

Multiplex detection of food allergens and gluten

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Abstract To help safeguard the food supply and detect the presence of undeclared food allergens and gluten, most producers and regulatory agencies rely on commercial test kits. Most of these are ELISAs with a few being PCR-based. These methods are very sensitive and analyte specific, requiring different assays to detect each of the different food allergens. Mass spectrometry offers an alternative approach whereby multiple allergens may be detected simultaneously. However, mass spectrometry requires expensive equipment, highly trained analysts, and several years before a quantitative approach can be achieved. Using multianalyte profiling (xMAP®) technology, a commercial multiplex test kit based on the use of established antibodies was developed for the simultaneous detection of up to 14 different food allergens plus gluten. The assay simultaneously detects crustacean seafood, egg, gluten, milk, peanut, soy, and nine tree nuts (almond, Brazil nut, cashew, coconut, hazelnut, macadamia, pine nut, pistachio, and walnut). By simultaneously performing multiple tests (typically two) for each analyte, this magnetic bead-based assay offers built-in confirmatory analyses without the need for additional resources. Twenty-five of the assays were performed on buffer extracted samples, while five were conducted on samples extracted using reduced-denatured conditions. Thus, complete analysis for all 14 allergens and gluten requires only two wells of a 96-well microtiter plate. This makes it possible to include in a single analytical

run up to 48 samples. All 30 bead sets in this multiplex assay detected 5 ng/mL of food allergen and gluten with responses greater than background. In addition, 26 of the bead sets displayed signal/noise ratios of five or greater. The bead-based design makes this 30-plex assay expandable to incorporate new antibodies and capture/detector methodologies by ascribing these new detectors to any of the unassigned bead sets that are commercially available.

Keywords Food allergens · Gluten · Detection · Multiplex · Luminex · BioPlex

Introduction

Over 11 million Americans are estimated to have either a food allergy or celiac disease (CD). Since, no cure exists for either ailment, individuals with food allergies or CD must rely on avoiding foods containing allergenic foods or gluten. To help the allergic or gluten-sensitive population, the Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) and the Gluten-free Regulation of 2013 were established [1, 2]. Enforcement of these legal mandates requires the availability of analytical methods that can reliably detect, identify, and quantify the presence of food allergens and gluten. To meet these needs antibody-based (e.g., ELISA and dipsticks/lateral flow devices) and PCR-based analytical methods have been developed and commercialized [3, 4].

The ability to detect allergenic proteins or biomarkers specific for allergenic foods and sources of gluten without the need to perform extensive purification and concentration procedures have made the use of antibody-based methods popular. Further increasing the popularity of these antibody methods are their ability to detect amounts of allergen and gluten consistent with the

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concentrations discussed as important for safeguarding the allergic or gluten-sensitive consumer [5–8]. However, the commercial immunodiagnostic methods available for the detection of food allergens and gluten are analyte specific with only a few efforts to develop assays capable of multiple analyses [9, 10]. As a result, when testing food for multiple food allergens and gluten, it becomes necessary to perform multiple analytical tests, a time-consuming and expensive process. With >30 % of the allergic population suffering from multiple allergies, the need to test foods associated with consumer complaints for multiple allergens is common [11, 12]. Further, homology between proteins in different foods make identifying some food allergens difficult. Both of these problems may be resolved by PCR and mass spectrometry (MS, and protein sequencing). PCR has been used extensively for the detection and the speciation between related allergens, but no single assay is available to test for all allergens [13–15]. Further, the selective fractionation for proteinaceous material makes PCR nonquantitative and of questionable utility when assessing exposure doses for products that include soy protein, gluten, casein, and whey. MS and protein sequencing is still in the research stage of development and requires expensive equipment and highly trained technicians [16–18].

An alternate approach is to determine the antigenic profile, which can be accomplished provided a sufficient number of antibodies are employed. Multianalyte profiling (xMAP®) technology provides a multiplex solution for the simultaneous detection, identification, and quantitation of as many as 100 different analytes. By conjugating different capture agents (e.g., antibodies, aptamers, and nucleotide sequences) to different color-coded bead sets, it is possible to run simultaneously the equivalent to 100 capture-based assays (e.g., ELISAs) in a single well of a microtiter plate [19, 20]. This technology has been successfully applied to the detection and identification of infectious agents, toxins, and biomarkers of health and disease. The availability of magnetic bead sets makes this platform particularly suitable for the analysis of food samples.

Using magnetic bead sets, a novel commercial assay for 14 food allergens plus gluten was developed incorporating 30 different established antibodies that were used already in other commercially available assays. The multiplex assay for food allergens and gluten employs two different extraction protocols and multiple assays (typically two) for most analytes. As a result, it is possible to analyze 12 different samples, each in triplicate, for the presence of 14 food allergens plus gluten within 6 h with LOD values comparable to that observed using analyte specific ELISA test kits. The power of this technology was illustrated in the analysis of cumin for the presence of undeclared allergens.

Materials and methods

Reagents

Antibodies were provided by Morinaga Institute of Biological Sciences, Inc. (MioBS, Yokohama-Shi, Japan), IEH Laboratories and Consulting Group (WA, USA), and Elution Technologies, Inc. (VT, USA). Phosphate-buffered saline (PBS) (catalog no. P3813), Tween® 20 (catalog no. P7949), and other reagents, unless specified otherwise, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Cumin (ground, roasted, and whole seeds) were purchased from local suppliers.

The responses generated by the multiplex assay were calibrated using established reference materials whenever possible. This included egg (RM 8445) and peanut butter reference materials (SRM 2387) purchased from NIST (Gaithersburg, MD, USA); nonfat dried milk powder (Carnation Milk, Nestlé® USA) demonstrated by multiple analysts using the Veratox® for Total Milk Allergen ELISA (Neogen Corp., Lansing, MI, USA), Morinaga Casein ELISA (Crystal Chem, Inc., Downers Grove, IL, USA), and the ELISA Systems Casein and β -Lactoglobulin Residue Detection Kits™ (Pi Biologique, Seattle, WA, USA) as indistinguishable from the no longer available nonfat dried milk standard reference material, NIST SRM 1549 (data not shown); wheat gluten (catalog no. G5004, Sigma-Aldrich Chemical Co.) was indistinguishable from the standard developed by the Prolamin Working Group and the wheat protein reference material developed for the government of Japan using both the R5 RIDASCREEN® Gliadin ELISA (R-Biopharm, Ag, Washington, MO, USA) and the Morinaga Gluten ELISA (US distributor Crystal Chem, Inc., Downers Grove, IL, USA) [21]; freeze-dried, powdered Black Tiger prawn, prepared from fresh prawn purchased from a local market, was demonstrated by ELISA analysis to be indistinguishable from the Black Tiger prawn protein standard material used to calibrate the crustacean ELISA manufactured by Maruha Nichiro Holdings, Inc. (US distributor, Wako Chemicals USA, Inc., Richmond, VA, USA [22, 23, data not shown]). Reference materials were not available for almonds, Brazil nuts, cashews, coconuts, hazelnuts, macadamia nuts, pine nuts, pistachios, walnuts, and soy. Therefore, all tree nuts were acquired as dried, not roasted, and unsalted from Nuts.com (Cranford, NJ, USA) with dried, raw soy beans, a product of ChoripDong (distributed by Seoul Shik Poom, Inc. Flushing NY, USA). Almond, cashew, hazelnut, pistachio, and soy beans were ground using an IKA A 11 Basic Analytical Mill (IKA® Works, Inc., Wilmington, NC, USA). Brazil nut, coconut, macadamia, pine nut, and walnut were first chopped then ground using an IKA Tube Mill.

Sample preparation

Food allergens were prepared as stock solutions individually or as a composite of all 14 food allergens plus gluten for both the nondenatured and denatured analyses [sodium dodecyl sulfate (SDS)/ β -mercaptoethanol]. In addition, a composite of egg, gluten, milk, and peanut was also prepared for the denatured analyses. The individual and composite allergen solutions were serially diluted to final concentrations of a particular food allergen of 100, 20, 10, 5, and 2 $\mu\text{g/mL}$. Unless specified otherwise, the samples were subsequently diluted a total of 200-fold during the sample extraction/preparation process to yield concentrations of 500, 100, 50, 25, 10, and 5 ng/mL in the analytical samples. Specifically, the samples analyzed using the denaturing antibody bead sets were diluted 2-fold with PBS containing 0.5 % SDS/2 % β -mercaptoethanol, incubated at 60 °C for 30 min, centrifuged at 2500 $\times g$ for 10 min, and diluted an additional 100-fold with assay buffer (PBS containing 0.05 % Tween[®] 20, PBS-T) prior to analysis. The samples analyzed using the nondenaturing bead sets were diluted 200-fold with assay buffer and incubated at room temperature for 2 h prior to analysis. To avoid possible errors, filtered, wide-bore pipette tips were used to pipette mixtures or suspensions of finely ground particles. Misinterpretations, regarding the concentrations of analyte, are common when dealing with complex mixtures that undergo multiple dilutions and extractions. To minimize such potential sources of error, whenever possible, the units used to describe the mixtures were chosen as descriptive of the preparation process (i.e., g/mL). In addition, the concentrations were expressed as the concentrations in the aliquot in the microtiter plate well (analytical sample) to which the bead sets were subsequently added. The use of this terminology also provides a better appreciation of the performance properties of the assay.

Cumin samples were prepared as described for the food allergen samples, except the denatured and nondenatured samples were centrifuged at 14,000 $\times g$ for 5 min prior to the final dilution step. The final dilutions were performed in 2-mL microtiter plates.

Multiplex analysis

The multiplex assay for food allergen detection by Radix[®] BioSolutions (Georgetown, TX, USA) analyzes samples simultaneously for the presence of 14 food allergens plus gluten representing seven of the eight major groups. The food allergens detected are crustacean seafood, egg, gluten, milk, peanut, soy, and nine tree nuts (almond, Brazil nut, cashew, coconut, hazelnut, macadamia, pine nut, pistachio, and walnut). All but crustacean seafood and egg were detected using two capture antibodies (bead sets). Two different extractions were performed to generate the analytical samples. The samples were extracted using PBS-T (nondenatured) and 0.5 % SDS/

2 % β -mercaptoethanol (denatured), which were subsequently analyzed using 29- and 9-plex, respectively, mixtures of bead sets. Included in each mixture were AssayCheX[™] Process Control microspheres (Radix[®] BioSolutions) to confirm instrument and reagent optimal performance. The various composite samples, blank controls to generate the background responses, denatured samples, and all cumin samples were analyzed as triplicates. The nondenatured samples of the tree nuts were analyzed as duplicates. All of the replicates displayed standard deviations or range/2 values (for duplicate analyses) <5 %, with most 2 % or less.

The protocol supplied by Radix[®] BioSolutions was followed to prepare the various assay reagents, standards, and perform the multiplex assay, including the parameters for running the Bio-Plex 200 (Bio-Rad Laboratories, Hercules, CA, USA). Specifically, 50 μL of sample were incubated in the dark with 50 μL of the conjugated MagPlex[®] microsphere bead mixture at 37 °C for 30 min with shaking using an SC20 orbital mixing chilling/heating dry bath (Torrey Pines Scientific, Carlsbad, CA, USA). The beads were then washed three times using PBS-T employing a Magnetic-Ring Stand (catalog no. AM10050, Applied Biosystems[®] Corp.) to prevent loss of the beads during inversion of the microtiter plate during washing. The beads were then mixed with 50 μL of the biotinylated detector antibodies (each at 1 $\mu\text{g/mL}$) for 30 min at 37 °C. After washing three times, 50 μL of streptavidin phycoerythrin (SAPE, supplied with the test kit) was added to each well and incubated for 15 min at 37 °C with shaking. The beads were then washed three times and suspended in 100 μL PBS-T prior to placing into the Bio-Plex for counting. The Bio-Plex was operated with the doublet discriminator gate set to 5000–25,000, PMT set to low, number of microspheres collected set to 100 per region, and number of microspheres collected set to 3500 total classified.

Results and discussion

Performance properties

The performance properties of the Radix[®] BioSolutions xMAP[®] multiplex assay for the detection of food allergens and gluten with ground allergens are depicted in Figs. 1 and 2 for samples prepared using the nondenatured and denatured extraction protocols. All 30 bead sets were able to detect their respective analytes at the lowest concentrations tested with observed responses greater than the background response plus three times the standard deviation (Bkgd+3D). This sensitivity, apparent limits of detection (LOD) <5 ng/mL , was observed using both the test kit standards and freshly ground allergenic foods without any form of fractionation. Further, all except four bead sets displayed signal/noise ratios >5 when detecting their respective analytes at 50 ng/mL . The beadsets

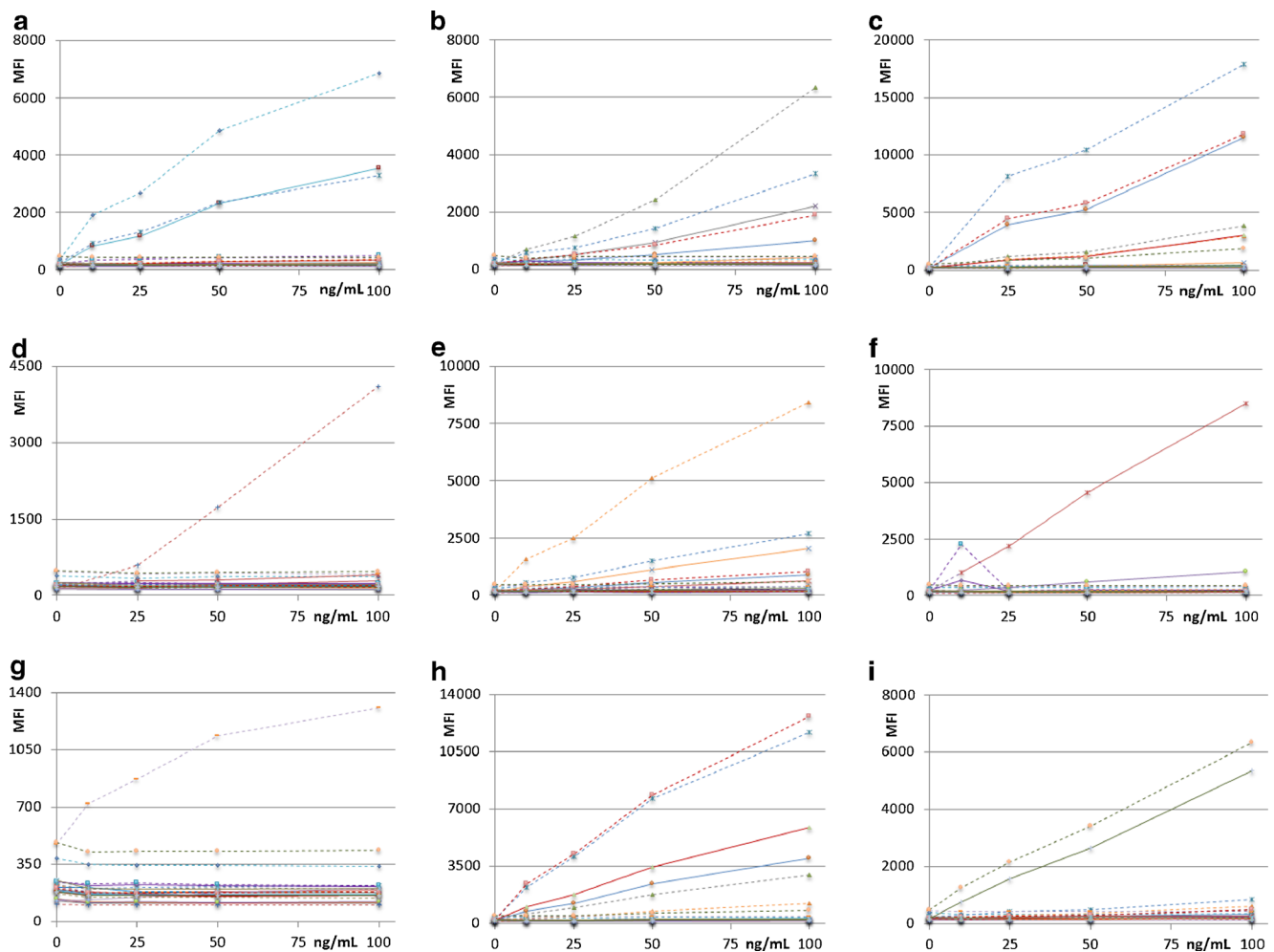


Fig. 1 Detection of tree nuts using the multiplex Radix® BioSolutions Food Allergen Detection Assay. Samples of **A** almond, **B** Brazil nut, **C** cashew nut, **D** coconut, **E** hazelnut, **F** macadamia, **G** pine nut, **H** pistachio, and **I** walnut at 0, 2, 5, 10, and 20 µg/mL (except cashew and coconut, which did not include 2 µg/mL samples) were analyzed using the nondenatured protocol that entailed a 200-fold dilution, resulting in concentrations of 0, 10, 25, 50, and 100 ng/mL in the analytical samples. The bead sets in each analysis were —◆— almond-12, —■— almond-13, —▲— Brazil nut-14, —×— Brazil nut-15, —*— cashew-18, —●— cashew-19, —+— coconut-20, —■— coconut-21, —■— crustacean, —◆— gluten-27,

—■— gluten-28, —▲— hazelnut-29, —×— hazelnut-30, —■— macadamia-33, —◆— macadamia-34, —■— peanut-37, —■— peanut-38, —■— pine nut-39, —■— pine nut-42, —■— pistachio-43, —■— pistachio-44, —■— soy-45, —■— soy-46, —■— walnut-47, —■— walnut-48, and the four AssayChex™ Process Control microspheres (IC-75, FC-76, AC-77, and NC-78) to confirm instrument and reagent optimal performance. The data are plotted as the average median fluorescence intensity (MFI) versus the concentration of tree nut in the analytical sample (ng/mL) in the microtiter plate prior to the addition of the bead sets. Error bars represent $\pm$ the range/2 between duplicate analyses

that displayed signal/noise ratios <5 were crustacean-22, coconut-21, pine nut-42, and macadamia-34 with ratios of <3 , <2 , <2 , and 4, respectively (Table 1).

Figure 1 depicts the responses observed with the various nondenatured bead sets with increasing titration of the nine different tree nuts. In all cases, the largest response (MFI) observed with a tree nut was by one of the bead sets specific for that tree nut. In addition, cross-reactivity was observed between some of the bead sets. Specifically, the almond sample displayed cross-reactivity with cashew-18; the Brazil nut sample with both cashew bead sets (18 and 19) and pistachio-43; the cashew sample with both pistachio beadsets (43 and 44), Brazil nut-14, and

walnut 47; the hazelnut sample with both cashew bead sets (18 and 19) and pistachio-43; the pistachio sample with Brazil nut-14, both cashew bead sets (18 and 19), and hazelnut-29; and the walnut sample with cashew-18, hazelnut-29, and pistachio-43. Analyzing the MFI responses normalized to the bead set displaying the most intense response for a particular tree nut, with a threshold of 2 %, indicated similar cross-reactivities with a few more low level cross-reactivities identified (Table 2). One of these, a cross-reactivity between Brazil nut and hazelnut-30 was particularly interesting. When a hazelnut sample was tested, hazelnut-30 displayed one fifth to one quarter the response of hazelnut-29 with preferential

interaction observed for hazelnut-29 in all other cross-reactivities that involved hazelnut antibodies. However, Brazil nut displayed no apparent cross-reactivity with hazelnut-29 except when the data were screened using a signal/noise criteria of only 1.3-fold (analysis not shown). Another feature of the cross-reactivity profiles was the observation that, while cashew-19 consistently displayed intensities at least half that displayed by cashew-18 with cashews, when both cross-reacted with other tree nuts, the ratio between the two bead sets significantly differed from 50 %, with no cross-reactivity observed between almonds and cashew-19 despite a strong cross-reactivity with cashew-18. Only by applying less stringent criteria, a signal/noise of 1.3, was a slight cross-reactivity between almond and cashew-19 observed.

Consistent with the cross-reactivity patterns observed (Figs. 1 and 2), the peanut and tree nut bead sets displayed greater responses when analyzing the composite of analytes than individual allergens (Table 1).

Cross-reactivity profiles

The ability to generate cross-reactivity profiles is an advantage of multiplex assays like xMAP® technology due to its ability to detect weak interactions. Cross-reactivity profiles provide a secondary form of assay confirmation. Cross-reactivity profiles are also often more sensitive to changes in antigenicity

that may arise from conformational changes, chemical modifications, or associated with the detection of homologous proteins. Indeed, changes in the relative intensities observed by two antibodies against the same antigen provide a powerful tool besides the appearance and disappearance of novel cross-reactivities.

Interpretation of cross-reactivity binding profile data is not simple. However, the increased globalization of the food supply, the growing number of individuals with multiple food allergies, and the demonstration that many allergic individuals display antibodies with cross-reactivity towards related foods make it critical that future analytical methods be able to detect and identify related possible sources of allergenicity; binding (antigen) profiles can serve such a purpose. For a binding profile to have utility, it must include a sufficiently large number of antibodies to ascertain the various nuances. Further, cross-reactivity is a function of the binding affinity and the number of binding sites (epitopes) on a molar basis. However, a detailed understanding of the binding affinities (avidities) of the antibodies and the properties of potential antigens were lacking. This made it necessary to express the profiles on a mass basis, a common practice. Expressing cross-reactivity on a per mass basis is potentially misleading. To compensate for this potentially misleading aspect, the cross-reactivity profile data were presented at several concentrations in Table 2. The cross-reactivities of the

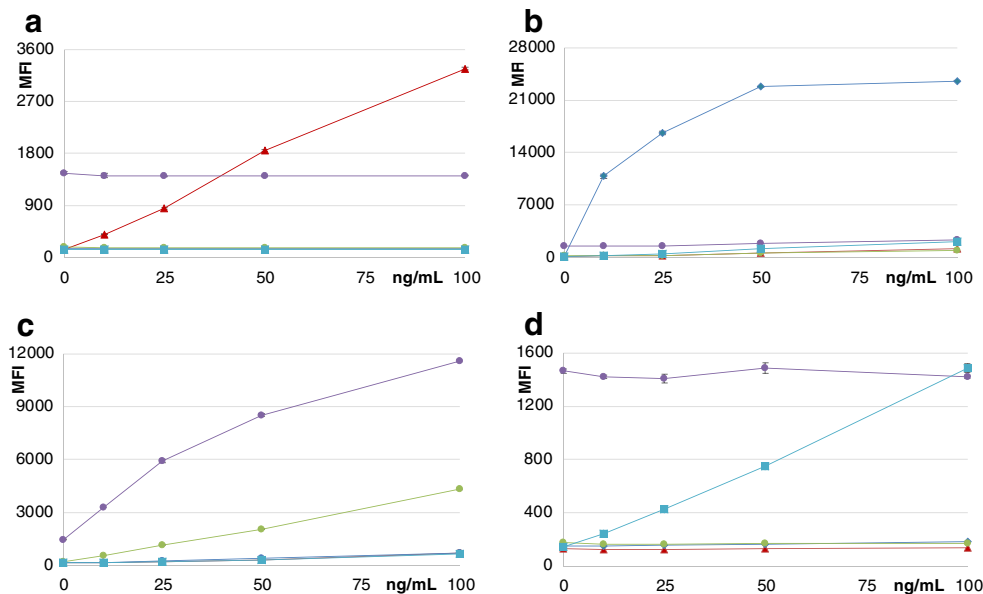


Fig. 2 Detection of egg, milk, gluten, and peanut using the multiplex Radix® BioSolutions Food Allergen Detection Assay. **A** egg RM 8445 (NIST), **B** gluten (Sigma Aldrich G5004), **C** nonfat milk (Carnation), and **D** peanut SRM 2387 (NIST) at initial concentrations of 0, 2, 5, 10 and 20 µg/mL were analyzed using the 0.5 % SDS/2 % β-mercaptoethanol (denaturing) protocol that entailed a 200-fold dilution to concentrations of 0, 10, 25, 50, and 100 ng/mL in the analytical sample. The bead sets in each analysis were —▲— egg-65, —◆— gluten-73, —●— milk-66,

—●— milk-67, —■— peanut-72, and the four AssayChex™ Process Control microspheres (IC-75, FC-76, AC-77, and NC-78) to confirm instrument and reagent optimal performance. The results are plotted as the average median fluorescence intensity (MFI) versus the concentration of egg, milk, gluten, and peanut (ng/mL) in the analytical sample in the microtiter plate prior to the addition of the bead sets. Analyses were performed in triplicate, and the error bars (typically within the size of the symbols) represent ± one standard deviation

Table 1 Comparison between analyte response and background

	Bead set	Extraction ^a	Bkgd	Composite 50 ng/mL	Individual 50 ng/mL	S/N ^b		Bead set	Extraction	Bkgd	Composite 50 ng/mL	Individual 50 ng/mL	S/N
Crustacean	22	buffer	208±8	620±40	— ^c	<3	Tree nuts Almond	12	Buffer	390±20	6500±100	4900±200	>
		—						13	Buffer	190±8	3400±100	2300±100	>
Egg	65	SDS/β-mer	131±2	1840±60	1850±10	>	Brazil nut	14	Buffer	200±20	6500±300	2410±30	>
								15	Buffer	128±5	1350±50	938±1	>
Gluten	27	buffer	250±10	2490±80	—	>	Cashew	18	Buffer	250±30	18,100±800	10,460±30	>
	28	buffer	241±5	14,600±300	—	>		19	Buffer	195±5	9000±400	5260±20	>
	73	SDS/β-mer	152±1	18,500±300	22,770±60	>	Coconut	20	Buffer	109±2	2170±70	1740±10	>
Milk	66	SDS/β-mer	176±2	2040±50	2060±10	>		21	Buffer	132±5	260±10	209±2	<2
	67	SDS/β-mer	1460±20	8000±100	8530±60	>	Hazelnut	29	Buffer	224±9	8500±300	5100±300	>
Peanut	37	buffer	199±8	1950±60	—	>		30	Buffer	141±5	2200±100	1100±100	>
	38	buffer	228±4	2500±60	—	>	Macadamia	33	Buffer	210±10	6700±200	4580±30	>
	72	SDS/β-mer	143±4	1160±20	750±10	>		34	Buffer	140±3	880±20	597±5	<5 ^d
Soy	45	buffer	173±5	2310±90	—	>	Pine	39	Buffer	470±10	1300±60	1137±6	<3
	46	buffer	250±10	1950±60	—	>		42	Buffer	130±3	220±10	178±2	<2
							Pistachio	43	Buffer	206±6	14,500±600	7900±500	>
								44	Buffer	182±6	5800±300	3400±200	>
							Walnut	47	Buffer	490±10	7000±800	3400±300	>
								48	Buffer	184±4	6000±1000	2600±400	>

Comparison of the MFI (median fluorescence intensity) generated by 50 ng/mL of analyte, either individually or mixed with the other analytes (composite), to the background response (MFI generated by samples lacking analytes, Bkgd). Values listed are the mean of triplicate analyses±standard deviation, except for the Individually analyzed tree nuts, which were analyzed in duplicate with the values listed as the mean±range/2

^a Extraction-sample preparation media used for the analysis. Buffer refers to nondenaturing conditions while SDS/b-mercaptoethanol refers to reducing-denaturing conditions, as described by the protocol accompanying the test kit

^b Signal/noise (S/N) expressed as either greater than five (>5 times Bkgd+3SD) or if less by the amount observed

^c “—” indicates not determined

^d The response of the composite exceeded a S/N of five but macadamia titrated individually had a S/N of 4

nondenatured bead set assays are presented in Table 2 as the responses measured (after subtracting the average background responses) normalized against the largest response observed with the sample. This approach compensated for the bead sets having different backgrounds, a problem illustrated by milk-67 (Fig. 2). Milk-67 displayed a consistently high MFI background of 1463±16 ($n=3$). Without correcting for background, milk-67 might be the source of numerous false positives. Gluten illustrates a different problem. Gluten-27, gluten-28, and gluten-73 displayed background MFIs of 247±14, 241±5, and 152±1 (all $n=3$), first nonzero standard (3.7 ng/mL) MFIs of 647, 2470, and 7100, and maximum responses (at 100 ng/mL in the analytical sample in the microtiter plate prior to bead set addition) of approximately 3200, 16,000, and 23,000, respectively (Figs. 1 and 2). As a result of the responses generated by gluten-28 and gluten-73 being enhanced, presumably due to multiple detector antibody binding sites per captured protein, insignificant responses

(for the gluten bead set) may be incorrectly interpreted when compared to other bead sets. An example of this problem was observed in the analysis of almond in the presence of gluten-28. The observed response by gluten-28 was insignificant and below the dynamic range for the bead set but appeared large when normalized relative to almond-12 (Table 2). Similar problems were observed with gluten-73 where data below the dynamic range were misinterpreted as significant (data not shown). Cashew-18 also displayed enhanced responses (Fig. 1h), which may explain the apparent high cross-reactivity with almond, Brazil nut, hazelnut, pistachio, and walnut (Fig. 1a, b, c, e, and f). The seriousness of this problem is debatable. Apparent cross-reactivities that may be enhanced due to an elevated signal have no negative effect on the use of profiles as a diagnostic tool to detect analytes, but may negatively impact mechanistic interpretations of antigen-antibody interactions and the biochemical properties of the analytical samples.

Table 2 Cross-reactivity profile patterns

Analyte	ng/mL ^a	Almond-12 ^b	Almond-13	Brazil nut-14	Brazil nut-15	Cashew-18	Cashew-19	Coconut-20	Coconut-21	Crustacean-22	Gluten-27	Gluten-28	Hazelnut-29
Almond	10	43		<	<	45	<	<	<	<	<	6 ^c	<
	25	44		<	<	47	<	<	<	<	<	6	<
	50	48		<	<	47	<	<	<	<	<	5	2
	100	52		<	<	47	<	<	<	<	<	4	3
Brazil nut	10	<		100	42	68	21	<	<	<	<	<	<
	25	<		100	41	56	15	<	<	<	<	<	<
	50	<		100	37	53	15	<	<	<	<	<	<
	100	<		100	34	50	13	<	<	<	<	<	<
Cashew	25	<		13	100	100	48	<	<	<	<	<	10
	50	2 ^d		14	100	100	50	<	<	<	<	<	10
	100	<		21	100	100	64	<	<	<	<	<	16
Coconut	25	<		<	<	<	<	100	4	<	<	<	13
	50	<		<	<	<	<	100	5	<	<	<	5
	100	<		<	<	<	<	100	4	<	<	<	5
Crustacean	10	2		<	<	<	<	2	<	100	<	2	<
	25	2		<	<	<	<	2	<	100	<	2	<
	50	3		2	<	<	2	3	2	100	2	2	<
	100	2		<	<	<	<	2	<	100	2	2	<
Gluten	10	<		<	<	<	<	<	<	<	100	<	<
	25	<		<	<	<	<	<	<	<	100	<	<
	50	<		<	<	<	<	<	<	<	100	<	<
	100	<		<	<	<	<	<	<	<	100	<	<
Hazelnut	10	<		<	<	22	5	<	<	<	<	100	18
	25	<		<	<	24	7	<	<	<	<	100	19
	50	<		<	<	26	7	<	<	<	<	100	20
	100	<		2	<	30	9	<	<	<	<	100	23
Macadamia	10	<		<	<	<	<	<	<	<	<	<	<
	25	<		<	<	<	<	<	<	<	<	<	<
	50	<		<	<	<	<	<	<	<	<	<	<
	100	<		<	<	<	<	<	<	<	<	<	<
Peanut	10	2		2	<	14	3	2	<	<	<	<	<
	25	4		3	<	18	5	4	<	<	<	<	<
	50	6		4	<	22	6	6	<	<	<	<	2
	100	10		7	<	27	8	10	2	2	2	2	5
Pine nut	10	<		<	<	<	<	<	<	<	<	<	<
	25	<		<	<	<	<	<	<	<	<	<	<
	50	<		<	<	<	<	<	<	<	<	<	<
	100	<		<	<	<	<	<	<	<	<	<	<
Pistachio	10	<		18	<	87	24	<	<	<	<	<	4
	25	<		19	<	95	26	<	<	<	<	<	5
	50	<		20	<	97	29	<	<	<	<	<	7
	100	<		22	<	92	30	<	<	<	<	<	8
Soy	10	<		5	<	22	3	<	<	<	<	<	2
	25	2		7	<	27	5	<	<	<	<	<	3
	50	3		9	<	32	6	2	<	<	<	2	5

Table 2 (continued)

Analyte	ng/mL ^a	Almond-12 ^b	Almond-13	Brazil nut-14	Brazil nut-15	Cashew-18	Cashew-19	Coconut-20	Coconut-21	Crustacean-22	Gluten-27	Gluten-28	Hazelnut-29
Walnut	100	5	<	14	<	43	9	3	<	<	2	4	8
	10	<	<	3	<	7	<	<	<	<	<	<	1
	25	<	<	5	<	11	<	<	<	<	<	<	5
	50	<	<	4	<	8	2	<	<	<	<	<	5
	100	<	<	5	<	10	2	<	<	<	<	<	7
Analyte	Hazelnut-30	Macadamia-33	Macadamia-34	Peanut-37	Peanut-38	Pine nut-39	Pine nut-42	Pistachio-43	Pistachio-44	Soy-45	Soy-46	Walnut-47	Walnut-48
Almond	<	2	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
Brazil nut	<	2	<	<	<	<	<	2	<	<	<	<	<
	2	2	<	<	<	<	<	2	<	<	<	<	<
	2	<	<	<	<	<	<	33	<	<	<	<	<
	4	<	<	<	<	<	<	32	<	<	<	<	<
	5	<	<	<	<	<	<	30	<	<	<	<	<
Cashew	4	<	<	<	<	<	<	27	<	<	<	<	<
	2	<	<	<	<	<	<	54	9	<	<	5	<
	2	<	<	<	<	<	<	55	10	<	<	6	<
	3	<	<	<	<	<	<	66	16	<	<	8	2
	<	<	<	<	<	<	<	<	<	<	<	<	<
Coconut	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
Crustacean	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
Gluten	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
Hazelnut	2	<	<	<	<	<	7	<	<	<	<	<	<
	3	<	<	<	<	<	9	<	<	<	<	<	<
	4	<	<	<	<	<	9	<	<	<	<	<	<
	5	<	<	<	<	<	10	<	<	<	<	<	2
	100	15	<	<	<	<	<	<	<	<	<	<	<
Macadamia	100	12	<	<	<	<	<	<	<	<	<	<	<

Table 2 (continued)

Analyte	Hazelnut-30	Macadamia-33	Macadamia-34	Peanut-37	Peanut-38	Pine nut-39	Pine nut-42	Pistachio-43	Pistachio-44	Soy-45	Soy-46	Walnut-47	Walnut-48
Peanut	100	10	<	<	<	<	<	<	<	<	<	<	<
	100	11	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	69	100	<	<	2	<	4	4	<	<
	<	3	<	70	100	<	<	4	<	6	6	2	<
Pine nut	2	4	<	71	100	2	<	5	2	8	7	4	<
	3	7	<	73	100	4	<	8	3	11	10	7	3
	<	<	<	<	<	100	2	<	<	<	<	<	<
	<	<	<	<	<	100	5	<	<	<	<	<	<
Pistachio	<	<	<	<	<	100	7	<	<	<	<	<	<
	<	<	<	<	<	100	9	<	<	<	<	<	<
	<	<	<	<	<	<	<	<	<	<	<	<	<
	<	<	<	<	<	<	<	100	38	<	<	<	<
Soy	<	<	<	<	<	<	<	100	39	<	<	<	<
	<	<	<	<	<	<	<	100	42	<	<	2	<
	<	<	<	<	<	<	<	100	45	<	<	2	<
	<	<	<	<	<	<	<	9	<	100	69	<	<
Walnut	<	<	<	<	3	<	<	12	<	100	67	<	2
	<	<	<	<	4	<	<	16	2	100	70	2	2
	<	2	<	2	8	<	<	25	2	100	74	3	4
	<	<	<	<	<	<	<	3	<	<	<	100	78
Walnut	<	<	<	<	<	<	<	4	<	<	<	100	83
	<	<	<	<	<	<	<	3	<	<	<	100	84
	<	<	<	<	<	<	<	5	<	<	<	100	88
	<	<	<	<	<	<	<	<	<	<	<	<	<

Responses of nondenatured analyses tabulated as average response minus background, normalized to the most intense bead set for the analyte, set to 100 (i.e., almond-12, cashew-18, coconut-20, crustacean-22, gluten-28, hazelnut-29, macadamia-33, peanut-38, pine nut-39, pistachio-43, soy-45, and walnut-47). “<” indicated a normalized response of ≤ 1 (1 % or less). Responses ≥ 2 % in italics except for analyses of almond by gluten-28; which were below the dynamic range of the bead set

^a Concentration of analyte in the sample following extraction and preparation (prior to adding bead sets)

^b Bead set identified as the immunogen used to generate the antibody and the number of the Mag Plex[®] microsphere color-coded bead set

^c Suspected artifact of gluten-28-these responses were within the noise and below the dynamic range of gluten-28. Although when normalized relative to almond appear as indicated

^d Suspected noise error in data generation

Cumin profiling

Multiplex profiling was recently applied to the analysis of ground cumin samples. Two of seven ground cumin samples, from unrelated suppliers, were found to generate positive ELISA results when analyzed using the Veratox® for Peanut Allergen and the Veratox® for Almond Allergen test kits, but negative results with the Veratox® for Egg Allergen ELISA test kit (Neogen Corp., Lansing, MI, USA). The other five products, three of whole seeds (ground in-house prior to analysis) and two purchased as ground (one roasted), tested negative with all three ELISA test kits. All seven samples were analyzed, in triplicate, using the xMAP® multiplex assay. The five samples that tested negative with the ELISAs were negative with the multiplex assay with average peanut-38 responses of 312 ± 9 , 343 ± 14 , 312 ± 9 , 324 ± 14 , and 662 ± 12 ($n=3$ for each product). The five samples also generated negative responses with the peanut-37 and peanut-72 bead sets (data not shown). In contrast, the two cumin products that tested positive with the ELISAs generated average peanut-38 MFI values of $15,900 \pm 300$ and $16,800 \pm 700$ ($n=3$ for each cumin), with peanut-37 and peanut-72 also generating positive responses. However, the responses observed with the other bead sets were not characteristic of peanut. Table 3 tabulates the responses with the various bead sets for the seven cumin samples as the average response generated by 5 mg/mL divided by the average background response for the particular bead set minus one ($S/N-1$). The $S/N-1$ values are inconsistent with any individual reference standard. To further explore the possibility that inadvertent cross-contact caused the positive ELISA results, the ratios between the responses observed with the bead sets were normalized to the response generated by peanut-38 and compared:

12:13:14:15:18:19:20:21:22:27:28:29:30:33:34:37:38:39:42:43:44:45:46:47:48—bead sets
58:26:30:09:40:22:20:09:06:05:31:28:09:20:11:76:100:06:08:25:11:14:15:02:07—cumin A
72:43:25:05:51:19:16:04:02:03:30:27:09:18:05:76:100:04:04:22:06:10:11:01:05—cumin B
10:02:07:01:27:08:10:01:02:02:02:05:03:07:01:73:100:04:01:08:03:11:10:07:03—peanut standard at 100 ng/mL.

The two cumins displayed cross-reactivities $\geq 20\%$ (italics) with almond-12, almond-13, Brazil-nut-14 cashew-18, cashew-19, coconut-20, gluten-28, hazelnut-29, macadamia-33, and pistachio-43 along with cross-reactivity with other tree nuts. All of these cross-reactivities were associated with elevated $S/N-1$ values (Table 3) and thereby indicative of properties of the two cumin samples versus random patterns characteristic of nonspecific binding and background noise.

Baking, roasting, and various forms of food processing are known to affect the antigenicity of peanuts [24, 25], and, as a result, profiling processed foods can be problematic if the standards are not identically processed. Fortunately, as there were no indications that the ground cumins (A and B) were processed, the standards used to generate the data in Table 2 should be directly applicable to these two cumin samples. Therefore, assuming the differences in the profiles of the cumin samples and peanut were due to another cause, the question became whether it was due to the presence of an analogous protein(s) or other artifacts. The two ground cumin samples that generated the positive results were ground in and labeled as products of Turkey. It is possible that homologous proteins specific to certain varieties were the cause of the results with cumin A and B and not cross-contamination by the various food allergens necessary to generate the complex profile observed. Further research is necessary to definitively resolve this ambiguity.

Interestingly, all seven cumin samples displayed elevated responses with soy-45 and soy-46 in the multiplex assay characteristic of soy in the analytical sample at 3–20 ng/mL. The five cumin samples (C–G) that tested negative for peanut displayed more intense responses with soy-46 than soy-45 as illustrated in Table 3. In contrast, the soy reference standard at concentrations of 10, 25, 50, and 100 ng/mL in the analytical sample displayed the opposite pattern. The MFI of soy-45 exceeded soy-46 for the soy reference standard at all concentrations examined with an overall average ratio between soy-46/soy-45 of 0.70 ± 0.03 (Table 2) or an overall average $S/N-1$ ratio of 0.60 ± 0.03 . The five cumin samples displayed an average $S/N-1$ ratio (soy-46/soy-45) of 1.3 ± 0.1 , significantly different from the soy reference standard. It is therefore unlikely that the cumin samples contained unprocessed soy but instead probably contained either soy that had undergone processing that changed its antigenicity or a homologous (cross-reactive) protein.

The apparent high cross-reactivity with gluten-28 is suspected of being due to the enhanced response of the bead set (see above).

The Food Allergen Detection Assay based on xMAP® technology provided an efficient, sensitive alternative to the use of analyte specific antibody-based assays when testing for multiple allergens. The ability to analyze 48 food samples using the equivalent of 30 different ELISAs within 6 h makes detailed survey and analytical testing for food allergens possible. Further, the ability to detect <5 ng/mL of allergen in the analytical sample makes the technology suitable for the analysis of complex foods where extensive dilution may be required to reduce potential artifacts associated with food matrices carried over during the extraction and preparation process. The availability of this multiplex method also makes profiling possible, an important tool that may help distinguish foods containing homologous proteins and help further our understanding of food allergies and celiac disease.

Table 3 Multiplex profile of cumin

	Country of origin	Cumin ^b												Walnut													
		Almond	Brazil nut	Cashew	Coconut	Cru ^a	Gluten	Hazelnut	Mac ^a	Peanut	Pine nut	Pistachio	Soy														
		-12	-13	-14	-15	-18	-19	-20	-21	-22	-27	-28	-29	-30	-33	-34	-37	-38	-39	-42	-43	-44	-45	-46	-47	-48	
Cumin ^b	A Ground	43	40	41	22	47	37	47	21	8	6	36	40	21	31	25	110	130	2	20	36	13	25	21	<	12	
	B Ground	56	69	36	11	63	33	40	11	2	4	37	41	21	29	13	110	140	2	10	34	8	19	17	<	8	
	C Seed	<	<	2	<	4	<	9	<	<	<	7	4	<	2	<	<	2	<	<	<	<	8	11	<	2	
	D Roasted, ground	<	2	4	2	5	3	4	<	<	<	6	2	<	2	2	2	2	<	<	2	<	10	14	<	2	
	E Seed	<	<	4	<	3	2	8	<	<	2	14	2	<	2	<	3	2	2	<	2	<	11	14	<	2	
	F Ground	2	<	6	<	9	2	10	<	<	9	49	6	2	2	<	4	2	2	<	3	<	14	16	<	3	
	G Seed	2	<	2	<	4	2	19	<	<	2	19	5	<	9	<	2	3	2	<	<	<	17	19	<	5	
	Reference standards ^c																										
Almond	17	18	<	<	12	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	
Brazil nut	<	<	31	16	13	4	<	<	<	<	<	<	2	<	<	<	<	<	<	8	<	<	<	<	<	<	
Cashew	<	<	18	<	71	58	<	<	<	<	<	<	12	4	<	<	<	<	<	<	56	16	<	<	3	2	
Coconut	<	<	<	<	<	<	37	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	
Crustacean	<	<	<	<	<	<	3	<	74	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	
Gluten	<	<	<	<	<	<	<	<	<	<	23	170	<	<	<	<	<	<	<	<	<	<	<	<	<	<	
Hazelnut	<	<	<	<	<	10	4	<	<	<	<	<	37	14	2	<	<	<	<	<	4	<	<	<	<	<	
Macadamia	<	<	<	<	<	<	<	<	<	<	<	<	<	<	39	7	<	<	<	<	<	<	<	<	<	<	
Peanut	11	4	11	4	40	16	33	4	4	4	3	4	9	9	13	4	130	170	<	3	15	4	26	21	4	5	
Pine nut	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	2	<	<	<	<	<	<	<	
Pistachio	<	<	14	<	46	19	<	<	<	<	<	<	4	<	<	<	<	<	<	<	60	31	<	<	<	<	
Soy	6	3	28	4	77	21	13	4	3	3	3	9	18	3	5	4	5	16	<	4	54	4	290	180	<	10	
Walnut	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	12	28	

Assays designated by the immunogen and the MagPlex[®] Microsphere color coded bead set. Responses tabulated as the average response divided by the average background minus one (S/N-1)

^a *Cru* and *Mac* are abbreviations for Crustacean Seafood (Black Tiger prawn) and Macadamia, respectively

^b Cumin listed as roasted, ground, or whole seeds with the country of origin and analyzed at 5 mg/mL. Cumin "C" was produced in either India, Turkey, or Syria

^c "<" Indicates a response < twice the background measured for the bead set ("S/N-1" ≤1)

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ERRATUM

Erratum to: Multiplex detection of food allergens and gluten

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In the original version of this article unfortunately, Table 2 contained some errors in the placement of the values. The corrected table is presented in this paper; in the electronic version, readers can easily enlarge it to meet their reading needs.

The online version of the original article can be found at <http://dx.doi.org/10.1007/s00216-015-8645-y>.

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Table 2 Cross-reactivity profile patterns^a

Analyte	ng/mL ^b	Almond-12°	Almond-13	Brazil nut-14	Brazil nut-15	Cashew-18	Cashew-19	Coconut-20	Crustacean-21	Crustacean-22	Gluten-27	Gluten-28	Hazelnut-29	Hazelnut-30	Macadamia-33	Macadamia-34	Peanut-37	Peanut-38	Pine nut-39	Pine nut-42	Pistachio-43	Pistachio-44	Soy-45	Soy-46	Walnut-47	Walnut-48
Almond	10	100	43	<	<	45	<	<	<	<	<	6 ^c	<	<	<	<	<	<	<	<	<	<	<	<	<	<
	25	100	44	<	<	47	<	<	<	<	<	6	<	<	<	<	<	<	<	<	<	<	<	<	<	<
	50	100	46	<	<	47	<	<	<	<	<	5	<	<	<	<	<	<	<	<	<	<	<	<	<	<
	100	100	52	<	<	47	<	<	<	<	<	4	3	2	2	2	<	<	<	<	2	<	<	<	<	<
Brazil nut	10	<	<	100	42	68	21	<	<	<	<	<	<	2	<	<	<	<	<	<	33	<	<	<	<	<
	25	<	<	100	41	56	15	<	<	<	<	<	<	4	<	<	<	<	<	<	32	<	<	<	<	<
	50	<	<	100	37	53	15	<	<	<	<	<	<	5	<	<	<	<	<	<	30	<	<	<	<	<
	100	<	<	100	34	50	13	<	<	<	<	<	<	4	<	<	<	<	<	<	27	<	<	<	<	<
Cashew	25	<	<	13	<	100	48	<	<	<	<	<	10	2	<	<	<	<	<	<	54	9	<	<	5	<
	50	<	<	14	<	100	50	<	<	<	<	<	10	2	<	<	<	<	<	<	55	10	<	<	6	<
	100	<	<	21	<	100	64	<	<	<	<	<	16	3	<	<	<	<	<	<	66	16	<	<	8	2
Coconut	25	<	<	<	<	<	<	100	4	<	<	<	<	<	13	<	<	<	<	<	<	<	<	<	<	<
	50	<	<	<	<	<	<	100	5	<	<	<	<	<	5	<	<	<	<	<	<	<	<	<	<	<
	100	<	<	<	<	<	<	100	4	<	<	<	<	<	5	<	<	<	<	<	<	<	<	<	<	<
Crustacean	10	2	<	<	<	<	<	2	<	100	<	<	2	<	<	<	<	<	<	<	<	<	<	<	<	<
	25	2	<	<	<	<	<	2	<	100	<	<	2	<	<	<	<	<	<	<	2	<	<	<	<	<
	50	3	<	2	<	<	2	3	2	100	2	2	2	<	<	<	<	2	2	<	2	2	2	2	2	<
	100	2	<	<	<	<	<	2	<	100	<	<	2	<	<	<	2	2	<	<	2	<	<	<	<	<
Gluten	10	<	<	<	<	<	<	<	<	<	15	100	<	<	<	<	<	<	<	<	<	<	<	<	<	<
	25	<	<	<	<	<	<	<	<	<	16	100	<	<	<	<	<	<	<	<	<	<	<	<	<	<
	50	<	<	<	<	<	<	<	<	<	16	100	<	<	<	<	<	<	<	<	<	<	<	<	<	<
	100	<	<	<	<	<	<	<	<	<	18	100	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Hazelnut	10	<	<	<	<	22	5	<	<	<	<	<	100	18	2	<	<	<	<	<	7	<	<	<	<	<
	25	<	<	<	<	24	7	<	<	<	<	<	100	19	3	<	<	<	<	<	9	<	<	<	<	<
	50	<	<	<	<	26	7	<	<	<	<	<	100	20	4	<	<	<	<	<	9	<	<	<	<	<
	100	<	<	2	<	30	9	<	<	<	<	<	100	23	5	<	<	<	<	<	10	<	<	<	<	2
Macadamia	10	<	<	<	<	<	<	<	<	<	<	<	<	<	<	100	15	<	<	<	<	<	<	<	<	<
	25	<	<	<	<	<	<	<	<	<	<	<	<	<	<	100	12	<	<	<	<	<	<	<	<	<
	50	<	<	<	<	<	<	<	<	<	<	<	<	<	<	100	10	<	<	<	<	<	<	<	<	<
	100	<	<	<	<	<	<	<	<	<	<	<	<	<	<	100	11	<	<	<	<	<	<	<	<	<
Peanut	10	2	<	2	<	14	3	2	<	<	<	<	<	<	<	<	69	100	<	<	2	<	4	4	<	<
	25	4	<	3	<	18	5	4	<	<	<	<	<	<	3	<	70	100	<	<	4	<	6	6	2	<
	50	6	<	4	<	22	6	6	<	<	<	<	<	2	4	<	71	100	2	<	5	<	8	7	4	<
	100	10	2	7	<	27	8	10	<	2	2	2	5	3	7	<	73	100	4	<	8	3	11	10	7	3
Pine nut	10	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	100	2	<	<	<	<	<	<
	25	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	100	5	<	<	<	<	<	<
	50	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	100	7	<	<	<	<	<	<
	100	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	100	9	<	<	<	<	<	<
Pistachio	10	<	<	18	<	87	24	<	<	<	<	<	4	<	<	<	<	<	<	<	100	38	<	<	<	<
	25	<	<	19	<	95	26	<	<	<	<	<	5	<	<	<	<	<	<	<	100	39	<	<	<	<
	50	<	<	20	<	97	29	<	<	<	<	<	7	<	<	<	<	<	<	<	100	42	<	<	2	<
	100	<	<	22	<	92	30	<	<	<	<	<	8	<	<	<	<	<	<	<	100	45	<	2	<	<
Soy	10	<	<	5	<	22	3	<	<	<	<	<	2	<	<	<	<	3	<	<	9	<	100	69	<	<
	25	2	<	7	<	27	5	<	<	<	<	<	3	<	<	<	<	4	<	<	12	<	100	67	<	2
	50	3	<	9	<	32	6	2	<	<	<	2	5	<	<	<	5	<	<	<	16	2	100	70	2	2
	100	5	<	14	<	43	9	3	<	<	2	4	8	<	2	<	2	8	<	<	25	2	100	74	3	4
Walnut	10	<	<	3	<	7	<	<	<	<	<	<	1	<	<	<	<	<	<	<	3	<	<	<	100	78
	25	<	<	3	<	11	<	<	<	<	<	<	5	<	<	<	<	<	<	<	4	<	<	<	100	83
	50	<	<	4	<	8	2	<	<	<	<	<	5	<	<	<	<	<	<	<	3	<	<	<	100	84
	100	<	<	5	<	10	2	<	<	<	<	<	7	<	<	<	<	<	<	<	5	<	<	<	100	88

^a Responses of non-densified analyses tabulated as average response minus background, normalized to the most intense bead set for the analyte, set to 100 (i.e., Almond-12, Cashew-18, Coconut-20, Crustacean-22, Hazelnut-29, Macadamia-33, Peanut-38, Pine nut-39, Pistachio-43, Soy-45, and Walnut-47). < Indicated a normalized response of ≤ 1 (1% or less). Responses $\geq 2\%$ in red except for analysis of almond by Gluten-28, which were below the dynamic range of the bead set.

^b Concentration of analyte in the sample following extraction and preparation (prior to adding bead sets).

^c Bead set identified as the immunogen used to generate the antibody and the number of the MagPlex[®] Microsphere color-coded bead set.

^d Suspected artifact of Gluten-28; these responses were within the noise and below the dynamic range of gluten-28. Though when normalized relative to Almond appear as indicated.

^e Suspected noise error in data generation.