produce relatively accurate results, they have limited applicability in high-throughput analyses of unintended allergenic contaminants in complex food systems. Therefore, a high-throughput, reliable, and highly sensitive method to trace potential allergenic ingredients in intensively processed food is urgently required for food traceability and food allergen risk management.

Next-generation sequencing (NGS) is a platform that enables the simultaneous analysis of multiple different genomic regions as well as detection of minor variants within heterogeneous mixtures, to enable the identification of all plant, animal, fungal, and microalgae ingredients in foods.^{23,24} NGS technologies have been widely used in the diagnosis of health disorders,²⁵ tumor mutation identification,²⁶ and traceability of aquafeed.²⁷ Hence, NGS based on single nucleotide polymorphism (SNP) detection has provided new opportunities for verifying trace allergenic ingredients in food products throughout the supply chain.²⁸ We hypothesized that NGS would overcome the limitations of other DNA-based methods and serve as a highly accurate method for the high-throughput analysis of intended and unintended allergenic ingredients in complex food systems.²⁹ To test this approach, public sequences of allergens were collected to design specific probe clusters to accurately identify allergenic ingredients from high-throughput metagenomic sequencing reads across plant and animal sources. We then provide a high-throughput strategy for allergen detection: the “hybridization probe cluster-targeted next-generation sequencing” (HPC-NGS) system. As a highly specific high-throughput method, HPC-NGS is able to screen the potential allergens in foods to address multiple issues in food traceability and food allergen risk management, which could be significantly advantageous for food processors, manufacturers, and food safety control departments.

In this paper, we provide a strategy for high-throughput screening of allergenic ingredients and unintended allergenic contaminants in food products by advancing HPC-NGS. First, using NCBI, we built a comprehensive genome collection containing 886 common plant and animal allergens, which enables accurate species detection directly from sequencing data. We then designed probes and screened them on total DNA extracted from food products for identifying each allergenic species. The highest-performing probe sets were chosen based on their ability to capture DNA hybridization and identify allergenic species. The probe set is a key component for use with high-throughput total DNA sequencing and simultaneous identification of multiple

allergenic ingredients and allergenic contaminants in food products. Finally, we demonstrate the applicability of HPC-NGS using the example of a panel of 24 raw single food ingredients, one mixed matrix containing 19 raw ingredients, and three highly processed foods relevant to food allergies.

MATERIALS AND METHODS

Construction of a Comprehensive Allergen Database. All sequences of the 886 allergens and variants from 175 species were downloaded from NCBI's RefSeq on March 13, 2019.

Total DNA Extraction. The total DNA of seven animal tissues (beef, chicken, shrimp, crab, oyster, grass carp, and tilapia) was extracted using a Rapid Animal Genomic DNA Isolation Kit (Sangong Biotech, Shanghai, China). The animal tissues (50 mg) were ground into a powder with liquid nitrogen and then mixed with 400 μ L of digestion buffer in a 1.5 mL centrifuge tube. After heating in a water bath for 1 h at 65 °C, 200 μ L of Buffer PA was added and mixed well for 5 min at −20 °C. Next, the extract was centrifuged in a Microfuge 22 R Centrifuge (Beckman Coulter, Mississauga, ON) at 10 000 rpm at 25 °C for 5 min. The supernatant was mixed well with an equal volume of isopropyl alcohol and centrifuged at 10 000 rpm at 25 °C for 5 min after stationary incubation for 3 min at 25 °C. The precipitate was rinsed three times with 70% ethanol and centrifuged at 10 000 rpm at 25 °C for 2 min. After removing the supernatant, the total DNA was dissolved in 100 μ L of TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at −20 °C after the residual ethanol had completely evaporated.

Total DNA extraction from 23 plant tissues (short ragweed, silvery wormwood, mugwort, turnip, apricot, birch, apple, cherry, peach, cashew, wheat, buckwheat, pea, pepper, soybean, mung bean, timothy, sesame, peanut, walnut, celery, hazelnut, and canary grass) was performed using a Rapid Plant Genomic DNA Isolation Kit (Sangong Biotech, Shanghai, China). The plant tissues (100 mg) were first ground into a powder with liquid nitrogen, and then 400 μ L of Buffer PCL and 8 μ L of β -mercaptoethanol were added before heating in a water bath for 45 min at 65 °C. Next, 200 μ L of Buffer PP was added and mixed well for 5 min at −20 °C before centrifugation at 10 000 rpm at 25 °C for 5 min. The supernatant was mixed well with 500 μ L of chloroform and centrifuged at 12 000 rpm at 25 °C for 5 min. The total DNA was dissolved in 100 μ L of TE Buffer and stored at −20 °C after rinsing with isopropyl alcohol and 70% ethanol.

Total DNA extraction from three highly processed food products (hazelnut chocolate, spaghetti, and cookies) was performed using a Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China). Each food sample (100 mg) was first ground into a powder with liquid nitrogen, and then 700 μ L of Buffer GP1 and 0.1% (v/v) β -mercaptoethanol were added before heating in a water bath for 20 min at 65 °C. Then, 700 μ L of trichloromethane was added and mixed well before centrifugation at 12 000 rpm at 25 °C for 5 min. The supernatant was mixed well with 700 μ L of Buffer GP2, transferred into a Spin Column CB3, and centrifuged at 12 000 rpm at 25 °C for 30 s. The precipitate was then washed with Buffer GD and Buffer PW in turn in a Spin Column CB3 and centrifuged at 12 000 rpm at 25 °C for 30 s. The total DNA was dissolved in 100 μ L of TE Buffer and stored at −20 °C after centrifugation at 12 000 rpm at 25 °C for 30 s. The DNA concentrations of each sample were listed in Table S1.

Sample Preparation. The mixed matrix was prepared by mixed total DNA from 19 raw allergenic ingredients (celery, peanut, short ragweed, silvery wormwood, mugwort, beef, turnip, chicken, soybean, shrimp, apple, canary grass, timothy, pea, apricot, cherry, canary grass, peach, crab) in equal volume. The spiked samples were prepared by mixing the total DNA of hazelnut chocolate and hazelnut in a volume ratio of 1:0, 1:1, 1:2, and 1:4. The contaminated sample was prepared by mixing the total DNA of hazelnut chocolate and birch pollen in a volume ratio of 1000:1.

DNA Library Construction. Sample DNA library construction was performed using DNA library preparation kits (Molbreeding, Shijiazhuang, China). The sample DNA (50 μ L) was first added into

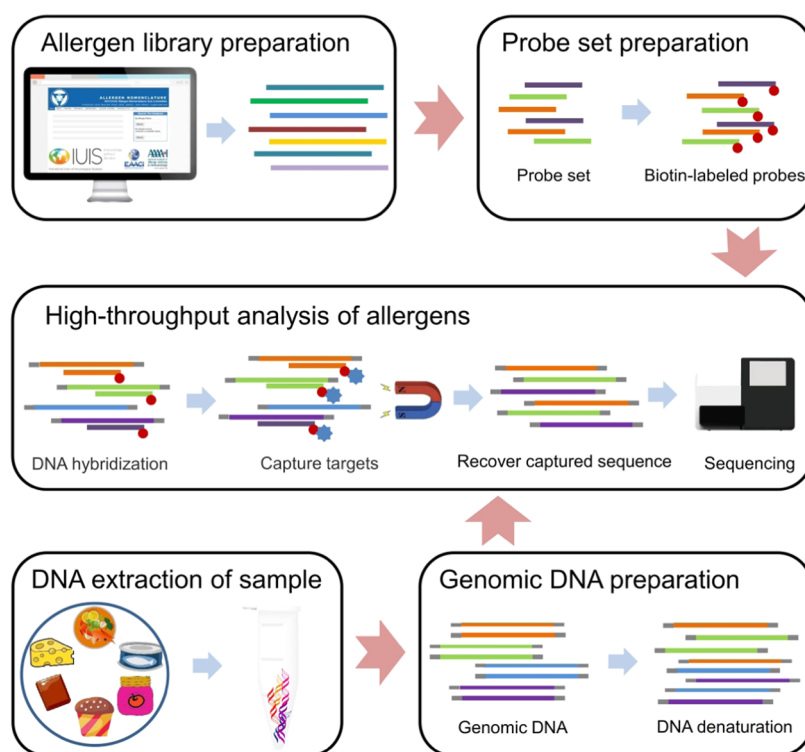


Figure 1. Workflow for analysis of allergens in sample DNA library using our high-throughput detection platform.

the system tube before the system tube was loaded into the adaptive connector. After placing the system tube in the tank, the DNA was fragmented into DNA fragments with target bp (200–300 bp) using S220 focused-ultrasonicator (Thermo Fisher Scientific, Massachusetts). Operating conditions were as follows: duty factor, 10%; peak power, 175; cycles per burst, 100; and treatment time, 60 s. Then, the DNA fragments were purified using immunomagnetic beads. The concentrations of purified DNA were determined using a Qubit RdsDNA HS Assay Kit and analyzed by agarose electrophoresis using AlphaView SA 3.4.0 software (ProteinSimple, California). The purified DNA (1–200 ng) was then mixed with 4 μ L of end repair buffer, 2.7 μ L of end repair enzyme, and nuclease-free water to make up a total volume of 20 μ L. End repair reactions were performed in a Veriti 96-well Thermal Cycler (Thermo Fisher Scientific, Massachusetts). The PCR was performed at 37 $^{\circ}$ C for 20 min and at 72 $^{\circ}$ C for 20 min with a final hold at 4 $^{\circ}$ C. Next, 2 μ L of DNA ligase, 8 μ L of Ultra DNA Ligase Buffer, 2 μ L of adapter, and 8 μ L of nuclease-free water were added in the same reaction tube. For joining reactions, the PCR was performed at 22 $^{\circ}$ C for 60 min, followed by a final hold at 4 $^{\circ}$ C. Then, 48 μ L of DNA clean beads were added in the same reaction tube for stationary incubation for 5 min before the precipitate was obtained using a magnetic shelf. The precipitate was rinsed with 100 μ L of 80% ethanol and incubated at 25 $^{\circ}$ C for 30 s. After removing the supernatant, the DNA was obtained after the residual ethanol evaporated completely. To amplify the DNA library, 20 μ L of a mixed reaction solution (10 μ L of PCR Master Mix, 1 μ L of unioliigo-i5, 1 μ L of DNA index-i7, 8 μ L of nuclease-free water) was added, and the DNA clean beads were washed and resuspended. Next, PCR amplification was performed; the PCR consisted of initial denaturation at 98 $^{\circ}$ C for 2 min, followed by 5–10 cycles at 98 $^{\circ}$ C for 30 s for denaturation, annealing at 65 $^{\circ}$ C for 30 s, extension at 72 $^{\circ}$ C for 40 s, and a final extension at 72 $^{\circ}$ C for 4 min. To purify the DNA library, 20 μ L of DNA clean beads was added in the same reaction tube for stationary incubation for 5 min before the precipitate was obtained using a magnetic shelf. The precipitate was rinsed with 100 μ L of 80% ethanol and incubated at 25 $^{\circ}$ C for 30 s. After removing the supernatant, the DNA library was dissolved in 30 μ L of resuspension buffer after the residual ethanol evaporated completely.

Hybrid Capture of Allergen DNA Library. We synthesized the 7482 probes in the probe set using the SeqCap EZ Developer platform (Roche, Basel, Switzerland). These biotinylated ssDNA probes were directly employed for hybrid capture experiments using a GenoBaits probe hybridization kit (Molbreeding, Shijiazhuang, China). According to the manufacturer's instructions, we first prepared a Library Master mix (LM) buffer (5000 ng of block I, 500 μ M of I7 for ILM/MGI 500, 500 μ M of I5 for ILM/MGI, 500 ng of DNA library), Hybridization Master mix (HM) buffer (20 μ L of RNA Hyb buffer I, 0.8 μ L of RNA Hyb buffer II, 8 μ L of RNA Hyb buffer III, 8 μ L of RNA Hyb buffer IV), and Capture Baits Master mix (CM) buffer (3.5 μ L of biotinylated probe set (4 pM), 0.5 μ L of block III, 2 μ L of ultrapure water). Hybridization was performed using PCR. LM buffer was heated at 98 $^{\circ}$ C for 5 min, followed by the addition of HM buffer at 65 $^{\circ}$ C for 3 min and then CM buffer at 65 $^{\circ}$ C for 2 min; final hybridization was performed at 65 $^{\circ}$ C for 16 h after adding 7 μ L of LM buffer and 13 μ L of HM buffer. To capture the hybrid DNA, probe beads were then mixed with the hybrid system and shaken at 25 $^{\circ}$ C for 30 min after rinsing with 200 μ L of RNA binding buffer three times. The precipitate was obtained using a magnetic shelf after rinsing with 200 μ L of RNA wash buffer I, followed by 200 μ L of RNA wash buffer II at 65 $^{\circ}$ C for 10 min. Next, 20 μ L of ultrapure water was added to resuspend the capture product. After hybridization and capture on streptavidin beads, we amplified the library pools using PCR. The PCR system consisted of 10 μ L of capture product, 20 μ L of PCR master mix, 1.6 μ L of primer mix for ILM/MGI, and 8.4 μ L of ultrapure water. The PCR consisted of initial denaturation at 98 $^{\circ}$ C for 1 min, followed by 18 cycles consisting of denaturation at 98 $^{\circ}$ C for 10 s, annealing at 65 $^{\circ}$ C for 30 s, and extension at 72 $^{\circ}$ C for 60 s, with a final extension at 72 $^{\circ}$ C for 4 min. To purify the captured library, we obtained the supernatant using a magnetic shelf and added 72 μ L of DNA clean beads to the supernatant. Removing the supernatant, the clean beads were rinsed with 200 μ L of 80% ethanol three times and resuspended in resuspension buffer. The captured libraries were obtained in the supernatant and then sequenced on HiSeq Xten platforms (Novogene, Tianjin, China). Next, the sequencing was matched with the DNA sequence of the allergens using bowtie2. The bowtie2

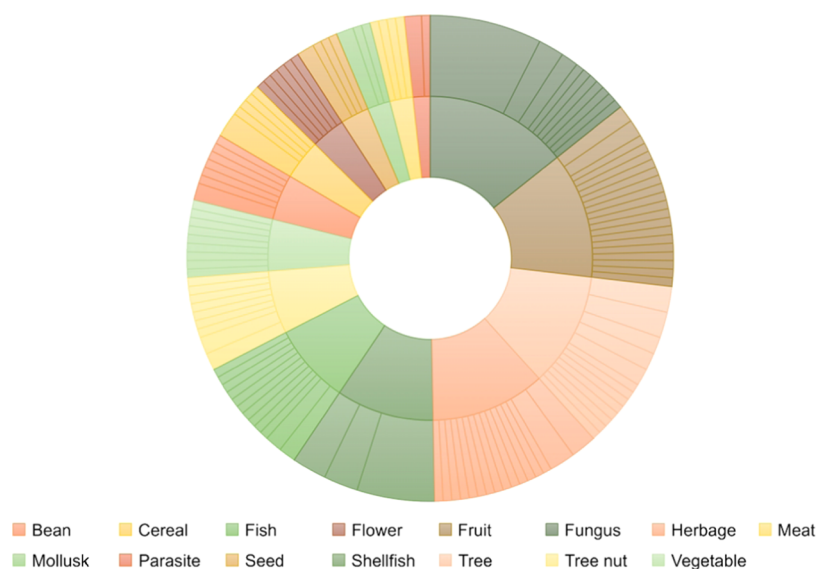


Figure 2. Classification of foodborne allergenic ingredients and nonfoodborne allergenic contaminants.

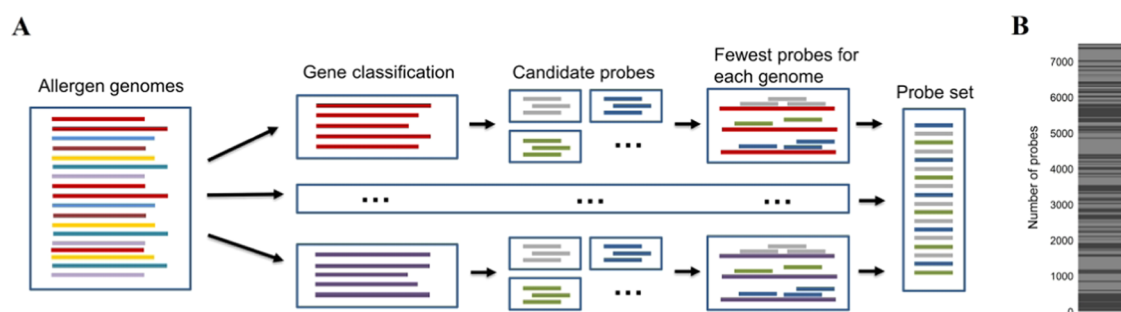


Figure 3. Probe design for capturing allergen genes. (A) Sketch of approach to probe design. (B) Number of probes designed for each allergen among all 7482 probes in the probe set.

search criteria applied were 95% identity over 100 bp of query length, and the read counts at the species level were summarized.

Statistical Analysis. The sequencing data of each biological sample were matched with the target allergen genes using bowtie2 (version 2.1.0), and then the number of reads matched on each target gene was calculated. GraphPad Prism version 7.0, Origin 2018, and R-3.6.1 were used for data analysis.

Data Availability. All other data supporting the findings of this study are available within the article and its Supporting Information files or are available from the authors upon request.

RESULTS

Outline of Method. We developed an experimental approach and provided a probe set to capture target DNA fragments and identify hidden allergenic ingredients in food products (Figure 1). Our approach consisted of NGS relying on the hybrid capture of allergen DNA and accurate sequence alignment using bowtie2 software (Molbreeding, Shijiazhuang, China).

To prepare samples for high-throughput analysis of potential allergens, genomic DNA must first be extracted from raw materials or highly processed foods and then be denatured into properly sized fragments with attached sequencing adaptors. To enhance the capture of diverse allergen DNA fragments, one or more probes were designed for each allergen gene. We synthesized the set of probe sequences as biotinylated single-stranded DNA (ssDNA), which are sensitive and therefore suitable for use in the hybrid capture of allergen DNA to form

a library. The enrichment of target sequences was achieved through hybrid capture, which improved the sensitivity of allergen detection. Finally, the captured DNA library was sequenced using NGS after enrichment, and the sequencing was matched with the DNA sequence of the allergens listed in Table S2 using bowtie2 software.

Specific Hybridization Probe Cluster for Capturing Allergen Diversity. To expand this approach, we designed a probe set to target allergen DNA fragments registered by WHO/IUIS. We first summarized all allergen genes of food ingredients and contaminants from the NCBI GenBank (Table S2), including 123 allergenic ingredients and 175 allergenic species. As shown in Figure 2, 175 allergenic species can be categorized into foodborne allergenic ingredients (bean, cereal, fish, fruit, meat, mollusk, shellfish, seed, tree nut, and vegetable) and nonfoodborne allergenic contaminants (flower, fungus, herbage, parasite, and tree). The probe set was designed based on food allergen genes and can be applied to achieve a more sensitive identification of allergenic ingredients, allergenic species, and contaminants through high-throughput metagenomic sequencing reads. The set of sequences used for oligonucleotide probes can determine whether a probe hybridizes to a target sequence region, and one or more probes are used to confirm the capture of the same target sequence (Figure 3A). The key to determining the final probe set is to find the optimal probes to identify a target sequence with both high sensitivity and high specificity.

Table 1. Allergen Determination in Known Samples from Single Species

sample no.	sequence type	sample name	expected species	observed expected species %	difference of observed from expected %	other species observed at >0.1%
1	total DNA	short ragweed	<i>Ambrosia artemisiifolia</i>	94.24	−5.76	<i>Artemisia argyi</i> 1.36%; <i>Artemisia vulgaris</i> 3.35%; <i>Brassica rapa</i> 0.63%; <i>Sinapis alba</i> 0.42%
2	total DNA	mugwort	<i>Artemisia vulgaris</i>	97.32	−2.68	<i>Artemisia annua</i> 1.35%; <i>Artemisia argyi</i> 1.33%
3	total DNA	birch pollen	<i>Betula verrucosa</i>	95.45	−4.55	<i>Alternaria alternata</i> 2.32%; <i>Cladosporium cladosporioides</i> 0.24%; <i>Cladosporium herbarum</i> 0.73%; <i>Corylus avellana</i> 1.26%
4	total DNA	peach	<i>Prunus persica</i>	99.85	−0.15	<i>Prunus persica</i> 0.15%
5	total DNA	canary grass	<i>Phalaris aquatica</i>	99.86	−0.14	<i>Phleum pratense</i> 0.14%
6	total DNA	pea	<i>Pisum sativum</i>	100	0	
7	total DNA	cashew	<i>Anacardium occidentale</i>	99.59	−0.41	<i>Pistacia vera</i> 0.41%
8	total DNA	apple	<i>Malus domestica</i>	96.84	−3.16	<i>Glycine max</i> 0.58%; <i>Gallus domesticus</i> 0.14%; <i>Prunus dulcis</i> 0.38%; <i>Prunus avium</i> 0.27%; <i>Prunus persica</i> 0.93%; <i>Pyrus communis</i> 0.86%
9	total DNA	sesame	<i>Sesamum indicum</i>	100	0	
10	total DNA	pepper	<i>Capsicum annuum</i>	99.62	−0.38	<i>Cladosporium herbarum</i> 0.38%
11	total DNA	timothy	<i>Phleum pratense</i>	99.61	−0.39	<i>Poa pratensis</i> 0.39%
12	total DNA	mung bean	<i>Vigna radiata</i>	100	0	
13	total DNA	wheat	<i>Triticum aestivum</i>	99.72	−0.28	<i>Triticum turgidum</i> ssp. durum 0.11%; <i>Hordeum vulgare</i> 0.17%
14	total DNA	buckwheat	<i>Fagopyrum esculentum</i>	100	0	
15	total DNA	peanut	<i>Arachis hypogaea</i>	100	0	
16	total DNA	walnut	<i>Juglans regia</i>	97.08	−2.92	<i>Carya illinoensis</i> 1.25%; <i>Juglans nigra</i> 1.67%
17	total DNA	soybean	<i>Glycine max</i>	100	0	
18	total DNA	shrimp	<i>Litopenaeus vannamei</i>	100	0	
19	total DNA	chicken	<i>Gallus domesticus</i>	100	0	
20	total DNA	crab	<i>Scylla paramamosain</i>	100	0	
21	total DNA	carp	<i>Cyprinus carpio</i>	100	0	
22	total DNA	beef	<i>Bos domesticus</i>	100	0	
23	total DNA	tilapia	<i>Oreochromis mossambicus</i>	100	0	
24	total DNA	oysters	<i>Crassostrea gigas</i>	100	0	

To avoid redundancy of probe sequences and to reduce the number of probes that target diversity across 175 allergenic species, we applied BLAST to test food allergen sequence datasets and classified the sequences with more than 120 bp repetitive sequences or at least 90% similarity into one category. For each category, clustalw2 software was employed for multisequence comparison, and then the BioPerl module of Perl language was used to select the consensus sequence of the target allergen genes as the candidate sequences. To reduce cross-hybridization and provide more sensitive detection, the length of the candidate sequences needed to be 120 bp, and the CG content of the candidate sequences was between 30 and 70% without simple repeated sequences. Finally, candidate probes from the target allergen genes were verified (mismatched base number less than 10), and the smallest set

of candidate probes that achieved full coverage of the allergen genes was obtained.

The probe set contained 7482 probes for 763 allergens from 230 species (Figure 3B), including 5624 probes for allergenic protein genes and 1858 probes for plant conserved genes. The sequence of each probe and its corresponding allergen gene is described in Table S3. This method represents a comprehensive coverage of the sequence diversity of allergenic species.

Specificity of HPC-NGS for Allergen Identification in Individual Raw Foods. To evaluate the accuracy of the pipeline across diverse allergenic ingredient types, we applied our designed probe set to detect 24 sequencing libraries using total DNA isolated from 24 known compositions of single plant- and animal-only tissue, respectively. The reads of each allergenic species analyzed from each sample DNA library are summarized in Table S4. Overall, these findings demonstrated

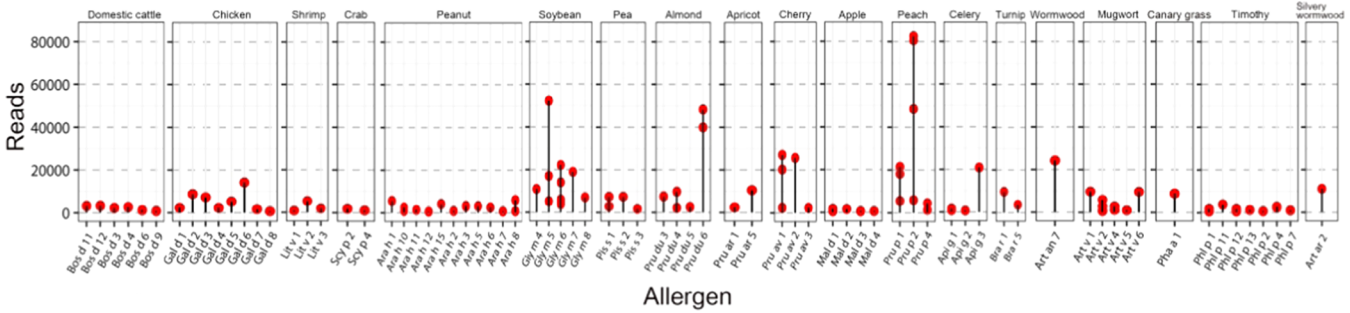


Figure 4. Allergen component analysis of a known mixed food matrix.

Table 2. Allergenic Component Detection in Highly Processed Food Products

sample name	expected component	expected species	match %	observed expected species %	difference of observed from expected %	other species observed
hazelnut	milk	<i>Bos domesticus</i>	0.08	99.87	−0.13	<i>Betula verrucosa</i> 0.11%; <i>Carpinus betulus</i> 0.02%
chocolate	hazelnut	<i>Corylus avellana</i>	8.98			
spaghetti	wheat	<i>Triticum aestivum</i>	1.08	99.97	−0.03	<i>Hordeum vulgare</i> 0.03%
	durum wheat	<i>Triticum turgidum</i> ssp. durum	0.02			
cookies	wheat	<i>Triticum aestivum</i>	0.66	99.98	−0.015	<i>Hordeum vulgare</i> 0.008%; <i>Triticum turgidum</i> ssp. 0.007%

consistent results between the observed and expected species, and one or more allergenic species were identified in each sample. The capture efficiency was up to 27.9% of the captured DNA mapping back to the allergen sequence, representing the increased specificity of longer reads. For example, the “top eight” food ingredients were successfully detected in the eight raw foods (Table 1). HPC-NGS reported a detection capability of 100% for *Arachis hypogaea* (peanut), *Bos domesticus* (milk), *Cyprinus carpio* (carp), *Gallus domesticus* (egg), *Glycine max* (soybean), and *Litopenaeus vannamei* (shrimp), 99.59% for *Anacardium occidentale* (cashew), and 99.72% for *Triticum aestivum* (wheat). For all datasets, HPC-NGS demonstrated 94.24–100% detection capability for the expected allergenic species. The detection capacity of HPC-NGS was shown as precision for expected species. However, we found that a fraction (>0.1%) of the reads were better matched to other unexpected species genes than to the original one. In the mugwort sample, 2.68% of the reads were matched to other mugwort species in the genus *Artemisia*, namely, *Artemisia annua* and *Artemisia argyi*. The main reason was that the sequence of the *Artemisia argyi* and *Artemisia annua* allergens showed 98.17 and 95.11% sequence similarity to the sequence of *Artemisia vulgaris* allergen, respectively (Table S5). These results suggested that high levels of false-positive results may be expected due to the high fidelity of plant genes within the same genus.³⁰ Hence, while this experiment’s results demonstrated a good specificity of HPC-NGS in capturing the target DNA fragment, the genotyping methods for accurately classifying allergens with high sequence similarity require improvement.

Evaluation of the Accuracy of HPC-NGS in a Complex System with Known Food Allergens. To prove that the HPC-NGS demonstrated an application of one-step multiple-allergen identification without interference, we next applied our HPC-NGS to distinguish allergens in a complex DNA library containing a total of 19 individual allergenic species. The use of the probe set improved our ability to capture the target sequences’ specific hybridization with the relevant probes. In this sample DNA library, a total of 75 280 112

reads were observed. As shown in Figure 4 and Table S6, 112 allergen DNA from 19 allergenic ingredients were captured, indicating that the species were in accordance with expectations in the complex sample. The capture efficiency of the allergen DNA ranged from 0.0007 to 0.1095%, demonstrating the high selectivity and sensitivity of this pipeline. These results illustrated that the probe set can be used to improve the sensitivity and accuracy of allergen detection, especially at low sequencing depths. Moreover, these results showed that HPC-NGS could target hundreds of allergen DNA in complex food matrices.

We also compared our HPC-NGS system with two hexaplex real-time PCR for allergen detection in the genomic DNA.³¹ The first system simultaneously determines six allergenic ingredients from cashew, peanut, hazelnut, celery, soy, and mustard, whereas the second system determines DNA contents from milk (beef), egg (chicken), almonds, sesame, pistachio, and walnut. In addition, a DNA hybridization-based optical thin-film biosensor chip was developed to simultaneously detect eight food allergens,³² including cashew, peanut, wheat, soybean, chicken, fish, shrimp, and beef. In this biosensor, aldehyde-labeled probes were arrayed and covalently linked on the chip surface; biotinylated target DNA amplification products combined with the probes could therefore change the interference pattern of light on the chip surface, producing a perceived color change from gold to purple. These results indicated that multiplex real-time PCR and DNA hybridization-based biosensors may not meet the requirements of high-throughput identification of allergens without prior knowledge. Conversely, our HPC-NGS functioned as an accurate high-throughput method for the one-step characterization of allergenic species in complex food systems.

Effective Identification of Allergens in Highly Processed Food Products. During food processing, especially thermal processing, DNA is prone to degradation, which can cause false-negative results. To further demonstrate the efficiency and accuracy of our HPC-NGS method for identifying any allergens present in food samples, we applied HPC-NGS directly to three highly processed food samples,

including hazelnut chocolate, spaghetti, and cookies (Tables 2 and S7). We observed two allergenic food ingredients and two allergenic contaminants in the hazelnut chocolate. The HPC-NGS achieved 99.87% detection capability of the expected food allergenic ingredients from *Bos domesticus* and *Corylus avellana*, with a small fraction of the reads yielding 0.11% hits from allergenic contaminant *Betula verrucosa* and 0.02% fewer false-positive hits, which better matched *Carpinus betulus*. The reason may be that the sequence similarity between *Carpinus betulus* allergen and *Betula verrucosa* allergen was 83.75% (Table S5). Similarly, detection capabilities of 99.97 and 99.98% of the expected allergic ingredients were observed in the spaghetti and cookies, respectively. However, a small fraction (<0.1%) of false-positive hits were observed because of the high fidelity of plant genes from the same genus. In addition, we used HPC-NGS to detect potential allergenic ingredients in DNA libraries of hazelnut chocolate spiked with variable amounts of hazelnut. As shown in Tables 3 and S8, the

Table 3. Allergenic Component Detection in the Hazelnut Chocolate Sample Spiked with Hazelnut

sample no.	background values (ng/ μ L)	spiked (ng/ μ L)	total count	observed species	match %
1	0.2	0.2	16 688	<i>Bos domesticus</i>	0.011985
				<i>Corylus avellana</i>	1.348274
2	0.2	1.35	43 062	<i>Bos domesticus</i>	0.032511
				<i>Corylus avellana</i>	1.449073
3	0.2	1.73	107 052	<i>Bos domesticus</i>	0.001868
				<i>Corylus avellana</i>	2.322236
4	0.2	2.04	147 438	<i>Bos domesticus</i>	0.006783
				<i>Corylus avellana</i>	2.465443

total reads of spiked samples and capture efficiency of hazelnut increased with the increase in the amounts of hazelnut. These results indicated that the DNA content of allergenic ingredients in samples has a positive correlation with its capture efficiency.

Multiplex real-time PCR has been applied to trace the allergens in hazelnut chocolate.³¹ Interestingly, both *Bos domesticus* and *Corylus avellana* exhibited negative signals.

Potential reasons why multiplex real-time PCR failed to fully distinguish potential allergens in hazelnut chocolate may be because the total DNA extraction of the sample may result in the dilution of the target DNA from low-level allergens with DNA from nontarget components or the presence of certain fats and additives may inhibit PCR or increase DNA degradation.³³ Milk chocolate was the most challenging matrix for LC-MS and ELISA because of its high content of polyphenols and lipids. Several recent studies reported the capability of LC-MS and ELISA in detecting hazelnut and milk in hazelnut chocolate. For example, the LC-MS method was used for multiallergen determination in five hazelnut chocolate samples,³⁴ although only two of the five chocolate samples were positively identified as containing hazelnut. In addition, commercial ELISA kits have been successfully applied in targeting casein and whey proteins in a chocolate dessert,³⁵ while their ability to detect casein and β -lactoglobulin in chocolate is limited.³⁶ In contrast, HPC-NGS showed an accuracy of 99.87–99.98% in hazelnut chocolate, spaghetti, and cookies, supporting its potential for analyzing hidden allergens in food products.

DISCUSSION

Food allergies, characterized by immune-mediated adverse systemic immune responses, are an increasing global health issue. The prevention of food-mediated allergic reactions relies heavily on appropriate labeling, especially of processed foods; identifying allergenic ingredients and any allergenic contaminants in a product can help allergic patients completely avoid their dietary triggers. Currently, specific multiplexed analysis for allergens is typically performed using either multiplex PCR,^{37,38} DNA sensors,³² or LC-MS (Table 4).³⁹ However, multiplex PCR demonstrates both low amplification efficiency and low overall efficiency, since oligonucleotide primer pairs of different allergens may interfere with one reaction, which could reduce the accuracy of multiple-allergen detection in one-step tests. DNA hybridization-based sensors are inferior in sensitivity to PCR but more accurate since no nonspecific amplification or primer dimerization would occur. LC-MS is limited in its application for distinguishing and identifying allergens in difficult food products since certain additives can react with food proteins, therefore masking the target allergenic proteins. Essentially, currently utilized technologies can accurately detect allergenic ingredients in a food system. However, it is difficult to accurately and completely estimate all of the allergenic contaminants in food products.

Table 4. Comparison of HPC-NGS with Typical Multiple-Allergen Detection Methods

methods	one-step detection capacity ^a	species-level accuracy ^b		probe cluster	no. of target allergenic ingredients	detection capability of unintended allergen
	allergenic ingredients	mixed food matrices	hazelnut chocolate	design strategy		
HPC-NGS	19	19/19	2/2	multiple probes for specific hybridization and the capture of individual target DNA fragments	123	yes
multiplex real-time PCR ³¹	6	6/6	0/2	a primer pair for amplifying target DNA and a fluorescent probe for detecting double-stranded DNA	6	no
DNA sensor ³²	8	8/8		a primer pair for amplifying target DNA and a probe for hybridizing PCR product	8	no
LC-MS ^{34,43}	12		2/5			no

^aOne-step detection capacity was shown by the number of allergic ingredients detected in one reaction. ^bSpecies-level accuracy was expressed by observed allergenic ingredients/expected allergic ingredients.

The high-throughput screening method described herein addresses the missed detection and sensitivity issues associated with conventional allergen identification. HPC-NGS is both highly selective and accurate in distinguishing potential allergenic ingredients, allergenic species, and allergenic contaminants in complex food systems. The detection capability of HPC-NGS was successfully demonstrated by targeting 112 allergen genes from 19 allergenic species within a complex food system. In addition, HPC-NGS was able to successfully identify expected allergenic ingredients and unintended allergenic contaminants present in hazelnut chocolate, spaghetti, and cookies, respectively. These results demonstrate that HPC-NGS can easily be adapted to fully detect present allergens in highly processed food products. Furthermore, HPC-NGS can be used to fully trace allergens for rapid monitoring of unintended contamination and food authenticity using a streamlined process amenable to high-throughput operations.

In an attempt to accurately determine the presence of allergens in food products, a new bioinformatic pipeline based on sequencing reads has recently been developed, although this pipeline requires laboratory-like conditions and further optimization. Based on the comprehensive food allergen database from NCBI's RefSeq, we designed a probe set for specific capture of target DNA sequences of allergens to assemble and enrich the genes of low-titer allergens to improve the high-throughput analysis of allergens.⁴⁰ This probe set improves the possibility of metagenomic sequencing in source-limited settings and can be employed to target allergen genes and relevant allergenic species and contaminants in samples by sequencing on a MiSeq (HiSeq).⁴¹ We applied HPC-NGS to analyze DNA libraries containing known compositions of single raw materials, demonstrating the high accuracy for species detection from a single DNA library. In addition, HPC-NGS was employed in a mixed DNA library consisting of 19 types of raw materials, illustrating that HPC-NGS can also enable the screening of expected and unintended allergenic species. Finally, three highly processed food products with low-titer allergens were examined. According to the list of ingredients on the food packages, all expected species were observed, showing the high sensitivity and good accuracy of HPC-NGS.

To the best of our knowledge, this is the first report of high-throughput screening of low-titer allergens from complex food systems using HPC-NGS, although there are still challenges and practical considerations to address. The first challenge is to improve genotyping methods for the accurate classification of allergens with high sequence similarity. Improvement in accuracy is required for more stringent alignment and overlap comparisons, which can also greatly reduce both the cost and the processing time of the procedure. The second challenge is to improve targeted methods for extracting the genome from highly processed food products. In intensively processed food products, observed matrix species' DNA correlates with the mass of that species.⁴² HPC-NGS can have higher accuracy and sensitivity for potential application identification of low-titer biological components in food systems.

In summary, this paper demonstrates the function of a high-throughput allergen detection approach consisting of NGS relying on the hybrid capture of allergen DNA and accurate sequence alignment using bowtie2 software. Our results showed that HPC-NGS could achieve between 99.97 and 99.98% detection capability of the expected low-titer allergenic

ingredients in highly processed food products (hazelnut chocolate, spaghetti, and cookies). In addition, our HPC-NGS method exhibited the capacity for one-step screening of 19 allergenic ingredients in a complex food system, exceeding the abilities of the current multiple-allergen detection methods. Obviously, HPC-NGS can have a meaningful, positive impact on the mass screening of allergens in food products to improve allergen risk management. Overall, due to its high accuracy and ability to perform high-throughput screening, HPC-NGS may have a much wider application in food safety testing procedures for fully distinguishing food adulteration and contamination.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c03595>.

Amounts of DNA in samples (Table S1); Comprehensive allergen database (Table S2); The probe set for each allergen gene (Table S3); Sequencing reads for every observed allergen gene in known samples from single species (Table S4); BLAST sequence alignment (Table S5); Sequencing reads for every observed allergen gene in known mixed samples from 19 species (Table S6); Sequencing reads for every observed allergen gene in highly processed food products (Table S7); Sequencing reads for every observed allergen gene in spiked samples (Table S8) (PDF)

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Notes

The authors declare no competing financial interest.

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